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# PHENOLOGY AND RESPONSES OF *ACACIA LONGIFOLIA* TO DROUGHT AND SALT STRESS: IMPLICATIONS FOR INVASIVENESS AND MANAGEMENT

Dissertação de Doutoramento na área científica de Biologia, especialidade Ecologia, orientada pela Professora Doutora Helena Freitas e apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra

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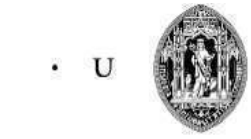

UNIVERSIDADE DE COIMBRA

Dissertação de Doutoramento apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra para obtenção do grau de Doutor em Biologia, especialidade de Ecologia

**RESUMO**

*Acacia longifolia* (Andrews) Willd., arbusto ou pequena árvore, é uma das espécies invasoras mais problemáticas nas regiões costeiras de Portugal continental, onde forma áreas extensas e muito densas. Esta espécie surge também ao longo de rios, vias de comunicação e áreas de montanha, reflectindo a sua capacidade em invadir diferentes habitats. A capacidade desta espécie em responder favoravelmente a várias condições ambientais pode determinar o seu desempenho face às espécies nativas e, consequentemente, o seu potencial invasor.

*Acacia longifolia* possui importantes atributos que lhe permitem sobreviver e reproduzir-se em solos pobres em nutrientes, mas os factores que afectam a distribuição e a presença desta espécie em condições de stress permanecem ainda pouco claros. Assim, um dos objectivos desta tese foi avaliar a resposta de *A. longifolia* a dois tipos de stress: hídrico e salino que são esperados que ocorram com maior frequência, nas regiões de clima Mediterrânico, nas próximas décadas. São também objectivos desta tese identificar o padrão fenológico de *A. longifolia* e avaliar a produção de biomassa da espécie.

A tese está estruturada em cinco capítulos (**capítulo 2 a 6**), cada um organizado como um artigo publicado ou submetido para publicação numa revista científica. Estes capítulos são precedidos pelo **capítulo 1** que corresponde à introdução geral e no qual é feita a revisão da literatura e seguidos pelo **capítulo 7** onde são apresentadas as considerações finais e perspectivas de trabalhos futuros.

O **capítulo 2** resume o primeiro estudo abrangente da fenologia de *A. longifolia*, as variações espacial e temporal e a correlação com o clima. Neste capítulo, pretendeu-se descrever a fenologia das fases vegetativa e reprodutiva (formação de gomos, floração e frutificação) de diferentes populações de *A. longifolia* distribuídas em Portugal continental segundo um gradiente de temperatura e precipitação, durante um período de 3 anos. Foi também estudada a influência dos factores climáticos, observados em cada ano e nos anos anteriores ao estudo, no desenvolvimento das fenofases consideradas. Os resultados mostraram diferenças significativas nas fenofases vegetativas e reprodutivas entre populações e anos de observação. Em geral, as fenofases reprodutivas tendem a ocorrer mais cedo na população a sul do que a norte, com cerca de 1 mês de diferença entre os 2 locais. Entre os factores

climáticos estudados, a temperatura e precipitação parecem exercer maior influência nas fenofases reprodutivas, enquanto a temperatura e a radiação afiguram-se mais associadas ao crescimento vegetativo.

No **capítulo 3**, foram desenvolvidas equações alométricas que permitem quantificar a produção de biomassa acima do solo de *A. longifolia* num ecossistema dunar. No total, foram seleccionadas, aleatoriamente, 18 plantas, nas quais foram medidos vários parâmetros morfológicos. A biomassa aérea de cada planta for determinada pelo método destrutivo e separada por componentes (madeira e folhagem). Os modelos de potência baseados no diâmetro na base, $d_b$, ou na combinação de $d_b$ e altura (h) na forma $d_b^2h$, revelaram ser os mais adequados para estimar com precisão a biomassa acima do solo de *A. longifolia*. Este estudo representa uma importante contribuição para o desenvolvimento de estratégias de gestão adequadas em áreas invadidas por esta espécie.

No **capítulo 4** foi estudado o efeito da disponibilidade de água nos parâmetros morfológicos, bioquímicos e fisiológicos de plantas de *A. longifolia* pertencentes a populações diferentes, uma localizada a norte, onde o clima é mais húmido, e outra a sul, de clima mais seco, de Portugal. As plantas de *A. longifolia* mostraram ser capazes de resistir à escassez de água, embora a resposta esteja muito dependente da duração do stress. Verificou-se, também, que as populações estudadas apresentaram estratégias diferentes relativamente à forma de lidar com o stress hídrico, sugerindo haver variabilidade genética entre ambas.

O **capítulo 5** resume os efeitos da salinidade na germinação de sementes de *A. longifolia* e *Ulex europaeus*, espécie nativa muito comum nos ecossistemas dunares de Portugal. A actividade das enzimas do sistema antioxidante (catalase, CAT; peroxidase do ascorbato, APX; peroxidase, POX e glutationa redutase, GR) foi igualmente avaliada nas sementes de ambas as espécies. Verificou-se que o aumento da concentração salina teve efeitos negativos na germinação das sementes, sendo que esse efeito foi mais acentuado nas sementes de *U. europaeus*, em que concentrações muito elevadas causaram um atraso ou inibição da germinação. Em relação à actividade enzimática, a maior actividade da APX observada em sementes de *A. longifolia* parece indicar que os mecanismos que protegem as sementes dos radicais que provocam danos metabólicos, inibindo a germinação, são mais eficientes nesta espécie do que na nativa, *U. europaeus*. Estes resultados sugerem que as sementes de *A. longifolia* são mais tolerantes à salinidade do que as sementes de *U.*

*europaeus* contribuindo, desta forma, para o seu potencial invasor em ecossistemas dunares.

No **capítulo 6**, a resposta de *A. longifolia* e *U. europaeus* à salinidade foi avaliada através da quantificação do crescimento da planta, conteúdo de solutos e actividade de enzimas do sistema antioxidante. Verificou-se que a salinidade teve efeito negativo no crescimento de *A. longifolia* mas o mesmo não se observou em *U. europaeus*. Em resposta à salinidade houve maior acumulação de $Na^+$ acompanhada por uma diminuição no teor de $K^+$ e na razão $K^+/Na^+$. Relativamente à actividade enzimática, esta foi mais elevada em *A. longifolia* quando comparada com a observada em *U. europaeus*. Os resultados obtidos mostraram que *A. longifolia* possui maior capacidade para suportar condições de salinidade, em parte devido a uma maior actividade da CAT e GR e da razão $K^+/Na^+$, o que pode representar uma vantagem adicional ao competir com espécies nativas em habitats salinos onde estejam presentes.

Uma melhor compreensão destes resultados (**Capítulo 7**) e de como *A. longifolia* será afectada pelo cenário de alterações climáticas previsto para a região Mediterrânea, permitirá prever a distribuição futura e o potencial invasor desta espécie em Portugal. Os resultados apresentados nesta tese contribuem para o maior conhecimento da biologia e ecologia da espécie, fornecem ideias e criam novas perspectivas de investigação. Além disso, constituem um importante contributo para a implementação de estratégias de gestão mais adequadas para controlar a invasão de *A. longifolia.*

**Palavras-chave**: *Acacia longifolia*, clima, crescimento, ecossistemas dunares, equações de biomassa, fenologia, germinação, invasão, NaCl, stress hídrico, *Ulex europaeus*

**SUMMARY**

*Acacia longifolia* (Andrews) Willd., an evergreen shrub or small tree, is one of the most widespread invasive species in the coastal regions of continental Portugal, where it forms extensive and monospecific stands. This species can also grow along rivers, roadsides and mountain slopes, reflecting its ability to invade multiple habitats. The ability of *A. longifolia* to respond to various environmental conditions could determine its performance relative to native species and consequently its invasiveness potential.

*Acacia longifolia* has important attributes that enable it to survive and spread in resource-poor soils, although the factors that affect its distribution and establishment under stressful environmental conditions remain unclear. One of the objectives of this thesis was to evaluate how *A. longifolia* responds to two stress types: drought and salt, that are expected to occur in the next decades in the Mediterranean region. A further goal was to identify the species' phenological pattern and to determine the biomass production of *A. longifolia*.

The thesis is divided into five chapters (**chapter 2 to 6**), each organised as a paper that has been either published or submitted to scientific journals for publication. These chapters are preceded by an introductory chapter (**chapter 1**) that corresponds to a general introduction and to the review of literature, and succeeded by a final chapter that integrates the final considerations of this thesis and suggestions for future research (**chapter 7**).

**Chapter 2** summarises the first comprehensive field study of the phenology of *A. longifolia*, its spatial and temporal variations and its correlation to climate. The intention of this chapter is to describe the vegetative and reproductive phenology (bud formation, flowering and fruiting) of *A. longifolia* populations from different locations, representing a temperature and precipitation gradient in mainland Portugal, over a period of 3 years. In addition, the influence of current and past climatic conditions on the development of each phenophase was investigated. The results highlighted significant variations in the vegetative and reproductive phenophases in different populations and years of observation. In general, the reproductive phenophases occurred earlier in the southern population than in the northern population, with an average delay of 1 month. Among the climatic factors, temperature and precipitation seemed to influence reproductive phenophases, whereas temperature and irradiance were associated more with vegetative growth.

In **Chapter 3,** allometric equations that quantify the aboveground biomass production of *A. longifolia* in a sand dune ecosystem were developed. A total of 18 plants was randomly selected and measured. In each plant, the aboveground biomass was determined by using the destructive method and was assessed by components (wood and foliage). The power function models based on diameter at base, $d_b$, or on a combination of $d_b$ and height (h) in the form $d_b^2h$, provide a reliable method for accurately estimating *A. longifolia* aboveground biomass. The results of this study represent an important contribution for developing adequate management strategies in areas invaded by this species.

**Chapter 4** presents the effects of contrasting water treatments on the morphological, physiological and biochemical parameters of *A. longifolia* plants belonging to different populations, one from the wet (northern) and another from the dry (southern) climate regions of Portugal. *Acacia longifolia* plants are capable of surviving under water-stressed conditions but the effects were greatly dependent on the severity of the water stress. The results also suggested a genetic difference between populations.

**Chapter 5** summarizes the effects of salt stress on germination of *A. longifolia* and *U. europaeus,* a native species very common in the sand dunes on the coast of Portugal. The activity of antioxidant enzymes (catalase, CAT; ascorbate peroxidase, APX; peroxidase, POX; and glutathione reductase, GR) on seeds was also studied. Results showed that the increase in salt concentration had an adverse effect on seed germination in both species. However, this effect was more prominent in *U. europaeus*, in which changes in salt concentration either delayed germination or inhibited it at high concentrations. In relation to the enzyme activity, the observed increase in APX activity in *A. longifolia* seeds indicates that the mechanisms which protect the seeds against radicals that cause metabolic damage, inhibiting seed germination, are more efficient in this species than in the native one, *U. europaeus*. These results suggest that the seeds of the invasive *A. longifolia* are more tolerant to salinity than *U. europaeus*, which may contribute to its invasive ability in sand dunes ecosystems.

In **chapter 6**, the response of *A. longifolia* and *U. europaeus* plants to salinity was evaluated by monitoring plant growth, ion content and antioxidant enzyme activities. The results showed that salinity reduced the plant height and the dry weight in *A. longifolia* whereas in *U. europaeus* the effect was not significant. Salt stress also caused a significant accumulation

of $Na^+$ and a decrease in $K^+$ content and $K^+/Na^+$ ratio. The activities of antioxidant enzymes were higher in *A. longifolia* compared to *U. europaeus*. The results suggest that the invasive species copes better with salinity stress in part due to a higher rates of CAT and GR activities and a higher $K^+/Na^+$ ratio, which may represent an additional advantage when competing with native species in co-occurring salty habitats.

A better understanding of these results (**chapter 7**) and how *A. longifolia* will be affected by the climate change scenario for the Mediterranean region will help to predict the future plant distribution and invasive capacity of this species in Portugal. The results of this thesis improve the current state of research, provide ideas and create new scientific links. Taken as a whole, the results will contribute to implement improved management strategies to control the invasion by *A. longifolia.*

**Keywords**: *Acacia longifolia*, biomass equations, climate, dune ecosystems, germination, invasion, NaCl, phenology, plant growth, *Ulex europaeus*, water stress

**ACKNOWLEDGEMENTS**

Pursuing a PhD is a difficult and sometimes a lonely path. In pursuing this path, first of all, I would like to thank my PhD supervisor, Professor Helena Freitas, for her support and encouragement through these last several years. I also thank Professor Helena Freitas for always believing in me and for lending her great critical scientific eye to my work and for her invaluable scientific guidance.

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Finally, and most importantly I am indebted to my family, in particular my parents and my brother who suffered much from the stresses associated with this academic dissertation. I would not have finished this thesis without their constant support, patience and love.

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| | |
|---|---|
| a.s.l. | Above sea level |
| ANOVA | Analysis of variance |
| APX | Ascorbate peroxidase |
| CAT | Catalase |
| CVG | Coefficient of germination velocity |
| DW | Dry weight |
| FAO | Food and Agriculture Organization |
| GEIB | Grupo Especialista en Invasiones Biológicas |
| GIS | Geographic Information System |
| GPS | Global Positioning System |
| GR | Glutathione reductase |
| GSSG | Oxidized gluthatione |
| IPCC | Intergovernmental Panel on Climate Change |
| LA | Leaf area |
| LAR | Leaf area rate |
| LW | Low-watered treatment |
| MS | Moderate-watered treatment |
| NaCl | Sodium chloride |

| | |
|---|---|
| $NADPH_2$ | Nicotinamide-adenine dinucleotide phosphate, reduced form |
| NAR | Net assimilation rate |
| *P* | P-value |
| POX | Peroxidase |
| r | Pearson´s correlation coefficients |
| *r* | Spearman rank correlation coefficients |
| RDW | Root dry weight |
| RGR | Relative growth rate |
| ROS | Reactive oxygen species |
| S.E. | Standard error |
| SD | Standard deviation |
| SDW | Shoot dry weight |
| SLA | Specific leaf area |
| U | Enzyme units |
| W | Aboveground biomass |
| $W_F$ | Biomass of foliage |
| WR:WRS | Root mass ratio |
| WR:WS | Root:shoot ratio |
| $W_W$ | Biomass of wood |
| WW | Well-watered treatment |

| | |
|---|---|
| Z | Synchrony of the population |
| $\delta^{13}C$ | Carbon isotope composition |
| $\delta^{15}N$ | Nitrogen isotope composition |
| ε | Extinction coefficient |

< 0.05).

# CHAPTER 1

# GENERAL INTRODUCTION

# 1. General introduction

## 1.1. Introduction

### 1.1.1. *Acacia longifolia*: Distribution and weed status

*Acacia longifolia* (Andrews) Willd. (Sydney golden wattle), belonging to the subfamily Mimosoideae of the family Fabaceae, is native to the south-eastern coast of Australia, and occurring naturally in coastland, rangelands, grasslands, shrublands, riparian zones, ruderal and/or disturbed areas (Global Invasive Species Database 2010). Although it can growth in different habitats, including nutrient poor-ecosystems, the Sydney golden wattle grows best in areas that receive at least 550 mm rainfall annually (Department of Primary Industries 2011). The species was originally used to stabilise sand dunes but *A. longifolia* is now an aggressive invader in many countries and is becoming a serious ecological problem, particularly by displacing native vegetation (Marchante *et al*. 2003). At present, *A. longifolia* has an invasive status in South Africa (Cronk and Fuller 1995), New Zealand (Parsons *et al*. 1998) and in Portugal (Marchante *et al*. 2003). *Acacia longifolia* has also been reported as invasive in India, United States, Israel (Elorza *et al*. 2004), Italy (GEIB 2006) and Brazil (Leão *et al*. 2011).

*Acacia longifolia* was first introduced in Portugal at the beginning of the 20$^{th}$ century to stabilise dunes and curb sand movements (Marchante *et al*. 2011). Besides intentional planting, its abundance and distribution has increased greatly by fire events (Marchante *et al*. 2003). After a disturbance event, such as fire or a mechanical clearing, *A. longifolia* rapidly spread to form extensive, dense stands that progressively reduce the high plant biodiversity and change the structure of native plants communities of the invaded areas (Marchante *et al*. 2003). At present, *A. longifolia* is very frequent in coastal sand dunes areas of continental Portugal, especially in the north and central coast where it is considered a priority problem (Ministério do Ambiente 1999; Marchante *et al*. 2003). However, it can also grow along the sides of rivers and roads and on mountain slopes (Marchante *et al*. 2008), reflecting its ability to invade multiple habitats which is partially due to its nitrogen fixing properties (Werner *et al*. 2010).

*Acacia longifolia*, ranging in size from a woody, perennial shrub to small tree (Fig. 1.1a), can live for up to 50 years and grow 7-15 m in height. This species has alternate linear to elliptic

phyllodes, which are 6-11 cm long x 10-35 mm wide, with 2-3 prominent primary veins (Fig. 1.1b). The inflorescence (Fig. 1.1c) consists of a 2-5 cm long spike, usually solitary or twinned, with 45-60 pale to golden-yellow flowers (Fig. 1.1d). The pods (Fig. 1.1e) appear 2 years after the first flowering and are cylindrical or subcylindrical, measuring 5-12 cm long x 4-10 mm wide and can be straight or curved, containing 4-11 brown elliptic seeds, sometimes irregularly shaped, 4-6 mm long, 20-25 mg, funicle folded several times into a thickened lateral skirt-like aril (Fig. 1.1f). The majority of seeds accumulate, beneath or very close to the canopies of the parent plants (Marchante *et al*. 2010) but can also be dispersed by animals (particularly ants), wind and water (Global Invasive Species Database 2010), favouring the establishment of new invasive spots (Cronk and Fuller 1995). *Acacia longifolia* produces a large number of seeds annually, which can remain viable in soil for long periods of time. Marchante *et al*. (2010) studied the seed bank of *A. longifolia* in a sand dune ecosystem, and showed that, on average, 12000 seeds/$m^2$ can fall under the trees in a single season. Although some of these seeds are lost through germination, decay and granivory, the seed bank is still considerable (500 to 1500 seeds/$m^2$) and more than 85% remains viable after 6 years.

A variety of management techniques have been used to control the spread of this species. In Portugal, the most common techniques include chemical and mechanical methods (*e.g.* felling, felling followed by herbicide application on stumps, and prescribed burning) but the success rate is generally low. The longevity of the seed bank, mass germination after removal of the overstory and the rapid replenishment of cleared areas, thus impeding the recovery of ecosystems, are some of the reasons for this poor performance. Biological control can be a highly effective and cost-efficient approach, as is the case in South Africa (Impson and Moran 2004), but is not yet an option in Portugal. Whatever the control method used, understanding the biology of *A. longifolia* is, therefore, the first step towards effective control of the species.

*Acacia longifolia* is characterised by its ability to respond favourably to various environmental conditions such as drought (Werner *et al*. 2010) and salinity (Marcar *et al*. 1995), but how these ecological constraints affect its distribution is not clear. Moreover, the reduced water availability anticipated in climate change scenarios for the Mediterranean region would inevitably affect its growth and performance and, consequently, its invasive

potential. Elucidating the factors that contribute to the success of this invasive species in variable environments may ease the prediction of future invasions and determine the best strategies to its control.


**Fig. 1.1.** Morphological features of *Acacia longifolia*: (a) plant pattern, (b) phyllodes, (c) flower buds, (d) inflorescences in anthesis, (e) mature pods, (f) seeds.

### *1.1.2. Objectives*

To reach a better understanding of the biology and the ecology of *A. longifolia* and, at the same time, the responses of this species under drought and salinity conditions, this thesis propose:

• To describe the phenological pattern of *A. longifolia* from different geographical origins and analyse how climatic factors affect this pattern;

• To determine the biomass production of *A. longifolia* plants in a characteristic sand dune ecosystem and to develop an allometric equation for its rapid and accurate calculation;

• To evaluate the influence of three water availability regimes (well-watered, moderate-watered and low-watered) on the morphology, physiology and biochemistry of *A. longifolia* plants from two contrasting populations growing under controlled conditions over three months;

• To compare the germination and growth response of *A. longifolia* and the native legume *Ulex europaeus*, which is very common in sand dune habitats, to four salt treatments (0, 50, 100 and 200 mM NaCl) and to analyse the antioxidative protection of both species in all treatments.

This thesis is structured in 7 chapters, including this general introduction (**Chapter 1**). **Chapters 2** to **6** correspond to scientific papers published or submitted to publication (Fig. 1.2) as follows:

Chapter 2, submitted to Biological Invasions:

**Morais MC,** Freitas H. Spatial and temporal variation in the phenology of the invasive plant *Acacia longifolia* in Portugal.

Chapter 3, submitted to Weed Research:

**Morais MC,** Freitas H. Aboveground biomass estimates of the invasive *Acacia longifolia* in Portuguese dunes: Contribution for its management

Chapter 4, published as:

**Morais MC,** Freitas H (2012) The acclimation potential of *Acacia longifolia* to water stress: Implications for invasiveness. Plant Science 196: 77-84.

Chapter 5, published as:

**Morais MC**, Panuccio MR, Muscolo A, Freitas H (2012) Does salt stress increase the ability of the exotic legume *Acacia longifolia* to compete with native legumes in sand dune ecosystems? Environmental and Experimental Botany 82: 74-79.

Chapter 6, published as:

**Morais MC**, Panuccio MR, Muscolo A, Freitas H (2012) Salt tolerance traits increase the invasive success of *Acacia longifolia* in Portuguese coastal dunes. Plant Physiology and Biochemistry 55: 60-65.

The final chapter (**Chapter 7**) includes the final remarks of this thesis.

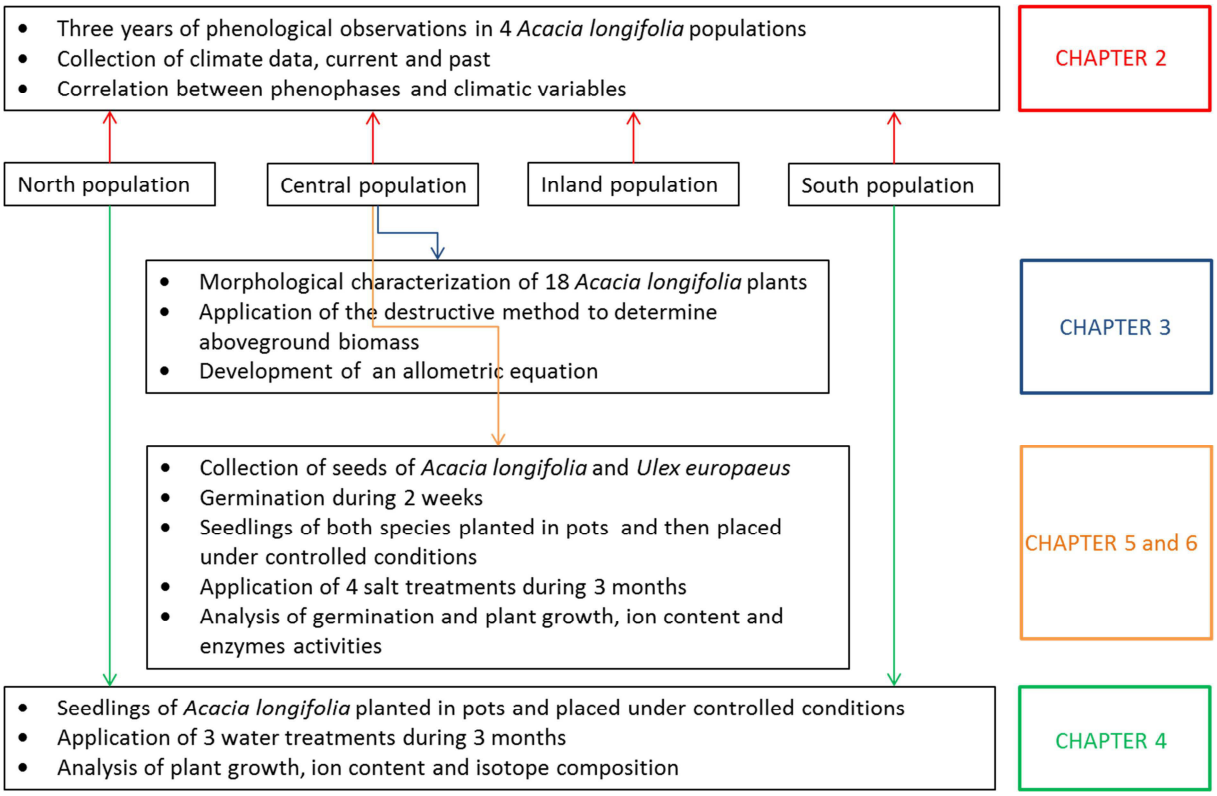


**Fig. 1.2**. A general overview of the thesis.

### *1.1.3. Study populations*

Four Portuguese *A. longifolia* populations were chosen for this study, representing a temperature and precipitation gradient (Fig. 1.3). Three populations were in the protected

coastal areas (North Littoral Natural Park, São Jacinto Dunes Natural Reserve and Sto. André and Sancha Lagoons Natural Reserve) and one population was in land (Coimbra). They will be referred to hereafter as the North, Central, Inland and South populations. A summary of the average climatic data observed at study populations for the 1971-2000 was given in Table 1.1.

**Table 1.1**. Summary of the average climatic data observed at study populations for the years 1971-2000.

| Variable | North[a] | Central[b] | Inland[c] | South[d] |
|---|---|---|---|---|
| Mean annual temperature (ºC) | 14.8 | 15.4 | 15.5 | 15.8 |
| Mean maximum temperature (ºC) | 19.8 | 21.1 | 21.2 | 18.5 |
| Mean minimum temperature (ºC) | 9.8 | 10.3 | 10.4 | 13.2 |
| Mean maximum temperature for the hottest month (ºC) | 38.6 | 39.0 | 41.6 | 37.1 |
| Mean minimum temperature for the coldest month (ºC) | -4.0 | -3.0 | -4.9 | 0.5 |
| Relative humidity (%) | 82.0 | 83.2 | 86.0 | 81.0 |
| Mean precipitation (mm) | 1470.2 | 906.7 | 905.1 | 511.0 |

[a]Weather station of Viana do Castelo, [b]Weather station of Aveiro, [c]Weather station of Coimbra/Bencanta, [d]Weather station of Sines.

The North population is in the protected coastal area of the North Littoral Natural Park. This Nature Park runs along the 16 km coast of the municipality of Esposende, in the north of Portugal, between the River Neiva and the region of Apúlia, most of which is less than 10 m above sea level. The climate is Mediterranean with a strong Atlantic influence. The Park, created in 2005 (Decreto-Regulamentar n.º 6/2005, de 21 de Julho), developed from the previous area named as "Protected Area of the Esposende Coastline" and is part of the Nature 2000 protected coastline of northern Portugal. Three quarters of the park is maritime and fluvial (next to the sea or river) and a quarter terrestrial (440 ha in total) and includes 15 different habitats. The area includes an extensive dune system with characteristic shrubs and herbs species, including: *Eryngium maritimum*, *Euphorbia paralias*, *Calystegia soldanella, Pancratium maritimum*, *Ammophila arenaria, Medicago marina*, *Lotus creticus* and *Othantus maritimus*. The interior of the dunes is usually covered by shrubby species

(*e.g. Ulex europaeus*, *Corema album*, *Cytisus grandiflorus*, *Agrostis curtisii*, *Erica cinerea, Rhamnus alaternus*) and wood plantations dominated by *Pinus pinaster*. In these areas, exotic invasive species also co-occur (*Acacia melanoxylon*, *A. longifolia, Carpobrotus edulis*, *Arctotheca calendula* and *Cortaderia selloana*. *Acacia longifolia* is the most prolific invasive species in the park. It was probably first introduced in the early 20$^{th}$ century together with *Pinus pinaster* to prevent dune wandering. For several decades the presence of *A. longifolia* was restricted to a small area located along the southern border of the park, mostly in the understory of *P. pinaster*. At the time, the wood of this species was used for fuel and for agricultural purposes, but the abandonment of these activities in the middle of the 80' led to the rapid spread of *A. longifolia,* which proliferated in several areas. At present, *A. longifolia* forms extensive, dense stands especially in the pine tree patches and the stabilised dune communities in the south of the park (A. Viana, unpublished data).

The Central population is in the São Jacinto Dunes Natural Reserve on the central-northern coast of Portugal on a strip of sand dunes bordered by the Atlantic Ocean in the west and the Ria de Aveiro in the east. This protected area was created in 1979 (Decreto-Lei n.º 41/79, de 6 de Março) and reclassified in 1993 (Decreto-Lei n.º 19/93, de 23 de Janeiro). Later in 1997 it was reclassified again (Decreto-Lei n.º 46/97, de 17 de Novembro). In 2004, the Reserve was reclassified again (Decreto Regulamentar n.º 24/04, de 12 de Julho) maintaining the protection status and extending its limits from 660 ha to 960 ha. The climate of the area is Mediterranean with an Atlantic influence. The vegetation of the primary dunes is characterized by several characteristic coastal sand dune species such as *Ammophila arenaria* ssp. *australis*, *Corema album*, *Eryngium maritimum*, *Cakile maritime*, *Euphorbia paralias*, *Helichrysum picardii* and *Otanthus maritimus*. Between the primary and secondary dunes grows *Corema album*, the most abundant bush in this area. The secondary dunes are covered by several woody species dominated by *Pinus pinaster*, *Eucalyptus globulus* and *Acacia longifolia* and the understorey is usually occupied by *Myrtus communis*, *Ulex europaeus* and *Juniperus turbinata,* among other species. Other exotic invasive species also co-occur with *A. longifolia* in different parts of the Reserve (*A. saligna*, *A. retinodes*, *Carpobrotus edulis* and *Cortaderia selloana*). *Acacia longifolia* was introduced to the Reserve in the first four decades of the 20$^{th}$ century to curb sand movement. It then spread, forming extensive, dense stands that thrived, particularly as a result of fire events. The most recent

fire in the reserve occurred in the summer of 1995, burning approximately 200 ha and eliminating all the aboveground vegetation in the affected area (Silva and Marchante 2012). Nowadays, approximately 2/3 of the reserve is occupied by this invasive species, which forms continuous closed stands, frequently with a cover of about 80% (Marchante *et al*. 2008).

The Inland population is in an urban green area called Quinta da Maia (2.2 ha) in the centre of Coimbra, in the central region of Portugal, approximately 45 km east of the coast. This green area is characterized by a Mediterranean climate and a podzol soil. The plant community is dominated by *Pinus pinaster* and *Quercus suber* with a shrubby understory occupied by many Mediterranean native shrubs such as *Rhamnus alaternus, Cistus salvifolius, Calluna vulgaris and Ulex minor*. In the area, the tree cover is also occupied by *Acacia* species, mainly *A. melanoxylon* and *A. dealbata*. *Acacia longifolia* occupies a small fraction of this green area and is restricted to the western slope. The date and reason for the introduction of *A. longifolia* to the area are unknown but it is reasonable to suppose that it was for soil stabilisation.

The South population is in the Sto. André and Sancha Lagoons Natural Reserve, a protected area on the Alentejo coast (SW Portugal) close to the town of Vila Nova de Santo André. This protected area was created in 2000 (Decreto Regulamentar n.º 10/2000, de 22 de Agosto), has a total extension of 5247 ha of which 3110 ha correspond to the onshore and 2137 ha correspond to marine part. The climate is Mediterranean. The Reserve includes an extensive dune system with characteristic dune vegetation dominated by *Ammophila arenaria and Linaria ficalhoana.* The stabilized dunes are occupied by shrubby dune formations dominated by *Juniperus turbinata* and *Corema album.* The inland dunes are occupied by dense shrubby thickets of *Juniperus navicularis*, *Ulex australis, Stauracanthus spectabilis* and *Santolina impressa*. The tree cover is mainly dominated by *Pinus pinaster* and *Eucalyptus globulus* and, in some areas, *Acacia* species*: A. longifolia*, *A. saligna*, *A. mearnsii* and *A. cyclops*. *Acacia longifolia* was introduced into the reserve in the middle of the 19$^{th}$ century (1950 to 1960) to curb sand movement after the abandonment of the dunes as agricultural land. It subsequently spread to forms continuous closed stands mainly at the southern end of the reserve, thriving in particular as a result of fire events (the lastest occurring in 1998)

and the industrial project in Sines (A. Vidal, unpublished data). At present, large invasive patches are often associated with deteriorating *Eucalyptus globulus* stands.

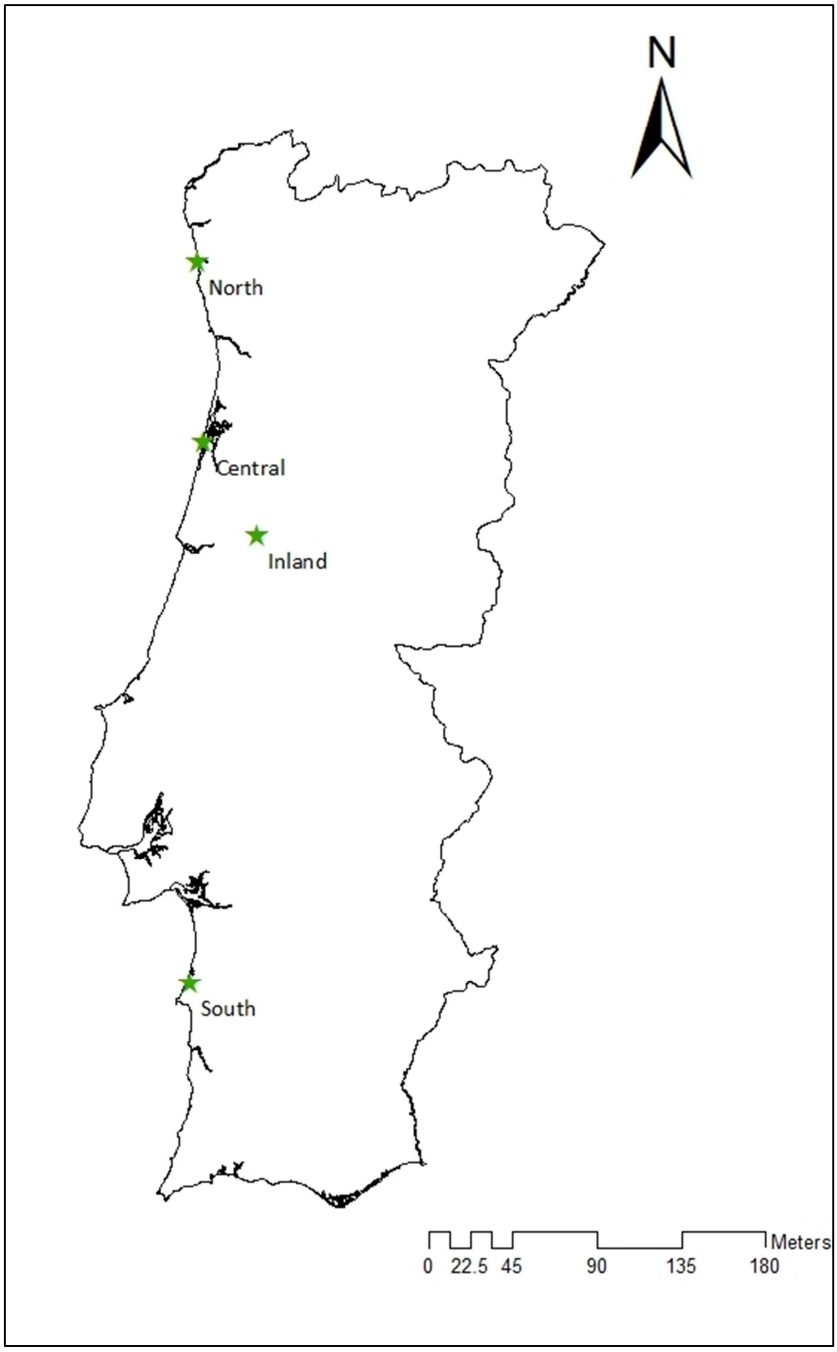


**Fig. 1.3.** Location of the four populations (marked with a green star) used in this study. From north to south (top to bottom), populations included in this study are: North Littoral Natural Park, São Jacinto Dunes Natural Reserve, Coimbra and Sto. André and Sancha Lagoons Natural Reserve).

## 1.2. Literature review

This section presents the general theoretical background of this thesis, which is organised in four main topics: the first synthesizes the key aspects associated with biological invasions (concepts, invasion process, species characteristics and invasive species impacts), the second concerns the importance of phenology to understand the invasive success, the third deals with biomass knowledge and its implications for management decisions, and the fourth summarises the effects of environmental stress on the distribution of invasive species.

### *1.2.1. Biological invasions: An overview*

The interest in biological invasions has increased in the last few decades (Engel *et al*. 2011) mainly due to their massive economic (Pimentel *et al*. 2005) and ecological (Levine *et al*. 2003) costs. Nowadays, biological invasions present one of the most serious threats to the ecosystem health and biodiversity, only after habitat destruction and fragmentation (Mack *et al*. 2000).

Biological invasions can be viewed as a progression of events (stages) in which exotic species are introduced to a new environment, establish a self-sustaining population and spread across the landscape (Theoharides and Dukes 2007). Invasion therefore involves four essential stages: transport, colonisation, establishment, and spread (Fig. 1.4). Although most introductions fail at the first hurdle, a select subset must pass through each stage of the introduction process to become successful invaders (Chapple *et al*. 2012). So, the number of plant species is progressively reduced, since exotic species must overcome biotic and abiotic barriers before becoming successful invaders (Richardson *et al*. 2000). At the end of the invasion process, the fraction of exotic plant species that become invasive is surprisingly small, often estimated at 1% (Richardson and Pyšek 2006).

Biological invasion begins with the **transport** (accidental or deliberate) of plant species (or their propagules) from their native ranges to new environments where it was not known to occur previously (Mack *et al*. 2000). In general, transport is mediated by humans over long distances or across a major geographic barrier (Richardson *et al*. 2000). The number of plant species that can cross this barrier is related to species traits that allow them to survive transport and to traits selected by humans before transport (Theoharides and Dukes 2007).

The second stage, **colonisation**, starts when the exotic species arrives in the new environment. The success of exotic species in new environments depends on three primary factors: propagule pressure (*i.e.* the number of individuals introduced), the abiotic barriers present in the new area (*e.g.* climate, soil type, availability of resources, etc.) and biotic barriers on a neighbourhood scale (*i.e.* interspecific competition, diseases, predators, etc.). In the **establishment** stage, the exotic species must colonise the new environment and develop self-sustaining populations over several life cycles (Richardson *et al*. 2000). So a successful establishment requires the incipient population to survive and reproduce (Blackburn *et al*. 2011). This stage is normally longer than the colonisation stage and occurs on a slightly larger spatial scale (Theoharides and Dukes 2007). During this stage, abiotic barriers as well as biotic barriers (through competition, mutualism, herbivory, parasitism, etc.) are relevant (Theoharides and Dukes 2007). The final, **spread** stage describes the process by which invasive species disperse across the new environments (Richardson *et al*. 2000). Once an invader has reached a new environment, its success depends on how it responds to the niche opportunities available (Shea and Chesson 2002). In other words, successful invasion success depends on environmental characteristics (*e.g.* connectivity of viable habitat patches, disturbance events, etc.), invasion characteristics, species traits and the mode and pattern of dispersal and reproduction (Hellmann *et al*. 2008).

The invasion process does not happen immediately. Rather, many alien species experience a lag phase between establishment and spread (Theoharides and Dukes 2007) which can be extremely variable. For example, Pyšek and Richardson (2008) reported lag phases of up to 80 years for herbaceous species and 150 or more years for woody plant species. A lag phase is often interpreted as an ecological phenomenon (Sakai *et al*. 2001) and usually reflects a lack of genetic variation, a lack of suitable local habitat, inclement environmental conditions, or a statistical artifact (Pyšek and Hulme 2005).

As said previously, only a small subset of individuals that is transported to new environments manage to pass successfully through all the different stages and overcoming all the ecological barriers (Diez *et al*. 2012) constraining invasion. In this multistage process, the barriers important at each stage can be different, with geographical barriers being generally important initially, and reproductive, dispersal later. Richardson *et al*. (2000), classify species that overcome each stage and its irrespective barrier as “exotic”, ”casual”, “naturalized” and

"invasive". According to this, exotic are species that have reached the first stage (transport), casual are species that have reached the second stage (colonization), naturalized are species that have taken the third stage (establishment) and invasive species are plants that complete the invasion process by overcoming all the stages (Fig. 1.4).

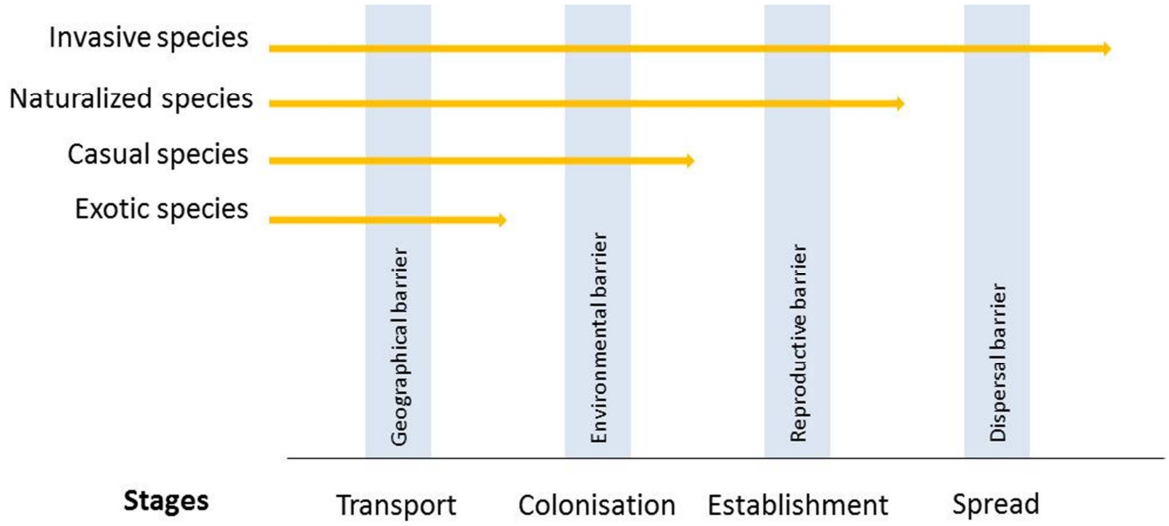


**Fig. 1.4.** Schematic depiction of the main phases in the invasion process, main barriers to invasion and the classification of species involved in each stage (Adpated from Richardson *et al*. 2000).

Beyond these stages, Levine (2008) and Catford *et al*. (2009) suggested one more stage: the **impact** stage, in which the invasive species produces harmful consequences on ecology and economy. In relation to this, many studies recognise that invasive species can provoke impacts at all levels of the ecological organisation: individuals, populations, species, communities and ecosystems (van der Velde *et al*. 2006). Invasive species can disrupt human well-being, affect biodiversity patterns, can have large effects on ecosystems processes (Pyšek and Richardson 2010) including the nutrient cycle, hydrology and fire regimes (Levine *et al*. 2003) and can affect crop production (Mack *et al*. 2000). As a result, the presence of invasive species threatens native biodiversity (Mack *et al*. 2000), can contribute to the extinction of species (van der Velde *et al*. 2006) and to a homogenization of global biodiversity (Catford *et al*. 2012). The severity of these impacts will depend, among other factors, on properties of the invasive species (invasiveness) and the susceptibility of a

community to be invaded (invasibility) (Huston 2004; Richardson *et al*. 2011). Despite the increasing attempts to link invasiveness to specific plant traits there is no simple response. One reason for this may be that the traits associated with invasiveness vary according to both habitat conditions and the stage of the invasion (Alpert *et al*. 2000). However, some plant characteristics, such as rapid and aggressive growth, great production of seeds with high viability, large plant size, early maturity, high phenotypic plasticity and rapid adaptation to disturbance (Kolar and Lodge 2001), have been pointed out as key traits that can contribute to plant invasiveness. Therefore, understanding the mechanisms of invasion and predicting invasion events is key to limit their impacts on ecosystems and requires a deep knowledge of the history of the species introduction, their attributes and the characteristics of the invaded ecosystems. This knowledge is not only a matter of scientific interest but of practical importance for management of invaded areas by invasive species.

### *1.2.2. Phenology in the context of invasions*

Phenology, defined as the study of the timing, duration and abundance of life cycle events (Sakai *et al*. 1999; Bustamante and Búrquez 2008), is perhaps the most important trait affecting species distribution (Chuine 2010). When applied to plants, phenology refers to the timing and duration of bud-burst, leaf expansion, flowering, fruiting, seed dispersal and germination (Fenner 1998). According to Milton and Moll (1982), the phenology of a species may be more related to site characteristics than species characteristics. In Mediterranean-type ecosystems, the occurrence of these phenophases is mainly driven by temperature, precipitation (Spano *et al*. 2003; Peñuelas *et al*. 2004) and photoperiod (Hunter and Lechowicz 1992).

The close link between phenological development and climatic factors makes phenology an excellent tool for identifying how plants species respond to favourable or stressful environmental conditions (Sekhwela and Yates 2007) and to climate changes (Beaubien and Johnson 1994; Menzel 2003) and, because this, interest in plant phenology has risen in the past decade (Schwartz *et al*. 2006). One implication of climate change is the advance in spring time phenology, which has been brought forward about 2.8 days per decade in the northern hemisphere (Parmesan 2007). This can has considerable effects on species

distribution, interactions, community patterns and opportunities for the establishment of invasive species (Bertin 2008).

It has been hypothesised that climate change will exacerbate the impacts of the naturalisation of invasive species and their subsequent invasion across communities (Thuiller *et al*. 2007). In particular, it is expected that invasive species will experience widespread changes in distribution in response to climate change, with many expanding their ranges into new areas (Smith *et al*. 2012). In general, invasive species have a different phenology from native species and are able to adjust their phenology to a new community or climate much more rapidly than natives species (Wolkovich and Cleland 2011), representing a significant competitive advantage over the latter in the area in which they are introduced (Cadotte and Lovett-Doust 2002). As a result, monitoring the phenology of invasive species can provide a better way of anticipating the impacts of these undesirable species and of protecting species and habitats that are supposedly at higher risk (Bertin 2008).

The ability of invasive species to occur in areas outside their natural ranges with remarkable success is often related to the timing of important phenological events such as flowering or fruiting (Pyšek and Richardson 2007). Previous studies (Godoy *et al*. 2009) demonstrate that invasive species tend to flower earlier and have an extended flowering period when compared to native species. This can be considered a critically important determinant for achieving high fecundity in these species and promotes invasiveness (Pyšek and Richardson 2007). A thorough understanding of the reproductive phenology of the invasive species is therefore essential to their effective management and control (Arshid and Wani 2011). In fact, phenological studies can be used to identify weak points in the life cycle of the invasive plants, in which the use of management or control methods are more effective (Pyšek *et al*. 2007). Marchante *et al*. (2011) also emphasised the importance of phenological knowledge for the efficient use of biological control in *Acacia longifolia*. For example, Milton and Moll (1982) have suggested that the ideal time for using herbicide to control *Acacia melanoxylon* in South Africa should be during summer. In addition, phenological information can also be used to map the distribution of invasive species on large spatial scales (Milton and Moll 1982). In this context, remote sensing can offer valuable information in terms of understanding the changing vegetation patterns associated with broad-scale plant invasions (Civille *et al*. 2005).

*1.2.3. Estimation of biomass and its importance in invasion*

FAO (2005) has defined biomass as "organic material both aboveground and belowground, and both living and dead, *e.g.* trees, crops, grasses, tree litter, roots etc". Aboveground biomass includes all living biomass above the soil including the stem, stump, branches, bark, seeds, and foliage, whereas belowground biomass refers to all fine and coarse litter biomass associated with the soil (Lu 2006).

The accurate estimation and mapping of the distribution of biomass is a prerequisite for many ecological applications, ranging from quantifying the amount of available resources (Zewdie *et al*. 2009) to monitoring and understanding climate change (FAO 2010). As argued by Puccinelli (2012), climate change can have profound ecological impacts, including changes in the distribution, abundance and diversity patterns of species, and the functioning of the ecosystem. Any changes in these factors will therefore increase the vulnerability of ecosystems to invasion (Gritti *et al*. 2006). Invasive species may have multiple consequences for an ecosystem by modifying ecosystem processes and altering the composition of the community and the structure ecosystem of the ecosystem (Gordon 1998; Mascaro and Schnitzer 2011). Such alterations lead to an increased search for control/management and restoration methods that may enhance ecological value (*e.g.* improve ecosystem functions and/or services or conserve or reduce biodiversity and resistance to further invasions (Gaertner *et al*. 2012). In most cases, the management practices applied to the invaded areas involve regulating biomass (Guo 2007), meaning that is fundamental to measure this variable to support management decisions (Guo 2007; Abdelkader *et al*. 2009).

A variety of methods can be used to estimate biomass, differing in terms of procedure, complexity and time requirement. Traditionally, biomass estimates are based on complete harvesting of selected stands or individual plants within the stand (Schmer *et al*. 2010) and the use of allometric equations that relate biomass to certain easily measured variables in the field (West 2004). The first method, known as the destructive method, involves cutting and measuring fresh biomass or the dry weight of the whole plant or its components and is considered the most accurate way to measure and monitor biomass, although it is a time-consuming, costly and laborious process (Flombaum and Sala 2007). Moreover it is difficult to implement in conservation areas (Vieira *et al*. 2008) and in large scale experiments (Seidel *et al*. 2011) and limits the possibilities of temporal studies (Van *et al*. 2000; Tackenberg

2007). The alternative, non destructive method does not require the destruction of the plant, provides estimates that can be as accurate as those obtained with destructive methods, enables more samples to be analysed, hence encompassing a large number of observations at relatively low cost (Montès *et al*. 2000) and allows for the follow-up of plant growth over time (FAO 2010). Allometric equations are also useful for estimating the biomass of species whenever destructive sampling is impractical or limited (Morote *et al*. 2012). Although these methods have enabled estimates to be produced for many areas, they have limitations, some of which are associated with the time required for collecting data and the sample size, although this can be overcome by using remote sensing techniques (Ghasemi *et al*. 2011). These techniques have received considerable attention due to their low cost and use as repeatable alternative for mapping biomass over a wide range of spatial and temporal scales (García *et al*. 2010). Remote sensing does not replace the need for good field data, but greatly increases the efficiency and usefulness of traditional methods (FAO 2010). The applications of these techniques can be very useful for example, in evaluating the impacts of management techniques used to control invasive species (Van *et al*. 2000).

### *1.2.4. The role of environmental stress in the invasion process*

Plant stress can be defined in various ways but is generally understood as any factor that inhibits the normal functioning of plants (Levitt 1972). During their growth, plants are confronted with various biotic and abiotic stress factors that weaken them and make them more susceptible to stress (Mahajan and Tuteja 2005). Among the environmental cues, the occurrence of disturbance or stress events such as drought and salt stress is an important abiotic filter that control plant distribution (Bartels and Sunkar 2005), including the spread of invasive species (Diez *et al*. 2012).

The potential for a plant species to become invasive is also determined by certain traits of the habitat being invaded, including the opportunity for colonisation, changes in atmospheric patterns and availability of resources, which may be affected by climate change (Capdevila-Argüelles and Zilletti 2010). According to the current climate projections for the Mediterranean region (IPCC 2007), the expected increment in the mean temperature and concurrent reduction in precipitation may contribute towards increasing soil salinity and lead to extreme events such as frequent and prolonged droughts. These events, acting as a

disturbance factor, can cause significant changes in vegetation structure and the functioning of the ecosystem (Thuiller *et al*. 2007) and could alter its resistance to invasion (Capdevila-Argüelles and Zilletti 2010). Two of the factors that can increase resilience to invasion in the native ecosystem are the high competitive ability of native species (Theoharides and Dukes 2007) and highly diverse plant communities (Werner *et al*. 2010). On the other hand, this can be severely compromised by the occurrence of various types of disturbances (Huston 2004). The magnitude, duration, frequency, and timing of these events also affect the resilience of the native community (Diez *et al*. 2012). In fact, the critical property of disturbances is that they kill part or all of some native plants, thus affecting their capacity to utilise resources and therefore limiting their growth and/or reproduction and making various types of resources (*e.g.* nutrients, water, space) available to other species (Huston 2004). This can provide windows of opportunity for invasive species which are better adapted to changing conditions (Diez *et al*. 2012). However, the invasive success of these species is largely dependent on their ability to cope with the new conditions (Garcia-Serrano *et al*. 2009), which is often attributed to their high phenotypic plasticity (Peperkorn *et al*. 2005; Richards *et al*. 2006; Theoharides and Dukes 2007; Droste *et al*. 2010), *i.e.* to the potential of specific traits of a genotype to modify growth and development in response to different environments (Richards *et al*. 2006), thus allowing them to become invasive (Hulme 2008; Davidson *et al*. 2011) and to successfully colonise a wide range of new environments (Mal and Lovett-Doust 2005). For example, Droste *et al*. (2010) demonstrated that when subjected to water stress the invasive grass *Microstegium vimineum* responded through plasticity in several ecophysiological traits (biomass production and specific leaf area) to limit stress and this may be positively associated with its successful invasion. The same finding was obtained by Molina-Montenegro *et al*. (2011), who demonstrated that phenotypic plasticity in an invasive plant, *Taraxacum officinale*, may be positively associated with the success of invasions. For this reason, phenotypic plasticity has been suggested as a common trait of plant invaders (Richards *et al*. 2006; Droste *et al*. 2010).

Water restriction limits ecophysiological potential in plants and has short and long term effects (Chaves *et al*. 2003) on different hierarchical levels, from molecular to whole plant level (Blum 1996). These effects are evident in anatomical and morphological alterations (Lei *et al*. 2006) as well as changes in physiological and biochemical processes, ranging from

photosynthesis to protein synthesis and solute accumulation (Lisar *et al*. 2012). Exposure to salt stress also triggers common reactions in plants (Bartels and Sunkar 2005).

In general, mechanisms avoidance (which enables plants to exclude the stress) and tolerance (which allows plants to maintain a thermodynamic equilibrium with the stress without suffering injury) are employed to protect plants from the damage caused by water or salt stress (Levitt 1972). In most plants, the response to water and salt stress is a combination of these survival mechanisms and this makes plant responses to both stresses complex, involving deleterious and/or adaptive changes (Chaves *et al*. 2002; Touchette *et al*. 2009). When exposure to water restriction, plants respond through plasticity in several physiological and morphological traits such as, high biomass allocation to roots, high water use efficiency, growth rate adjustment (Yin *et al*. 2005), reduced leaf area, stomatal closure and osmotic adjustment (Garcia-Serrano *et al*. 2009) that allow them to survive under these adverse conditions. In several species, high leaf plasticity, *i.e.* variations in morphology, anatomy and physiology, can also be observed as a response to water stress (Li *et al*. 2011) and salt stress (Yousif *et a*l. 2010). Specific leaf area, in particular, has been shown to be related to invasiveness (Grotkopp and Rejmánek 2007). However, the plant response depends on stress severity and the period of time in which the plants have grown under water stress (Chaves *et al*. 2003) or salt stress (Nawaz *et al*. 2010). Short periods of drought reduce growth rates although plants can manage this to survive and complete their growth cycle (Harb 2010). In contrast, prolonged droughts can limit plant growth (Ogbonnaya *et al.* 1998), reduce biomass production (Osório *et al*. 1998), alter the allocation pattern of biomass (Gallacher and Sprent 1978), influence reproductive capacity (Rodiyati *et al.* 2005) and even cause plant death (Ogbonnaya *et al*. 1998). Moreover, when plants are exposed to short periods of time under salinity, there will be a significant decrease in growth rates followed by a gradual recovery, whereas if the salinity level is high enough, growth slows down dramatically and plants may died (Munns 2002). The plant species and features, including the age and stage of development, organ and cell type, and genotype, also influence the plant response to stress (Bray 1997). Within the stages of the plant life cycle, seed germination and seedling emergence and establishment are critical development stages that may depend greatly on water availability (Al-Taisan 2010) and salinity (Ungar 1996).

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**CHAPTER 2**

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# SPATIAL AND TEMPORAL VARIATION IN THE PHENOLOGY OF THE INVASIVE PLANT *ACACIA LONGIFOLIA* IN PORTUGAL

Maria C. Morais, Helena Freitas

[Submitted to Biological Invasions]

## 2. Spatial and temporal variation in the phenology of the invasive plant *Acacia longifolia* in Portugal

### 2.1. Abstract

Phenological shifts may play an important role in the success of invasive species. Although *Acacia longifolia* is one of the most widespread invasive species in the coastal regions of continental Portugal, there are significant gaps in our understanding and interpretation of its phenology. This study aimed to investigate the phenology of *A. longifolia*, its spatial and temporal variations and its association with climate. Vegetative growth and reproductive phenology (bud formation, flowering and fruiting) were studied in four *A. longifolia* populations, representing a temperature and precipitation gradient with mainland Portugal, over a period of 3 years. Current and past climatic conditions were used to examine their relevance to the development of each phenophase. Overall, bud formation took place between summer and spring, anthesis occurred between late winter and the beginning of spring and fruiting started in late winter. Vegetative growth was observed throughout the year, but it was more pronounced in the spring/summer period. Temperature and precipitation seemed to influence reproductive phenophases, whereas temperature and irradiance were associated more with vegetative growth. Compared all populations, the reproductive phenophases occurred earlier in the southern population than in the northern population, with an average delay of 1 month. According to this and considering the climate change scenario for the Mediterranean region, an increase in air temperature and unsteady precipitation may cause an earlier display of flowering which may affect the future reproductive success and the invasive capacity of this species.

**Keywords**: *Acacia longifolia*, climate, invasive species, phenology, synchrony

### 2.2. Introduction

Plant phenology, involving the timing, duration and abundance of vegetative and reproductive phases (phenophases) in the life cycle of plants (Sakai *et al*. 1999; Haugaasen and Peres 2005; Bustamante and Búrquez 2008) is crucial to understanding species interaction (Okullo *et al*. 2004), community function and diversity (Fenner 1998; Eddy and

Judd 2003). As an indicator of plant responses to stressful or favourable environmental conditions, phenology is thus of fundamental importance in monitoring, managing and conserving ecosystems (Sakai *et al*. 2005). Phenophases may reflect the physiological and morphological adaptive strategies of species and plant communities in utilising resources (van Schaik *et al*. 1993) and can be critical to plant survival and reproduction (Rathcke and Lacey 1985; Haugaasen and Peres 2005; Sakai *et al*. 2005; Torimaru and Tomaru 2006).

The timing and intensity of each phenophase can be affected by pollination (Sakai *et al*. 1999), competition, herbivory, and seed dispersal factors (van Schaik *et al*. 1993) and by predictable temporal variations in rainfall, solar radiation, temperature (Wielgolaski 2001; Lobo *et al*. 2003) and photoperiod length (Badeck *et al*. 2004). However, the influence of each factor on a particular phenophase varies from year to year (Richardson and O'Keefe 2009) and according to location (Jolly and Running 2004). In mid and high latitudes, with a vegetation rest in winter and an active growing period in summer, air temperature has the greatest influence on phenology (Chmielewski *et al*. 2004). On the other hand, in the tropics and/or in (semi)-arid regions, phenology is mainly driven by seasonal variations in rainfall and soil water availability (Ghazanfar 1997; Morellato *et al*. 2000; Bruns *et al*. 2003; Forrest and Miller-Rushing 2010). In Mediterranean-type of ecosystems, the start and length of the flowering phenology is explained by variations in precipitation (Godoy *et al*. 2009b), whereas in the same ecosystems, maximum temperature is responsible for leaf unfolding and spring flowering (Gordo and Sanz 2005).

The close link between phenological development and climatic factors makes phenology an excellent instrument for detecting and measuring the biotic response to climate change (Beaubien and Johnson 1994; Roetzer *et al*. 2000; Menzel 2003; Bertin 2008). Furthermore, the potential use of these data in other fields like remote sensing has added value to phenological data (Chen *et al*. 2000). According to Clarke (2007), climate change will increase the rate of species loss and provide opportunities for the establishment of invasive species. The ability of these species to establish and spread in areas outside their natural ranges is associated with a wide variety of traits that give them a competitive edge over the native species in their introduced community (Cadotte and Lovett-Doust 2002). In particular, invasive species have a different phenology from native species, and are able to adjust their phenology to a new community or climate much more rapidly than native species

(Wolkovich and Cleland 2011). Among the reproductive traits observed in invasive species, appropriate flowering phenology is a key trait for achieving high fecundity and can affect the success of naturalization and invasion (Pyšek and Richardson 2007).

*Acacia longifolia* is an invasive species that was introduced to Portugal at the beginning of the 20$^{th}$ century to stabilise dunes and curb sand movement (Marchante *et al*. 2011). This species commonly invades coastal dunes where it forms extensive, nearly monospecific stands (Marchante *et al*. 2003), but it can grow along the sides of rivers, roads and on mountain slopes (Marchante *et al*. 2008), confirming its ability to adapt to diverse habitats in the region it invades. Most of the studies on *A. longifolia* phenology have been carried out in Australia and South Africa (Milton and Moll 1982; Lindsay and French 2005). Recently, Morais and Freitas (2008) studied geographical variation in *A. longifolia* phenology, but this is the first study to compare the phenological pattern of this species in populations located in sites with marked environmental differences over an extended period of time, and with a sufficient number of samples to investigate the influence of climate on the development of phenophases. This approach is valuable as it helps us to understand the extent of phenological variability as a survival strategy in different environments. Knowledge of the phenology of this species is crucial for its effective management and can be used to identify weak points in the life cycle, and support long term management. In addition, recent studies (Marchante *et al*. 2011) have shown the importance of phenological knowledge for efficient biological control.

Hence, this study examined the phenology of *A. longifolia* in four populations in northern, central and southern locations in Portugal, in order to assess the spatial and temporal variations in the phenology of *A. longifolia*. Specifically, the following objectives were addressed: (i) a description of the phenological pattern of *A. longifolia*; (ii) a comparison of the timing and duration of vegetative and reproductive phenology in the different populations, and (iii) an evaluation of the effect of current and past climatic conditions on *A. longifolia* phenology.

## 2.3. Material and Methods

### *2.3.1. Plant description*

*Acacia longifolia* is an evergreen shrub or small tree which grows 7-15 m tall. It has alternate linear to elliptic phyllodes, which are 6-11 cm long x 10-35 mm wide, with 2-3 prominent primary veins. The inflorescence forms spikes, which are usually solitary or twinned and 2-5 cm long. The pods are cylindrical or subcylindrical, measuring 5-12 cm long x 4-10 mm wide and can be straight or curved, containing 4-11 brown elliptic seeds (M. Morais personal observation). Preliminary observations in two populations in central Portugal revealed that flower buds set between June and March of the following year, flowering occurs from late winter to the beginning of spring, and the pods develop between March and July (Morais and Freitas 2008).

*2.3.2. Study populations*

This study was carried out in four populations in Portugal representing a temperature and precipitation gradient. They will be referred to hereafter as the North, Central, Inland and South populations. The distance between the populations varied from 100 Km to 500 Km. The North population (41°31'N, 8°47'W, 0-20 m a.s.l.) is in the protected coastal area of the North Littoral Natural Park near the municipality of Esposende in the north of Portugal. The vegetation in this area includes several shrubs and herbs species typical of coastal ecosystems and most dunes in the interior are usually covered by wood plantations in which the main introduced species are *Pinus pinaster* and *Acacia longifolia*. The nearest weather station at Viana do Castelo (41°42' N, 8°48' W) reports a mean annual precipitation of 1470.2 mm for the period 1971-2000. The mean monthly temperature is 14.8°C, the mean maximum temperature 38.6°C in June, and the mean minimum temperature is -4.0°C in December. The Central population (40°39' N, 8°44' W, 10-15 m a.s.l.) is in the São Jacinto Dunes Natural Reserve on the central-northern coast of Portugal. The climate is Mediterranean with an Atlantic influence. Data (1971-2000) from the University of Aveiro weather station (40°38' N, 8°40' W) indicate average temperatures ranging from 10.2°C (January) to 20.2°C (August), and a mean annual precipitation of 906.7 mm. The soils are arenosols and the vegetation is characterized by randomly distributed small trees and more abundant shrubs, subshrubs, and herbs and contains characteristic coastal sand dune species such as *Ammophila arenaria* ssp. *australis*, *Corema album*, *Eryngium maritimum*, *Cakile maritime*, *Euphorbia paralias*, *Helichrysum picardii* and *Otanthus maritimus*.

The Inland population (40°12’ N, 8°24’ W, 35 m a.s.l.) is in Coimbra, in central Portugal, approximately 45 km east of coast. The soil is classified as a podzol and the vegetation consists mainly of *Pinus pinaster*. Other exotic species such as *Acacia dealbata* are also present. The mean annual precipitation is 905.1 mm and the mean monthly temperatures range from 9.6°C (January) to 21.6°C (July) (Coimbra/Bencanta weather station, 40°12’ N, 8°27’ W, 1971-2000).

The South population (38°03' N 8°48' W, 0-47m a.s.l.) is in the Sto. André and Sancha Lagoons Natural Reserve, a protected area on the Alentejo coast (SW Portugal) close to the town of Vila Nova de Santo André. The population includes an extensive dune system with characteristic dune vegetation and some woody plantations dominated by *Pinus pinaster*, *Eucalyptus globulus* and *Acacia longifolia*. The climate is Mediterranean with an Oceanic influence. According to the Sines weather station (37°57’ N, 8°53’ W, 1971-2000), the annual precipitation for the South population totals 511 mm and the average air temperature is 15.8°C, with a maximum of 37.1°C in July and a minimum temperature of 0.5°C in January.

### *2.3.3. Climate data*

The average monthly figures for temperature, relative humidity, irradiance and precipitation from March 2008 to March 2011 and one year before the beginning of this study were compiled from records obtained from the weather station closest to each population. To evaluate the effects of climate, we considered not only the effects of the climatic values on the phenophases in the current month, but also in the preceding months, up to 12 months before the current month. In order to determine which past climatic variables were important, we divided the climatic data into 3-month period, involving the calculation of climatic means for the 1-3, 4-6, 7-9 and 10-12 months before the beginning of each phenophase.

### *2.3.4. Collection of phenological data*

In early 2008 *A. longifolia* plants in the North (n = 40), Central (n = 40), Inland (n = 20) and South populations (n = 40) were randomly selected, positioned using GPS and marked with a coloured flag and a tag with a unique identification number. Only plants with visible and

healthy crowns were included. The distance between marked plants ranged from 2 m to 1600 m in the North, 2 m to 1910 m in the Central, 2 m to 100 m in the Inland and 2 m to 3700 m in the South population. Phenological observations were carried out over 3 consecutive years, from March 2008 to March 2011 at all populations, involving visits at intervals of 1-2 months. In total, 100 censuses were conducted and phenological data were collected from 140 plants from all four populations.

During each census the reproductive and vegetative phenology was registered and the following phenophases were recorded: vegetative growth, flower bud formation, open flowers (flowers in anthesis), flower senescence (brown or abscising flowers), immature pods (growth of pods until full expansion), mature pods (brown and closed pods) and dehiscent pods (mature and open pods). A phenophase was considered to be present when displayed in more than 5% of the plant crown; otherwise it was recorded as an "infrequent event" or absent (Castro-Díez and Montserrat-Martí 1998). The intensity of each reproductive phenophase was estimated in terms of percentages using the scale described by Goulart *et al*. (2005): 0 - absence of phenophase, 1 - presence of phenophase in 1%-25% of the crown, 2 - presence of phenophase in 26%-50%, 3 - presence of phenophase in 51%-75% and 4 - presence of phenophase >76%. These percentages were estimated on the basis of the proportion of the crown that showed each phenophase. For the vegetative phenophase, the presence or absence of vegetative growth was recorded. All the data were collected by the first author and some volunteers.

As no observations were made during the interval between the sampling dates (usually 30 days), it was assumed that a particular phenophase began in a plant 15 days before the date on which the phenophase was recorded for the first time and finished 15 days after the date on which the phenophase was recorded for the last time (Singh and Kushwaha 2006).

### *2.3.5. Phenological variables*

In order to characterise, quantify and compare the phenology, five phenological variables were estimated for each phenophase in each population and year: (1) start date; (2) end date; (3) duration (difference between start and end date); (4) peak date; and (5) synchrony. The measurement of synchrony was calculated as a standard deviation around its mean

value (SD). A low SD indicates a high level of synchrony (Augspurger 1983). For the purpose of simplification, each date is expressed in numerical values of months, *e.g.* 6.5 for the middle of June.

Additionally, synchrony indices based on the duration of flower bud formation, flowering (considered as open flowers and flower senescence) and fruiting (considered as immature, mature and dehiscent fruit) were quantified at both individual and population levels. The individual index of synchrony (Xi) was calculated using Augspurger´s method (1983), modified from Primack (1980) as follows:

$$Xi = \left(\frac{1}{N-1}\right)\left(\frac{1}{fi}\right)\sum_{j=1}^{n} ej \neq i$$

where, ej is the number of months on which the plants i and j presented flower bud/flower/fruit synchronously (j ≠ i), fi is the number of months during which plant i presented flower bud/flower/fruit, and N is number of plants in the population considered. Xi varies from 1 (perfect synchrony) to 0 (no synchrony). A measure of the overall synchrony of the population (Z) is obtained by averaging the synchrony of all plants.

*2.3.6. Statistical analysis*

As most of the data distributions were not normal (Shapiro-Wilk test), even after data transformations, a two-way ANOVA on data ranks was used to test the effect of population and year on the phenological variables. The relationship between the phenophase start date and its duration, as well as between the phenophase duration and total duration of the reproductive phenology, were analysed with Spearman rank correlations (*r*). The effect of current and past climatic conditions on the development of each phenophase was also tested. Spearman rank correlations were therefore calculated for each phenophase and the climatic variables (mean monthly temperature, maximum temperature, minimum temperature, precipitation, relative humidity and irradiance) as follows: (i) the correlation between the intensity of each phenophase and climatic variables occurring in the same month, (ii) the correlation between each phenological event and climatic variables occurring up to 1 year prior to the beginning of each phenophase, by using the climatic means over

successive 3-month period as described previously. All the statistical analyses were carried out at $P < 0.05$ significant level using a PASW Statistics 18 software package.

## 2.4. Results

### *2.4.1. General phenological pattern*

The phenological diagram for *A. longifolia* is summarised in Fig. 2.1. In general, the formation of flower buds started in June and they gradually increased in size until March, peaking in summer (August). Anthesis began in winter (mainly in January) and finished in early spring (April), with a peak in February. The senescence of flowers started in January and occurred until April, reaching its maximum in late February. Pod development, which became visible in late February, continued for several months and the appearance of mature pods started to be observed in April, when dehiscence began. Pod dehiscence peaked in July and in most plants ended in September. However, its presence was still detected up to January. Vegetative phenology was observed in all months of the year, but was more pronounced in the spring/summer period.

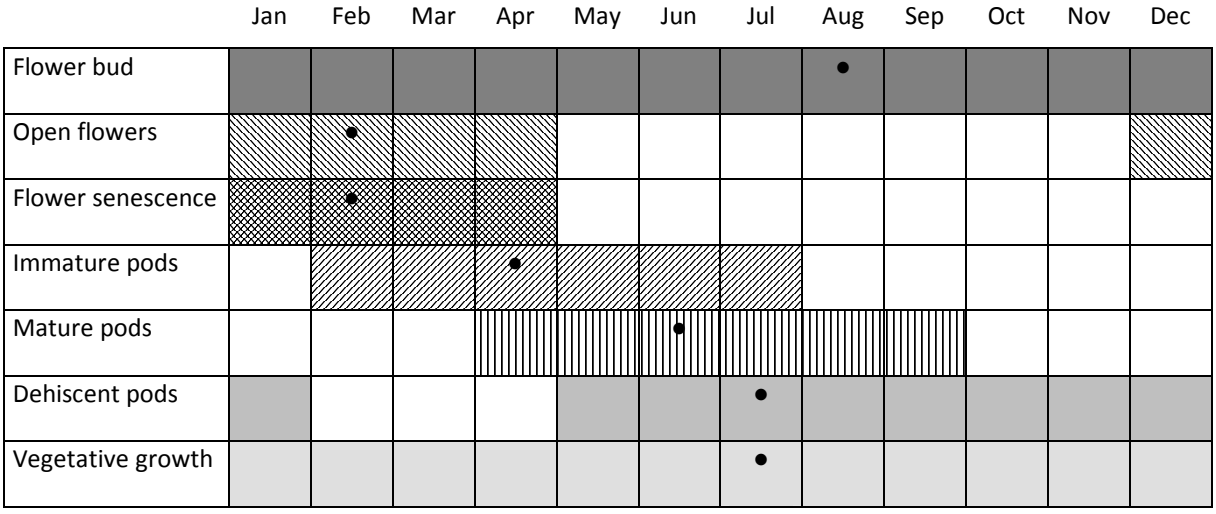


**Fig. 2.1**. Phenological diagram for *Acacia longifolia* during 2008-2011. The symbol ● represents the peak month.

*2.4.2. Variability of the phenological pattern*

The timing and duration of the reproductive phenophases in the different populations over the years revealed a geographic and temporal influence on the phenological pattern (Fig. 2.2 to Fig. 2.5).

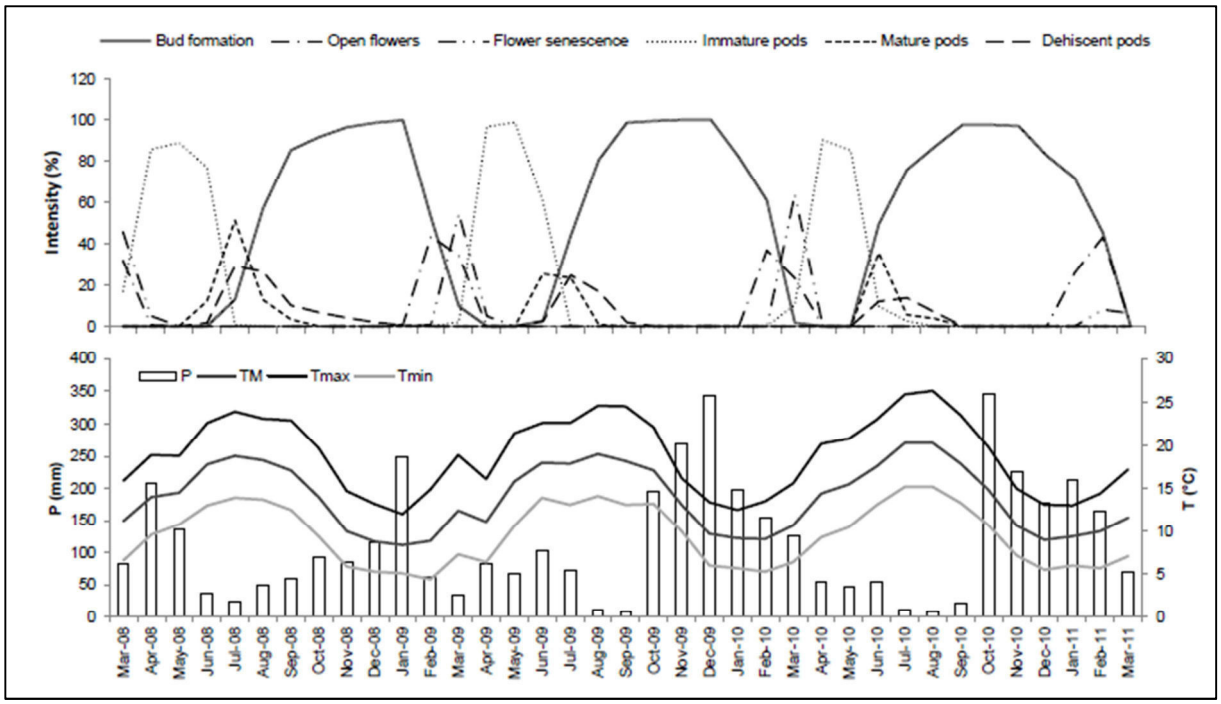


**Fig. 2.2**. Phenological pattern of *Acacia longifolia* (top) and climatic conditions (bottom) observed in the North population during the 3-year observation period (2008-2011). P (mm) is the total monthly precipitation (mm), TM (°C) is the monthly mean temperature, Tmax (°C) is the monthly mean of the daily maximum temperature and Tmin (°C) is the monthly mean of minimum temperature.

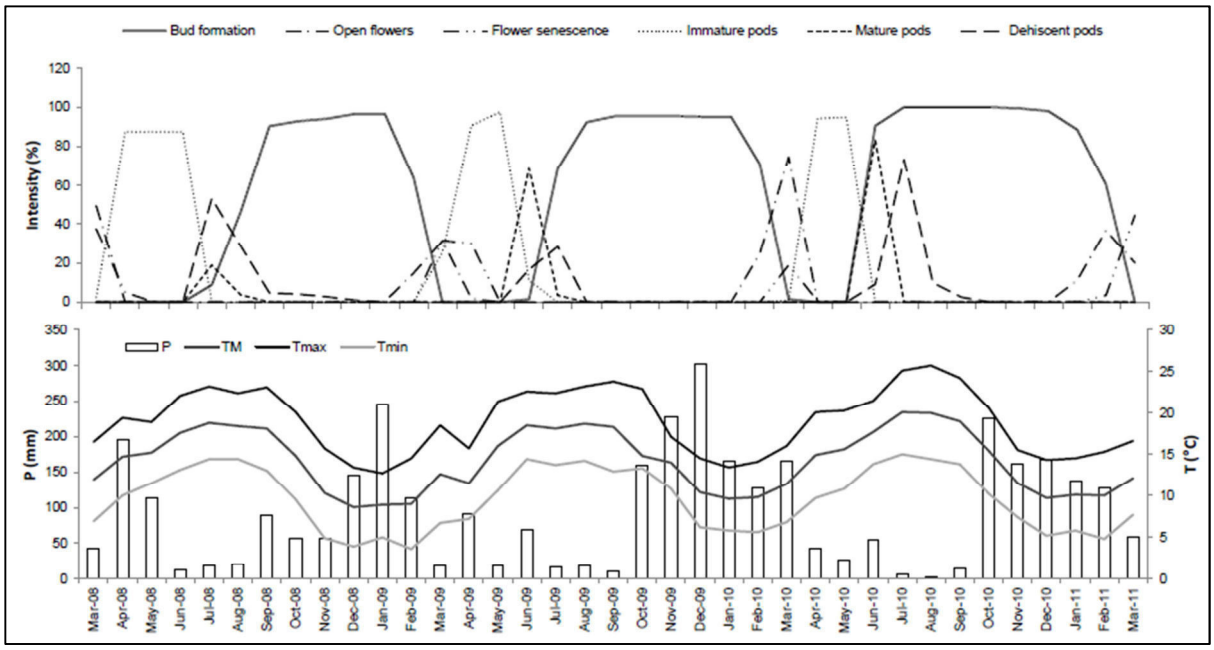


**Fig. 2.3**. Phenological pattern of *Acacia longifolia* (top) and climatic conditions (bottom) observed in the Central population during the 3-year observation period (2008-2011). P (mm) is the total monthly precipitation (mm), TM (°C) is the monthly mean temperature, Tmax (°C) is the monthly mean of the daily maximum temperature and Tmin (°C) is the monthly mean of minimum temperature.

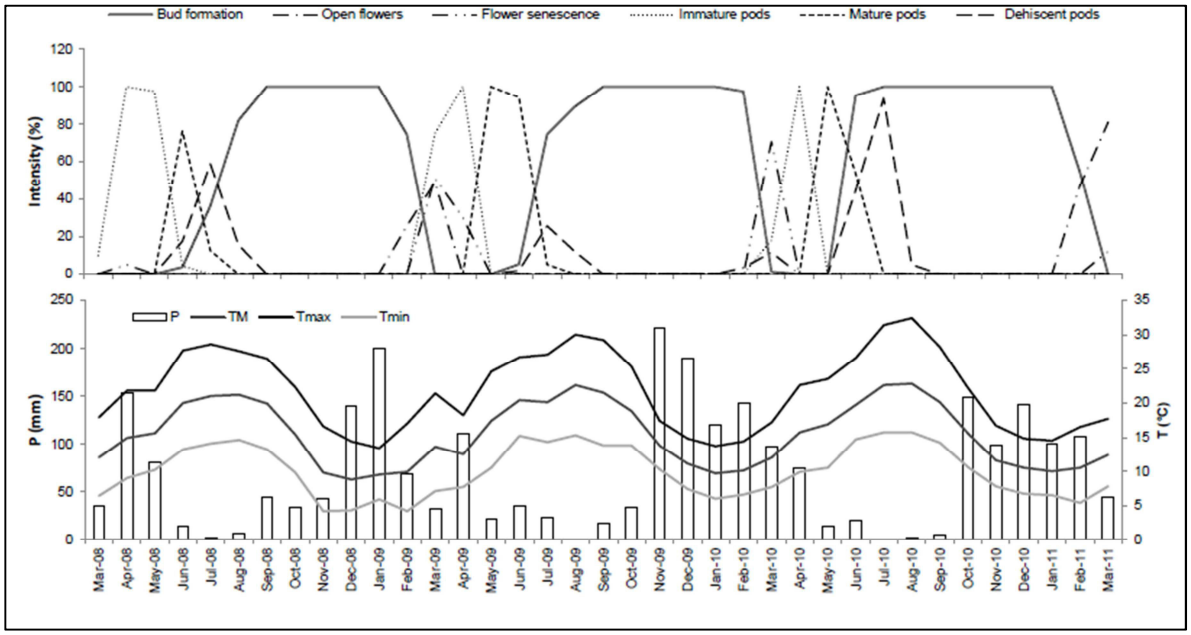


**Fig. 2.4**. Phenological pattern of *Acacia longifolia* (top) and climatic conditions (bottom) observed in the Inland population during the 3-year observation period (2008-2011). P (mm)

is the total monthly precipitation (mm), TM (°C) is the monthly mean temperature, Tmax (°C) is the monthly mean of the daily maximum temperature and Tmin (°C) is the monthly mean of minimum temperature.

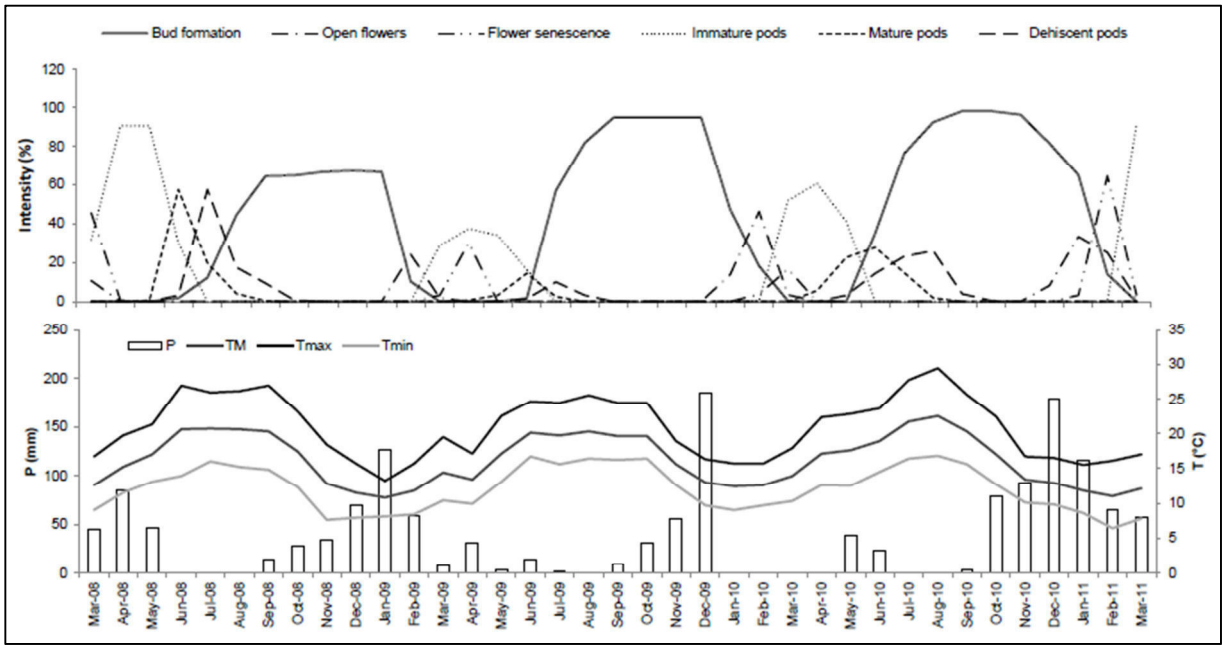


**Fig. 2.5**. Phenological pattern of *Acacia longifolia* (top) and climatic conditions (bottom) observed in the South population during the 3-year observation period (2008-2011). P (mm) is the total monthly precipitation (mm), T (°C) is the monthly mean temperature, Tmax (°C) is the monthly mean of the daily maximum temperature and Tmin (°C) is the monthly mean of minimum temperature.

Taking all the flower bud formation variables (Table 2.1) into account (start date, duration, peak date, end date and index of synchrony), the standard deviation (SD) values indicated a strong variation at plant level, mainly in the peak date (1.26< SD <1.71). On the contrary, the end date seemed to be less variable (0.32< SD <0.57) between plants. Comparing the population index of synchrony (Z), the Inland population presented the highest values (Z = 0.49), indicating a greater synchrony among plants. Our results also showed a significant variation between populations and years ($P$ < 0.001). The Inland population, where plants presented flower buds at the beginning of June, had the longest flower bud phenophase (approximately 9 months), finishing in late January. In contrast, flower bud formation started

later in the South population, but had a shorter duration (7 months), finishing by mid January. In all the populations, this phenophase started earlier in the third year but continued over a long period of time.

**Table 2.1.** Flower bud formation phenological variables of each population. Values are mean ± SD. The results of the two-way ANOVA are at the bottom of the table.

| Population | Year | First date | Duration | End date | Peak date | Index of synchrony (Z) |
|---|---|---|---|---|---|---|
| North | 1 | 7.4 ± 0.63 | 8.2 ± 0.82 | 2.6 ± 0.53 | 10.3 ± 1.18 | 0.28 ± 0.12 |
| | 2 | 6.7 ± 0.44 | 8.0 ± 0.79 | 2.1 ± 0.46 | 8.5 ± 0.88 | 0.17 ± 0.12 |
| | 3 | 6.2 ± 0.48 | 8.9 ± 0.74 | 2.8 ± 0.39 | 7.4 ± 1.52 | 0.28 ± 0.17 |
| | mean | 6.8 ± 0.70 | 8.4 ± 0.86 | 2.5 ± 0.55 | 8.8 ± 1.71 | 0.24 ± 0.15 |
| Central | 1 | 7.5 ± 0.75 | 7.2 ± 0.75 | 2.0 ± 0.00 | 9.3 ± 1.35 | 0.21 ± 0.08 |
| | 2 | 6.8 ± 0.43 | 7.9 ± 0.52 | 2.0 ± 0.49 | 7.7 ± 0.69 | 0.26 ± 0.12 |
| | 3 | 6.0 ± 0.00 | 7.5 ± 0.16 | 1.0 ± 0.17 | 6.1 ± 0.32 | 0.89 ± 0.22 |
| | mean | 6.8 ± 0.80 | 7.5 ± 0.61 | 1.7 ± 0.57 | 7.8 ± 1.61 | 0.44 ± 0.34 |
| Inland | 1 | 6.0 ± 0.00 | 8.7 ± 0.55 | 1.9 ± 0.39 | 8.8 ± 0.41 | 0.37 ± 0.19 |
| | 2 | 6.4 ± 0.51 | 8.5 ± 0.51 | 2.0 ± 0.00 | 8.5 ± 0.51 | 0.48 ± 0.05 |
| | 3 | 6.0 ± 0.00 | 8.8 ± 0.39 | 1.8 ± 0.39 | 6.0 ± 0.22 | 0.63 ± 0.25 |
| | mean | 6.1 ± 0.36 | 8.7 ± 0.50 | 1.9 ± 0.32 | 7.8 ± 1.31 | 0.49 ± 0.21 |
| South | 1 | 7.5 ± 1.11 | 6.5 ± 1.32 | 1.4 ± 0.55 | 9.2 ± 1.14 | 0.12 ± 0.07 |
| | 2 | 7.0 ± 0.97 | 7.0 ± 0.99 | 1.4 ± 0.64 | 8.2 ± 0.13 | 0.17 ± 0.10 |
| | 3 | 6.2 ± 0.71 | 7.6 ± 0.80 | 1.6 ± 0.50 | 7.7 ± 1.05 | 0.19 ± 0.07 |
| | mean | 6.9 ± 1.07 | 7.0 ± 1.14 | 1.5 ± 0.57 | 8.3 ± 1.26 | 0.16 ± 0.08 |
| Two-way ANOVA (*P*-values) | | | | | | |
| Site (S) | | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| Year (Y) | | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| S x Y | | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |

Flowering patterns also varied considerably between populations and years (Table 2.2). On average, the South population started to flower in early January, whereas the other populations only started between late January and early February. In addition, the timing of the open flower peak differed among populations. In the South population, the peak occurred in mid January, whilst in the other populations it was always in mid February. Open

flowers were visible for a short period of time, ranging from 1.0 (Inland population) to 1.6 (North population) months and finishing between early February (South population) and early March (Inland population), when flower senescence started. Flower senescence also occurred over relatively short periods of time (approximately 1 month), finishing in mid March. In total, the length of the flowering period (from the first open flower to the last flower senescence) lasted 1.5 (Inland population) to 2.0 (North population) months. In general, the North population had an average delay in flowering of 1 month in relation to the South population. The distribution of each flowering phenological variables differed over the years ($P < 0.001$); in the third year, for example, the first open flowers appeared early (in December) and peaked early in comparison with the previous years. The phenological variables also differed in terms of synchrony. The flowering duration was less synchronous (SD = 0.47), whilst the first date for open flowers was the most asynchronous among plants (SD = 0.67). Since some of the phenological variables (duration and end date of open flowers and start date of flower senescence) occurred at the same time during the study period, the Inland population showed the highest phenological synchrony (0.00< SD <0.18) whilst the opposite trend was evident in the South population (0.48< SD <0.78). This trend was confirmed by the high figures for the site index of synchrony (Z) which revealed that in the Inland population there was a perfect overlap between plants (Z = 1.0).

The results for fruiting variables (Table 2.3) showed significant differences between populations and years of observation. For example, the development of pods started earlier in the South population (early March) but the fruiting amplitude was higher in comparison with the other three populations (5.5 months). Moreover, the start of this phenophase occurred about one month later in the North population than in the South population. Comparing the 3 years observed, the first pods appeared and finished early in the third year. The fruiting phenophase was also shorter in the third year in comparison with previous years. As a result of these differences, the synchrony was higher in the third year for most of the fruiting variables. Taking all the sites into consideration, the South population displayed a low site synchrony index (Z = 0.18) and the Inland population gave the highest values (Z = 0.37). The first date of immature pod and the total duration of the fruiting phenophase were, respectively, the most (0.23< SD <0.50) and the less (0.69< SD <1.46) synchronous variables.

The duration of each phenophase (flower bud formation, flowering and fruiting) was significantly influenced by the start date of each phenophase. In general, the plants which started early tended to have longer phenophases, as suggested by the negative correlation between the start date and the duration of flower bud formation ($r$ = -0.527, $P$ < 0.001), flowering ($r$ = -0.414, $P$ < 0.001) and fruiting ($r$ = -0.334, $P$ < 0.001). The length of the flower bud formation and fruiting phenophase also had an important effect on the total duration of the reproductive phenology ($r$ = 0.354, $P$ < 0.001 and $r$ = 0.750, $P$ < 0.001, respectively). Conversely, the total duration of the reproductive phenology was not affected by the flowering amplitude ($r$ = 0.031, $P$ > 0.05).

Within the study period, the percentage of individuals with vegetative growth ranged from 22% to 100%, with pattern differing between populations (Fig. 2.6). In the North, 96% of the plants presented vegetative growth during the entire study period with the maximum observed in July and August. In the Central population, the percentage of individuals presenting vegetative growth fell to 78% and the maximum occurred in August. The Inland and the South populations presented similar percentages of individuals with vegetative growth (87.0%), with the maximum values observed in April and July, respectively. The two-way analysis of variance also revealed significant differences between the years ($P$ < 0.001); the second year presented the highest values (90.7%) whereas the first year had the lowest percentage of individuals with vegetative growth (84.3%).

**Table 2.2.** Flowering phenological variables of each population. Values are mean ± SD. The results of the two-way ANOVA are at the bottom of the table.

| Population | Year | First date Open flower | Duration Open flower | End date Open flower | Peak date Open flower | First date Flower senescence | End date Flower senescence | Total duration Flowering | Index of synchrony Flowering(Z) |
|---|---|---|---|---|---|---|---|---|---|
| North | 1 | 2.0 ± 0.36 | 1.4 ± 0.13 | 3.0 ± 0.00 | 2.4 ± 0.49 | 3.0 ± 0.22 | 3.3 ± 0.48 | 2.2 ± 0.27 | 0.49 ± 0.14 |
| | 2 | 2.1 ± 0.34 | 1.7 ± 0.38 | 2.8 ± 0.39 | 2.4 ± 0.49 | 3.0 ± 0.00 | 3.0 ± 0.16 | 1.9 ± 0.34 | 0.72 ± 0.30 |
| | 3 | 1.4 ± 0.49 | 1.4 ± 0.39 | 2.0 ± 0.17 | 2.0 ± 0.00 | 2.5 ± 0.51 | 3.5 ± 0.87 | 1.9 ± 0.54 | 0.26 ± 0.14 |
| | mean | 1.9 ± 0.51 | 1.5 ± 0.35 | 2.6 ± 0.48 | 2.3 ± 0.44 | 2.9 ± 0.35 | 3.3 ± 0.56 | 2.0 ± 0.41 | 0.50 ± 0.28 |
| Central | 1 | 2.4 ± 0.54 | 1.2 ± 0.41 | 2.7 ± 0.65 | 2.5 ± 0.51 | 3.3 ± 0.56 | 3.4 ± 0.54 | 1.9 ± 0.41 | 0.51 ± 0.28 |
| | 2 | 1.9 ± 0.41 | 1.9 ± 0.38 | 3.0 ± 0.16 | 2.8 ± 0.37 | 3.0 ± 0.24 | 3.0 ± 0.24 | 2.0 ± 0.41 | 0.33 ± 0.15 |
| | 3 | 1.5 ± 0.51 | 1.8 ± 0.51 | 2.8 ± 0.41 | 2.1 ± 0.38 | 2.9 ± 0.33 | 2.9 ± 0.33 | 1.9 ± 0.50 | 0.40 ± 0.15 |
| | mean | 1.9 ± 0.62 | 1.6 ± 0.54 | 2.8 ± 0.47 | 2.5 ± 0.52 | 3.1 ± 0.44 | 3.1 ± 0.45 | 1.9 ± 0.44 | 0.41 ± 0.22 |
| Inland | 1 | 2.0 ± 0.00 | 1.0 ± 0.00 | 3.0 ± 0.00 | 2.3 ± 0.49 | 3.0 ± 0.00 | 3.0 ± 0.00 | 1.5 ± 0.00 | 1.00 ± 0.00 |
| | 2 | 2.0 ± 0.00 | 1.0 ± 0.00 | 3.0 ± 0.00 | 3.0 ± 0.00 | 3.0 ± 0.00 | 3.0 ± 0.00 | 1.5 ± 0.00 | 1.00 ± 0.00 |
| | 3 | 2.1 ± 0.32 | 1.0 ± 0.00 | 3.0 ± 0.00 | 2.7 ± 0.46 | 3.0 ± 0.00 | 3.0 ± 0.00 | 1.5 ± 0.00 | 1.00 ± 0.00 |
| | mean | 2.0 ± 0.18 | 1.0 ± 0.00 | 3.0 ± 0.00 | 2.7 ± 0.47 | 3.0 ± 0.00 | 3.0 ± 0.00 | 1.5 ± 0.00 | 1.00 ± 0.00 |
| South | 1 | 2.0 ± 0.00 | 1.0 ± 0.22 | 2.1 ± 0.27 | 2.0 ± 0.00 | 2.2 ± 0.43 | 2.5 ± 0.51 | 1.3 ± 0.29 | 0.44 ± 0.09 |
| | 2 | 1.3 ± 0.52 | 1.0 ± 0.41 | 2.2 ± 0.37 | 2.0 ± 0.18 | 2.4 ± 0.49 | 2.4 ± 0.50 | 1.7 ± 0.52 | 0.28 ± 0.13 |
| | 3 | 0.6 ± 0.69 | 1.6 ± 0.47 | 1.8 ± 0.53 | 1.2 ± 0.40 | 1.9 ± 0.43 | 2.2 ± 0.43 | 2.3 ± 0.36 | 0.37 ± 0.12 |
| | mean | 1.2 ± 0.78 | 1.2 ± 0.48 | 2.0 ± 0.45 | 1.7 ± 0.48 | 2.2 ± 0.49 | 2.4 ± 0.48 | 1.8 ± 0.58 | 0.36 ± 0.13 |
| Two-way ANOVA (*P*-values) | | | | | | | | | |
| Site (S) | | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| Year (Y) | | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| S x Y | | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |

**Table 2.3.** Fruiting phenological variables of each population. Values are mean ± SD. The results of the two-way ANOVA are at the bottom of the table.

| Population | Year | First date Immature pod | First date Mature pod | Duration Mature pod | End date Mature pod | Peak date Mature pod | First date Dehiscent pod | End date Dehiscent pod | Total duration Fruiting | Index of synchrony Fruiting(Z) |
|---|---|---|---|---|---|---|---|---|---|---|
| North | 1 | 3.4 ± 0.50 | 6.8 ± 0.42 | 1.1 ± 0.81 | 7.3 ± 0.89 | 6.8 ± 0.38 | 7.2 ± 0.71 | 9.1 ± 1.86 | 6.3 ± 1.80 | 0.09 ± 0.05 |
| | 2 | 3.8 ± 0.40 | 6.5 ± 0.50 | 1.3 ± 0.59 | 6.7 ± 0.62 | 6.5 ± 0.51 | 7.0 ± 0.49 | 8.0 ± 0.70 | 5.1 ± 0.55 | 0.48 ± 0.26 |
| | 3 | 3.5 ± 0.51 | 6.0 ± 0.00 | 1.1 ± 0.40 | 6.6 ± 0.66 | 6.0 ± 0.00 | 6.5 ± 0.57 | 7.0 ± 1.00 | 4.5 ± 0.83 | 0.22 ± 0.07 |
| | mean | 3.6 ± 0.50 | 6.4 ± 0.50 | 1.2 ± 0.63 | 6.9 ± 0.82 | 6.4 ± 0.50 | 6.9 ± 0.67 | 8.1 ± 1.59 | 5.4 ± 1.46 | 0.25 ± 0.22 |
| Central | 1 | 3.9 ± 0.32 | 6.6 ± 0.50 | 0.8 ± 0.32 | 6.7 ± 0.58 | 6.6 ± 0.49 | 7.0 ± 0.00 | 8.4 ± 1.68 | 5.3 ± 1.47 | 0.18 ± 0.09 |
| | 2 | 3.4 ± 0.59 | 6.0 ± 0.23 | 1.5 ± 0.24 | 7.0 ± 0.40 | 6.5 ± 0.55 | 6.6 ± 0.49 | 7.8 ± 0.41 | 4.9 ± 0.71 | 0.17 ± 0.06 |
| | 3 | 3.9 ± 0.28 | 6.0 ± 0.16 | 0.8 ± 0.28 | 6.0 ± 0.00 | 6.0 ± 0.16 | 6.0 ± 0.16 | 7.4 ± 0.86 | 4.0 ± 0.88 | 0.30 ± 0.18 |
| | mean | 3.7 ± 0.49 | 6.2 ± 0.42 | 1.1 ± 0.43 | 6.6 ± 0.58 | 6.3 ± 0.51 | 6.5 ± 0.50 | 7.8 ± 1.14 | 4.7 ± 1.16 | 0.22 ± 0.13 |
| Inland | 1 | 3.0 ± 0.00 | 5.0 ± 0.22 | 1.0 ± 0.23 | 6.0 ± 0.22 | 6.0 ± 0.00 | 6.0 ± 0.40 | 7.8 ± 0.42 | 4.9 ± 0.59 | 0.33 ± 0.16 |
| | 2 | 3.0 ± 0.00 | 5.0 ± 0.00 | 2.4 ± 0.11 | 6.9 ± 0.22 | 5.0 ± 0.00 | 7.0 ± 0.48 | 7.7 ± 0.48 | 5.7 ± 0.48 | 0.53 ± 0.17 |
| | 3 | 3.3 ± 0.49 | 5.0 ± 0.00 | 1.6 ± 0.18 | 7.0 ± 0.00 | 5.0 ± 0.00 | 6.0 ± 0.00 | 8.0 ± 0.00 | 4.9 ± 0.70 | 0.28 ± 0.14 |
| | mean | 3.1 ± 0.32 | 5.0 ± 0.13 | 1.7 ± 0.62 | 6.7 ± 0.47 | 5.3 ± 0.47 | 6.3 ± 0.58 | 7.8 ± 0.38 | 5.1 ± 0.69 | 0.37 ± 0.19 |
| South | 1 | 3.0 ± 0.00 | 5.5 ± 0.51 | 1.3 ± 0.69 | 6.7 ± 0.74 | 6.1 ± 0.27 | 6.4 ± 0.55 | 7.9 ± 1.07 | 5.1 ± 1.21 | 0.18 ± 0.12 |
| | 2 | 3.0 ± 0.21 | 5.9 ± 0.88 | 1.1 ± 0.35 | 6.2 ± 0.77 | 6.0 ± 0.84 | 6.6 ± 0.65 | 7.4 ± 0.96 | 5.3 ± 1.09 | 0.27 ± 0.17 |
| | 3 | 2.9 ± 0.35 | 5.3 ± 0.64 | 1.7 ± 0.84 | 6.1 ± 1.10 | 5.3 ± 0.64 | 6.4 ± 0.75 | 7.9 ± 1.32 | 6.0 ± 1.45 | 0.16 ± 0.48 |
| | mean | 3.0 ± 0.23 | 5.5 ± 0.67 | 1.4 ± 0.73 | 6.4 ± 0.93 | 5.8 ± 0.66 | 6.4 ± 0.65 | 7.8 ± 1.17 | 5.5 ± 1.34 | 0.18 ± 0.11 |
| Two-way ANOVA (*P*-values) | | | | | | | | | | |
| Site (S) | | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.424 | <0.001 | <0.001 |
| Year (Y) | | 0.022 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.006 | <0.001 | <0.001 |
| S x Y | | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |

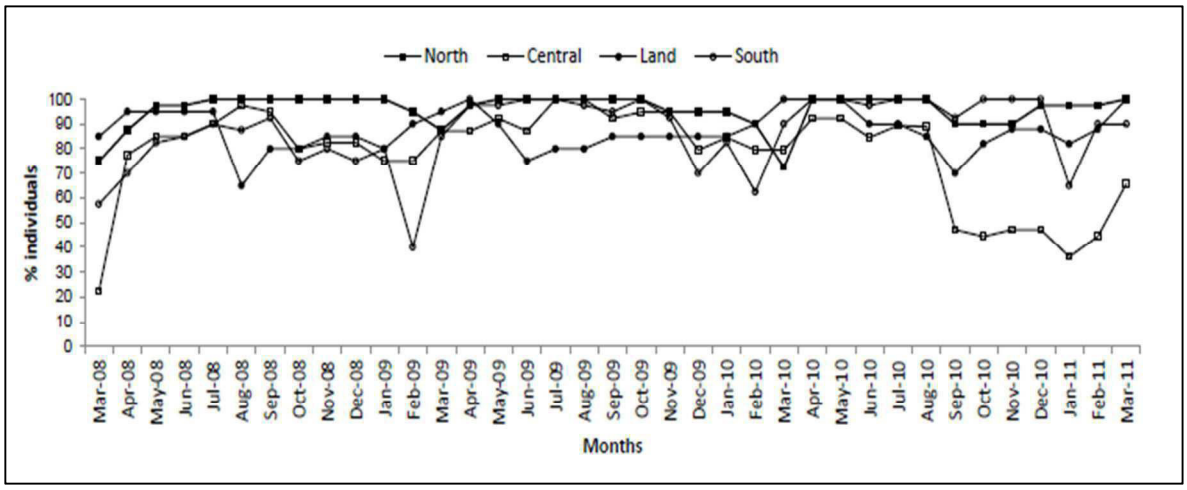


**Fig. 2.6**. Percentage of individuals with vegetative growth observed during 2008-2011 in each population.

*2.4.3. Influence of climate on phenology*

The phenological pattern observed in each site during the study period was influenced by climatic conditions (Fig. 2.2 to 2.5). In fact, a number of significant correlations were found between the intensity of each phenophase in a given month and climatic variables observed in the same month and in previous months (Table 2.5). Most of the flower bud development occurred during the cold season, which was accompanied by low temperatures and increasing precipitation. As a consequence, the minimum temperature and precipitation in the previous cold season (7 to 9 months earlier) were important in determining flower bud intensity. The increase in air temperature, mainly the maximum temperature, in the previous season (4 to 6 months earlier) may have been responsible for the high intensity of flowering. Since peak flowering tended to occur in mid February, this suggested that maximum temperature in the previous August to October was a key factor in the development of flowering. Depending on the fruiting pattern, three relationships with climatic variables were clear. First, the beginning and development of pods was greatly influenced by high temperatures in the previous summer (7-9 months earlier). Secondly, the intensive development of mature pods depended on an increase in water availability and lower minimum temperatures in the 3 months prior this phenophase (1-3 months earlier). Finally, the end of this phenophase was clearly determined by maximum temperature and irradiance observed in the previous winter (4-6 months earlier). On the other hand, the

occurrence of vegetative growth was associated more with lower temperatures and irradiance observed in the 4-6 months prior to this phenophase.

**Table 2.4.** Summary of the average climatic data observed at study populations for the years 2008-2011.

| Variable | North[a] | Central[b] | Inland[c] | South[d] |
|---|---|---|---|---|
| Mean annual temperature (ºC) | 13.9 | 14.5 | 15.4 | 16.2 |
| Mean maximum temperature (ºC) | 18.8 | 19.0 | 21.7 | 20.9 |
| Mean minimum temperature (ºC) | 9.6 | 9.6 | 10.0 | 12.1 |
| Mean maximum temperature for the hottest month (ºC) | 37.5 | 37.4 | 40.3 | 37.5 |
| Mean minimum temperature for the coldest month (ºC) | -2.7 | -6.2 | -2.5 | -7.6 |
| Relative humidity (%) | 79.5 | 84.2 | 76.0 | 76.5 |
| Irradiance (h) | 188.5 | 211.8 | 192.6 | 251.9 |
| Cumulative precipitation (mm) | 4266.5 | 3528.8 | 2624.1 | 1491.3 |

[a]Weather station of Viana do Castelo, [b]Weather station of Maceda Air Base, [c]Weather station of Sines Thermoelectric Power Station, [d]Weather station of Agrarian High School of Coimbra.

**Table 2.5.** Spearman rank correlation coefficients (*r*) between phenophases and climatic conditions.

| Phenophase | Months | TM (ºC) | Tmax (ºC) | Tmin (ºC) | Tmaxa (ºC) | Tmina (ºC) | H (%) | I (h) | P (mm) |
|---|---|---|---|---|---|---|---|---|---|
| Flower bud | 0 | -0.079$^{n.s.}$ | -0.049$^{n.s.}$ | -0.096n.s. | -0.032$^{n.s.}$ | -0.180$^{n.s.}$ | 0.094$^{n.s.}$ | -0.260** | 0.214* |
| | 1-3 | 0.414*** | 0.470*** | 0.303** | 0.400*** | 0.198* | -0.305** | 0.264** | -0.355*** |
| | 4-6 | 0.126$^{n.s.}$ | 0.187$^{n.s.}$ | 0.006$^{n.s.}$. | 0.266** | -0.048$^{n.s.}$ | -0.307** | 0.246* | -0.201* |
| | 7-9 | 0.476*** | -0.451*** | -0.470*** | -0.382*** | -0.562*** | 0.076$^{n.s.}$ | -0.428*** | 0.466*** |
| | 10-12 | -0.384*** | -0.326** | -0.380*** | -0.411*** | -0.453*** | 0.273** | -0.588*** | 0.351*** |
| | 0 | 0.068$^{n.s.}$ | 0.104$^{n.s.}$ | -0.072$^{n.s.}$ | 0.019$^{n.s.}$ | 0.021$^{n.s.}$ | -0.090$^{n.s.}$ | -0.030$^{n.s.}$ | -0.191$^{n.s.}$ |
| | 1-3 | 0.136$^{n.s.}$ | 0.260$^{n.s.}$ | 0.001$^{n.s.}$ | 0.145$^{n.s.}$ | 0.218$^{n.s.}$ | -0.002$^{n.s.}$ | -0.021$^{n.s.}$ | -0.111$^{n.s.}$ |
| | 4-6 | 0.258$^{n.s.}$ | 0.347* | 0.189$^{n.s.}$ | 0.396* | 0.280$^{n.s.}$ | -0.377* | 0.208$^{n.s.}$ | -0.286$^{n.s.}$ |
| | 7-9 | 0.130$^{n.s.}$ | 0.191$^{n.s.}$ | -0.006$^{n.s.}$ | 0.248$^{n.s.}$ | 0.016$^{n.s.}$ | -0.248$^{n.s.}$ | 0.099$^{n.s.}$ | -0.194$^{n.s.}$ |
| | 10-12 | -0.012$^{n.s.}$ | 0.047$^{n.s.}$ | -0.086$^{n.s.}$ | 0.107$^{n.s.}$ | 0.034$^{n.s.}$ | -0.225$^{n.s.}$ | -0.105$^{n.s.}$ | -0.128$^{n.s.}$ |
| Flower senescence | 0 | -0.119$^{n.s.}$ | -0.153$^{n.s.}$ | -0.116$^{n.s.}$ | -0.189$^{n.s.}$ | -0.269$^{n.s.}$ | -0.190$^{n.s.}$ | -0.029$^{n.s.}$ | -0.118$^{n.s.}$ |
| | 1-3 | 0.242$^{n.s.}$ | 0.239$^{n.s.}$ | 0.105$^{n.s.}$ | 0.101$^{n.s.}$ | 0.120$^{n.s.}$ | -0.077$^{n.s.}$ | 0.086$^{n.s.}$ | -0.154$^{n.s.}$ |
| | 4-6 | 0.245$^{n.s.}$ | 0.298$^{n.s.}$ | 0.194$^{n.s.}$ | 0.357$^{n.s.}$ | 0.179$^{n.s.}$ | -0.387* | 0.343$^{n.s.}$ | -0.400* |
| | 7-9 | 0.078$^{n.s.}$ | 0.028$^{n.s.}$ | 0.113$^{n.s.}$ | 0.074$^{n.s.}$ | -0.019$^{n.s.}$ | -0.143$^{n.s.}$ | 0.149$^{n.s.}$ | 0.068$^{n.s.}$ |
| | 10-12 | -0.065$^{n.s.}$ | -0.156$^{n.s.}$ | -0.034$^{n.s.}$ | -0.248$^{n.s.}$ | -0.115$^{n.s.}$ | -0.170$^{n.s.}$ | -0.017$^{n.s.}$ | -0.098$^{n.s.}$ |
| Immature pods | 0 | 0.120$^{n.s.}$ | 0.116$^{n.s.}$ | 0.090$^{n.s.}$ | 0.280$^{n.s.}$ | 0.053$^{n.s.}$ | 0.103$^{n.s.}$ | 0.085$^{n.s.}$ | 0.204$^{n.s.}$ |
| | 1-3 | -0.141$^{n.s.}$ | -0.110$^{n.s.}$ | -0.144$^{n.s.}$ | 0.058$^{n.s.}$ | -0.151$^{n.s.}$ | 0.188$^{n.s.}$ | -0.079$^{n.s.}$ | 0.107$^{n.s.}$ |
| | 4-6 | -0.005$^{n.s.}$ | 0.082$^{n.s.}$ | -0.085$^{n.s.}$ | 0.131$^{n.s.}$ | -0.074$^{n.s.}$ | 0.208$^{n.s.}$ | -0.154$^{n.s.}$ | 0.067$^{n.s.}$ |
| | 7-9 | 0.478** | 0.458** | 0.398** | 0.423** | 0.374* | -0.156$^{n.s.}$ | 0.219$^{n.s.}$ | -0.412** |
| | 10-12 | 0.132$^{n.s.}$ | 0.204$^{n.s.}$ | 0.110$^{n.s.}$ | 0.322* | 0.064$^{n.s.}$ | 0.085$^{n.s.}$ | 0.084$^{n.s.}$ | 0.085$^{n.s.}$ |

**Table 2.5.** Spearman rank correlation coefficients (*r*) between phenophases and climatic conditions (cont.).

| Phenophase | Months | TM (ºC) | Tmax (ºC) | Tmin (ºC) | Tmaxa (ºC) | Tmina (ºC) | H (%) | I (h) | P (mm) |
|---|---|---|---|---|---|---|---|---|---|
| Mature pods | 0 | -0.113$^{n.s.}$ | -0.117$^{n.s.}$ | -0.179$^{n.s.}$ | 0.112$^{n.s.}$ | -0.184$^{n.s.}$ | -0.057$^{n.s.}$ | -0.091$^{n.s.}$ | 0.406* |
| | 1-3 | -0.304$^{n.s.}$ | -0.199$^{n.s.}$ | -0.392* | -0.001$^{n.s.}$ | -0.415* | -0.079$^{n.s.}$ | -0.125$^{n.s.}$ | 0.177$^{n.s.}$ |
| | 4-6 | -0.157$^{n.s.}$ | -0.184$^{n.s.}$ | -0.109$^{n.s.}$ | -0.191$^{n.s.}$ | -0.178$^{n.s.}$ | 0.313$^{n.s.}$ | -0.272$^{n.s.}$ | 0.2307$^{n.s.}$ |
| | 7-9 | 0.202$^{n.s.}$ | 0.241$^{n.s.}$ | 0.177$^{n.s.}$ | 0.409* | 0.145$^{n.s.}$ | -0.208$^{n.s.}$ | 0.151$^{n.s.}$ | 0.022$^{n.s.}$ |
| | 10-12 | 0.057$^{n.s.}$ | -0.110$^{n.s.}$ | 0.037$^{n.s.}$ | 0.246$^{n.s.}$ | 0.000$^{n.s.}$ | -0.056$^{n.s.}$ | 0.039$^{n.s.}$ | 0.083$^{n.s.}$ |
| Dehiscent pods | 0 | 0.491*** | 0.457** | 0.452** | 0.292* | 0.370** | -0.076$^{n.s.}$ | 0.431** | -0.408** |
| | 1-3 | -0.068$^{n.s.}$ | 0.043$^{n.s.}$ | -0.134$^{n.s.}$ | 0.052$^{n.s.}$ | -0.163$^{n.s.}$ | -0.087$^{n.s.}$ | 0.009$^{n.s.}$ | 0.076$^{n.s.}$ |
| | 4-6 | -0.538*** | -0.511*** | -0.538*** | -0.492*** | -0.546*** | 0.210$^{n.s.}$ | 0.561*** | 0.288* |
| | 7-9 | -0.160$^{n.s.}$ | -0.001$^{n.s.}$ | -0.211$^{n.s.}$ | -0.046$^{n.s.}$ | -0.155$^{n.s.}$ | 0.030$^{n.s.}$ | -0.306* | -0.054$^{n.s.}$ |
| | 10-12 | 0.519*** | 0.577*** | 0.393** | 0.432** | 0.354* | -0.201$^{n.s.}$ | 0.407** | -0.508*** |
| Vegetative growth | 0 | 0.243** | 0.259** | 0.274** | 0.292*** | 0.315*** | -0.209* | 0.271** | -0.144$^{n.s.}$ |
| | 1-3 | 0.001$^{n.s.}$ | -0.008$^{n.s.}$ | 0.042$^{n.s.}$ | 0.047$^{n.s.}$ | 0.093$^{n.s.}$ | -0.154$^{n.s.}$ | 0.128$^{n.s.}$ | -0.040$^{n.s.}$ |
| | 4-6 | -0.350*** | -0.3936*** | -0.274** | -0.348*** | -0.283*** | 0.128$^{n.s.}$ | -0.320*** | 0.315*** |
| | 7-9 | -0.059$^{n.s.}$ | -0.058$^{n.s.}$ | -0.046$^{n.s.}$ | -0.112$^{n.s.}$ | -0.045$^{n.s.}$ | -0.102n.s. | -0.202* | 0.021$^{n.s.}$ |
| | 10-12 | 0.250** | 0.253** | 0.284*** | 0.243** | 0.296*** | -0.155$^{n.s.}$ | 0.209* | -0.113$^{n.s.}$ |

Notes: TM, monthly mean temperature; Tmax, monthly mean maximum temperature; Tmin, monthly mean minimum temperature; Tmaxa, monthly highest maximum temperature; Tmina, monthly lowest minimum temperature; H, relative humidity; I, irradiance; P, monthly precipitation.

$^{n.s.}$ $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

## 2.5. Discussion

This study was conducted in four populations over three years, and it represents the first attempt to document spatial and temporal variation in *A. longifolia* phenology in Portugal. The results indicate that the phenological pattern of *A. longifolia* varies across populations and years but, in general, this species displays a sequential development of phenophases, with phenological activity extending throughout the year. In all populations, the reproductive phenophases occurred in the following sequence: summer to winter: appearance and growth of flower buds in axils of phyllodes; winter to early spring: flowering; spring to summer: fruiting. Conversely, vegetative growth occurred throughout the year, peaking during the summer period. The development of flower buds generally started in the previous summer (June) and continued for approximately 7 months before bud burst, in winter (February). Pod formation occurred in early spring (March) and the first mature pods appeared early, in April, tending to coincide with the start of seed dispersal, which could last from 0.5 to 5.5 months. On average, the reproductive phenophase lasted 14 months and was significantly influenced by the length of the fruiting phenophase. On the whole, this phenological sequence is quite similar to the one observed for the same species in the S. W. Cape, South Africa (Milton and Moll 1982) and Australia (Lindsay and French 2005).

Among the potentially important traits observed in invasive species, early flowering or long blooming is a recurrent property associated with plant invasiveness (Pyšek and Richardson 2007). Previous studies in different parts of the Mediterranean region have shown that successful invaders generally flower early in comparison with native species, whose peak flowering occurs in spring (Godoy *et al*. 2009b). One possible advantage of the early flowering is the reduced competition for both abiotic resources (*e.g.* water and nutrients) and the biotic resources (*e.g.* pollinators) required for flowering (Godoy *et al*. 2009a). In the case of *A. longifolia*, flowering occurred predominantly in winter, which has also been reported for other invasive species (Godoy *et al*. 2009b). This strategy of flowering earlier associated with a high seed bank (Marchante *et al*. 2011), may certainly contribute to its invasive potential in Portuguese ecosystems.

Our results also show that the four selected populations differed significantly in terms of the start, end, and duration of their reproductive phenophases, suggesting a geographical variation in the reproductive phenology. This was particularly evident when comparing the

flowering and fruiting phenophases in the South and the North populations. On average, plants started and ended flowering about 1 month earlier in the South population (from early January to early February) than in the North population (late January to late February). Moreover, the South population had a shorter flowering period, while the North population extended its blooming (1.8 months and 2.0 months in the South and in the North population, respectively). This trend suggests that extended blooming is probably restricted by limited precipitation (Bustamante and Búrquez 2008). In relation to the fruiting phenophase, the first date for immature, mature and dehiscent pods also occurred early in the South population compared to the North population. In this case, the differences ranged from 0.5 months (the first date for dehiscent pods) to 1 month (the first date for mature pods). The variability observed in phenological variables in different populations has also been found in other species (Eddy and Judd 2003; Haugaasen and Peres 2005; Bustamante and Búrquez 2008) and this could reflect not only genetic variation but also year-to-year climatic variability (Fenner 1998).

Not all the plants developed at the same speed in each population. In fact, the low plant synchrony observed in each phenological variable (high values of standard deviation around the mean) suggested strong variations on a plant level. This heterogeneity can be attributed to intraspecific differences in plant traits, such as morphology, anatomy or physiology, which affect the phenological pattern (Rathcke and Lacey 1983). Moreover, it illustrates the plasticity of the individuals and this trait can contribute to their success and expansion in ecosystems (Singh and Kushwaha 2006). When comparing the reproductive phenophases (flower bud formation, flowering and fruiting), flowering was the most synchronous phenophase. As pointed out by Buide *et al*. (2002) this is particularly important since greater synchrony of flowering tends to increase plant reproductive success. Reproductive synchrony was also not perfect among populations, as indicated by the diversity patterns found here. The South population displayed the lowest levels of synchrony and the Inland population showed the highest degree of overlap among plants. This low variability observed in the Inland population may be related to its size. In relation to the other populations, the Inland population was smaller and had fewer plants, meaning that they lived in a very homogenous habitat. Therefore, all the plants were probably closely related and the synchrony may reflect genetic similarity (Buide *et al*. 2002). Genetic differences, in turn, are

more likely to be evident in a stressful habitat. In terms of climatic factors, the South population may be considered a more stressful environment than the other sites (*i.e.* less precipitation, extreme temperatures). This result agrees with that of Goulart *et al*. (2005), who also observed greater individual variability in phenology under less favourable conditions.

The recent literature emphasises that changes in phenological events also occur over the years, suggesting that climatic conditions are an important factor in determining within-year patterns of phenology (Ramírez and Briceño 2011). Moreover, the phenophase response of plants may be sensitive to the conditions at the time of the phenophase, or to the conditions in the preceding months (Chapman *et al*. 1999) and the results of this study are consistent with those previous findings. However, climate do not always influence phenology in a similar way or act synergistically and, in some cases, even have a null or opposite effect on reproductive and vegetative phenophases. In Mediterranean ecosystems, climatic factors such as temperature and precipitation (Spano *et al*. 2003; Peñuelas *et al*. 2004) and light availability (Hunter and Lechowicz 1992) have been cited as the main factors that can affect the timing and intensity of phenophases. This corroborates the results of this study in which the close correlations between temperature, irradiance and precipitation and reproductive and vegetative phenology indicated that they are important climatic cues for phenological development of *A. longifolia*.

The minimum temperatures and precipitation for the coldest previous season proved to be the most influential climate parameter for flower bud formation. This result is in agreement with the findings of Sekhwela and Yates (2007) for different *Acacia* species. Our study also showed that warmer autumn temperatures lead to greater flowering. The dependence of flowering on past temperatures is quite well documented in other studies (Eddy and Judd, 2003; Lesica and Kittelson 2010). In the South population, flowering started earlier in relation to the other sites and this could be attributed to the warmer temperatures observed during the monitoring period. Furthermore, the advance in the flowering date in relation to increasing temperatures in the months prior to anthesis is well established in many plant species (Orlandi *et al*. 2010; Lesica and Kittelson 2010). Several studies have also indicated that flowering is mainly influenced by precipitation and irradiance (Rathcke and Lacey 1985; Eddy and Judd 2003) but we did not find any significant relationship to these two climatic

parameters in our study. In relation to the fruiting phenophase, Peñuelas *et al*. (2004) stated that its pattern clearly correlates with variations in temperature and precipitation in the preceding months. However, in *A. longifolia*, these factors only became relevant in terms of the development of mature pods. For dehiscent pods, temperature and irradiance proved to be the most important factors.

In many plants species in the Mediterranean region, vegetative growth usually occurs during the most appropriate time of the year (Milla *et al*. 2010) but this trend was not observed in this study. In fact, vegetative growth in *A. longifolia* occurred all year round with major activity in the spring/summer period, suggesting that the vegetative phenology of this species is more flexible than its reproductive phenology. A similar result has been documented for other *Acacia* species (Milton 1987). Among the climatic factors, temperature seems to be the most relevant factor in triggering vegetative growth. On the contrary, there was a weak relationship between vegetative growth and air humidity and precipitation, which contrasts with results for other *Acacia* species (Sehkwela and Yates 2007).

To summarise, the phenological pattern of *Acacia longifolia* in Portugal generally follows a geographical variation and differed principally in the timing and duration of the phenophases. Significant differences were also found between years, which may be attributed to differences in climatic conditions. Climate, particularly, temperature, precipitation and irradiance, is likely to be the major influence on the reproductive and vegetative phenophases in *A. longifolia*. Considering the predicted climate change scenario for the Mediterranean region, an increase in air temperature and unsteady precipitation could lead to earlier flowering of *A. longifolia* as recorded in the South population and this will have serious implications for the future reproductive success of this species.

The results of this study also represent an important step towards the development of remote sensing and GIS applications for mapping the distribution of this invasive species across large spatial extents.

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**CHAPTER 3**

# ABOVEGROUND BIOMASS ESTIMATES OF THE INVASIVE *ACACIA LONGIFOLIA* IN PORTUGUESE DUNES: CONTRIBUTION FOR ITS MANAGEMENT

Maria C. Morais, Helena Freitas

[Submitted to Weed Research]

## 3. Aboveground biomass estimates of the invasive *Acacia longifolia* in Portuguese dunes: Contribution for its management

### 3.1. Abstract

*Acacia longifolia* (Andrews) Willd., an evergreen shrub or small tree, is one of the most widespread invasive species in the coastal regions of continental Portugal, where it forms dense monospecific stands. Quantifying biomass production and partitioning into wood and foliage is important for developing appropriate management strategies in these areas. However, studies on *A. longifolia* biomass are scarce and there are no estimates for the specific condition of sand dunes. Thus the main purpose of this study was to develop allometric equations for estimating aboveground biomass (W, Kg) of *A. longifolia* at the São Jacinto Dunes Natural Reserve on the central-northern coast of Portugal. A total of 18 plants were randomly selected for measurements, separated by size and destructively sampled and aboveground biomass was assessed by components (wood and foliage). The power function models based on diameter at base, $d_b$ ($W = 0.399 + d_b^{1.492}$, $R^2 = 0.841$, $P < 0.001$, $S_{y,x} = 0.360$) or on combination of $d_b$ and height (h) in the form $d_b^2h$ ($W = 115.230 + d_b^2h^{0.626}$, $R^2 = 0.926$, $P < 0.001$, $S_{y,x} = 0.246$) provide a reliable method for accurately estimate *A. longifolia* aboveground biomass. The results of this study will contribute to implement improved management strategies to control the invasion by *A. longifolia* and could even be applied to other invasive species with analogous biological characteristics that grow in the same ecosystems.

**Keywords**: Invasive species, biomass equations, destructive sampling, power models, dune ecosystems

### 3.2. Introduction

Knowledge of the quantity and distribution of aboveground biomass is important in ecological studies (Flombaum and Sala 2007). Estimating the aboveground biomass has been used for several purposes, including the assessment of the structure and function of ecosystems (Brown *et al*. 1999), the amount and distribution of available resources (Zewdie *et al*. 2009) and the primary productivity of ecosystems (Carvalho 2003; Sağlam *et al*. 2008).

There is evidence that invasive species may have important implications for ecosystem productivity by altering the community composition and ecosystem structure, which may change the aboveground biomass storage and dynamics (Mascaro and Schnitzer 2011). For these reasons, the measurement of biomass offers invaluable information to support management decisions (Northup *et al*. 2005; Sağlam *et al*. 2008; Abdelkader *et al*. 2009).

Generally two methods are considered for estimating biomass: destructive and non-destructive. The conventional destructive method involves complete harvesting of selected plots or individual plants within the plot (Schmer *et al*. 2010) and weighing each component, such as stem or trunk, branches or foliage. Even though considered to be the most precise way to measure and monitor biomass, this method is clearly time-consuming (Flombaum and Sala 2007), costly (Houghton 2005; Flombaum and Sala 2007; Vieira *et al*. 2008) and a laborious process (West 2004; Flombaum and Sala 2007; Vieira *et al*. 2008; Schmer *et al*. 2010). Besides, it may be difficult to undertake in conservation areas (Vieira *et al*. 2008) and it limits the possibilities of temporal studies (Flombaum and Sala 2007). The non-destructive method is based on mathematical (allometric) equations that relate biomass to some easily measured variables in the field (West 2004). This method provides estimates that can be as accurate as those obtained with destructive methods and enables the analysis of more samples, hence encompassing a large number of observations at relatively low cost (Montès *et al*. 2000). Allometric equations are also useful to estimate biomass of species whenever destructive sampling is impractical or limited (Morote *et al*. 2012). In addition, recent interest in these equations has focused on their application to remote sensing (Abdelkader *et al*. 2009) and to estimate carbon pools and fluxes between vegetation and the atmosphere (Chave *et al*. 2005).

Several authors have published relatively simple equations for estimating biomass in shrubs, herbs and trees in different ecosystems around the world (*e.g.* Smith and Brand 1983; Harmand *et al*. 2004; Zianis *et al*. 2005; Abdelkader *et al*. 2007; Sağlam *et al*. 2008; Basuki *et al*. 2009). In Portugal, much of the work on allometric equations has involved the most widespread tree species, such as *Pinus pinaster* (Lopes 2005), *Quercus pyrenaica* (Carvalho 2003), *Eucalyptus globulus* (António *et al*. 2007), *Quercus suber* (Paulo and Tomé 2006) and *Pinus pinea* (Correia *et al*. 2008). Allometric equations have also been developed for some shrub species present in the Montado, such as *Cistus lanadifer*, *Genista hirsuta*, *Helichrysum*

*stoechas*, *Lavandula stoechas* and *L. viridis* (Castro and Freitas 2009). With regard to *Acacia* species, allometric equations have commonly been used to determine biomass production in tropical and arid areas (*e.g.* Milton and Siegfried 1981; Okello *et al*. 2001; Harmand *et al*. 2004; Barichello *et al*. 2005; Vélez and del Valle 2007). However, at present, no allometric equations are available for *Acacia longifolia* (Andrews) Willd. (Sydney golden wattle) in Portugal, in spite of its increasing dominance of many coastal areas. This lack of information complicates the management of invaded stands. The present study fills this gap by focusing on determining the aboveground biomass of *A. longifolia* at the São Jacinto Dunes Natural Reserve, one of the areas in Portugal where this species is largely widespread. The main objective of this study was to estimate the aboveground biomass and partitioning by plant components using the destructive method. A second objective was the development of a practical model for the prediction of *A. longifolia* aboveground, based on easily measurable variables in the field. This study is the first of its kind to examine the aboveground biomass of *A. longifolia*. Our results will be helpful to produce more accurate estimates of aboveground biomass and serve as a vital tool for species use and management of invaded ecosystems in the future.

## 3.3. Material and Methods

### *3.3.1. Study area*

This study was conducted at the São Jacinto Dunes Natural Reserve, which is on the central-northern coast of Portugal (40°39´N, 8°44´W; 10-15 m a.s.l.) and covers an area of 960 ha. The climate is of Mediterranean type with an Atlantic influence. Long-term (1971-2000) data from the University of Aveiro weather station (40°38'N, 8°40'W) indicates an average temperature between 10.2°C (January) and 20.2°C (August) and a mean annual rainfall of 906.7 mm. It also indicates that most rainfall occurs in autumn and winter. The soils are arenosols and the vegetation is characterized by random small trees and more abundant shrubs, subshrubs, and herbs. At the beginning of the 20$^{th}$ century, several *A. longifolia* seedlings were planted in the area for dune stabilisation. In 1995, approximately 1/3 of the vegetation in the Reserve was burned in a natural fire and the *A. longifolia* population increased dramatically. Nowadays, approximately 2/3 of the Reserve is occupied by this

invasive species, which forms continuous closed stands, frequently with a cover of about 80% (Marchante *et al*. 2008).

*3.3.2. Aboveground biomass measurements*

Fieldwork was carried out in the summer of 2009 and autumn of 2010. Morphological measurements and destructive sampling (cutting and weighing) were performed in 18 plants which were randomly selected, encompassing the range of heights and canopy forms observed in the field. Plants were separated into three classes according to height, namely: class I [0-2 m], class II [2-4 m] and class III [>4 m], *i.e.* six plants for each size class. Measurements (to the nearest cm) were taken for each of the selected plants before they were harvested: plant height ($h_t$); 2 crown diameters ($C_d$), taken at right angles to each other across the canopy of the plant; diameter at the base ($d_b$) at 10 cm above ground level, as the diameter of the single largest stem or a sum of individual stem diameters in case of multiple-stem plants, crown depth ($C_l$) and total number of stems ($n_s$). The average, maximum, and minimum values and standard errors for each variable are listed in Table 3.1.

**Table 3.1.** Descriptive statistics of all morphological variables measured. Values are mean ± S.E. (n = 6). Values in parentheses represent the minimum and maximum values.

| Size class | $h_t$ (m) | $d_b$ (cm) | $C_d$ (m) | $C_l$ (m) | $n_s$ |
|---|---|---|---|---|---|
| Class I | 1.67 ± 0.07 | 8.65 ± 1.77 | 2.86 ± 0.47 | 1.60 ± 0.10 | 2.5 ± 1.15 |
| | (1.55-2.00) | (3.48-13.36) | (1.80-4.90) | (1.20-1.68) | (1-8) |
| Class II | 2.41 ± 0.10 | 9.40 ± 1.31 | 3.04 ± 0.22 | 1.94 ± 0.15 | 3.0 ± 1.44 |
| | (2.10-2.76) | (4.37-14.05) | (2.35-3.75) | (1.60-2.60) | (1-10) |
| Class III | 5.40 ± 0.54 | 14.07 ± 3.21 | 2.22 ± 0.13 | 3.02 ± 0.18 | 2.8 ± 1.14 |
| | (4.20-7.45) | (5.43-25.00) | (1.7-2.50) | (2.30-3.45) | (1-8) |

$h_t$, plant height; $d_b$, diameter at the base; $C_d$, crown diameter; $C_l$, crown depth; $n_s$, number of stems

Some authors (Ludwig *et al*. 1975; Abdelkader *et al*. 2007) consider that canopy dimensions such as canopy area and canopy volume are very good predictors of aboveground biomass. However, in the case of these *Acacia* plants, which appeared either single- or multiple-

stemmed shrubs or small trees and with a less consistent architecture, these dimensions incorporated significant measurement errors. The same problems were found for *Elaeagnus angustifolia* (Zhou *et al*. 2007) and for other *Acacia* species, such as *Acacia aneura* (Harrington 1979). For this reason, canopy area and volume were not considered in this study.

Each randomly selected plant, cut at 10 cm above the ground surface, was manually partitioned into two components - wood and foliage (including leaves, young shoots and immature pods). The fresh weight of wood and foliage for each plant was determined directly in the field. Representative sub-samples of each component per plant were placed in separate bags and taken to the laboratory to oven-dry at 65°C until a constant weight was attained. For each plant, the fresh weight values of each component were multiplied by their absolute dry weight to fresh weight ratio, to obtain the biomass component. The total aboveground biomass per plant (W) was calculated by summing the biomass values for the two components, wood ($W_W$) and foliage ($W_F$). The distribution of W between components was calculated on a percentage basis.

### *3.3.3. Statistical analysis*

#### *3.3.3.1. Regression analysis*

Regression analysis was used to estimate the aboveground biomass based on morphological measurements (independent variables). The $h_t$, $d_b$, $C_d$, $C_l$, $n_s$ and a combination of $d_b$ and $h_t$ (as diameter squared times height, $d_b^2h$) were used as independent variables. Pearson´s correlation coefficients (r) were calculated in order to select the independent variables to be used in the regression analysis. Using these predictors, linear, logarithmic, exponential, quadratic, power and cubic regression models relating W and combinations of the selected variables were fitted using the general expression: $W = f(X, \beta) + \varepsilon$, where W represents the aboveground biomass, X the independent variables and ε the error term.

The goodness-of-fit statistics were based on the coefficient of determination ($R^2$), adjusted coefficient of determination ($R^2_{adj}$), standard errors of the estimate ($S_{y,x}$), *P*-values and the distribution of residuals. When more than one model presented a similarly good fit to the data, the equation with the fewest parameters was chosen as the best model.

*3.3.3.2. Data analysis*

Differences in mean aboveground biomass in the different class sizes were analysed using Student´s test. All statistical analyses were performed using a PASW Statistics 18 software package.

## 3.4. Results

*3.4.1. Aboveground biomass production and partitioning*

Table 3.2 shows the distribution of aboveground biomass ($W_F$, $W_W$ and W) and its components for *Acacia longifolia*, considering the effect of size class, as suggested by the significant differences between size classes ($P < 0.05$). W increased proportional with the increase in plant size. The aboveground biomass increased from 8.9 kg plant$^{-1}$ for the class of small plants (class I) to 11.0 Kg and 26.9 kg plant$^{-1}$ for the medium (class II) and large plants (class III), respectively. This pattern of variation was similar for $W_W$ ($P < 0.05$), whereas $W_F$ did not seem to be dependent on size class ($P > 0.05$).

**Table 3.2.** Distribution of aboveground biomass ($W_F$, $W_W$ and W) by plant components for the three size classes. Values are mean ± S.E. (n = 6).

| Size class | $W_F$ (kg) | $W_W$ (Kg) | W (Kg) |
|---|---|---|---|
| Class I | 2.30 ± 0.60a | 6.65 ± 2.11b | 8.94 ± 2.66b |
| Class II | 3.17 ± 0.64a | 7.82 ± 1.64b | 10.99 ± 2.17ab |
| Class III | 2.17 ± 0.90a | 24.71 ± 6.78a | 26.88 ± 7.62a |

The same letter on each column indicates no significant different at $P < 0.05$.

Allocation of biomass into different components, as a percentage of the total aboveground biomass for each size class, is illustrated in Fig. 3.1. As expected, plants allocated more biomass to wood than to foliage. Comparisons of the size classes revealed that the allocation of $W_F$ and $W_W$ was significantly different between size classes. In fact, in class III the wood constituted 93% of the total aboveground biomass, which was significantly different ($P <$

0.001) from the mean proportion of aboveground biomass allocated to wood in class I and II (approximately 70%).

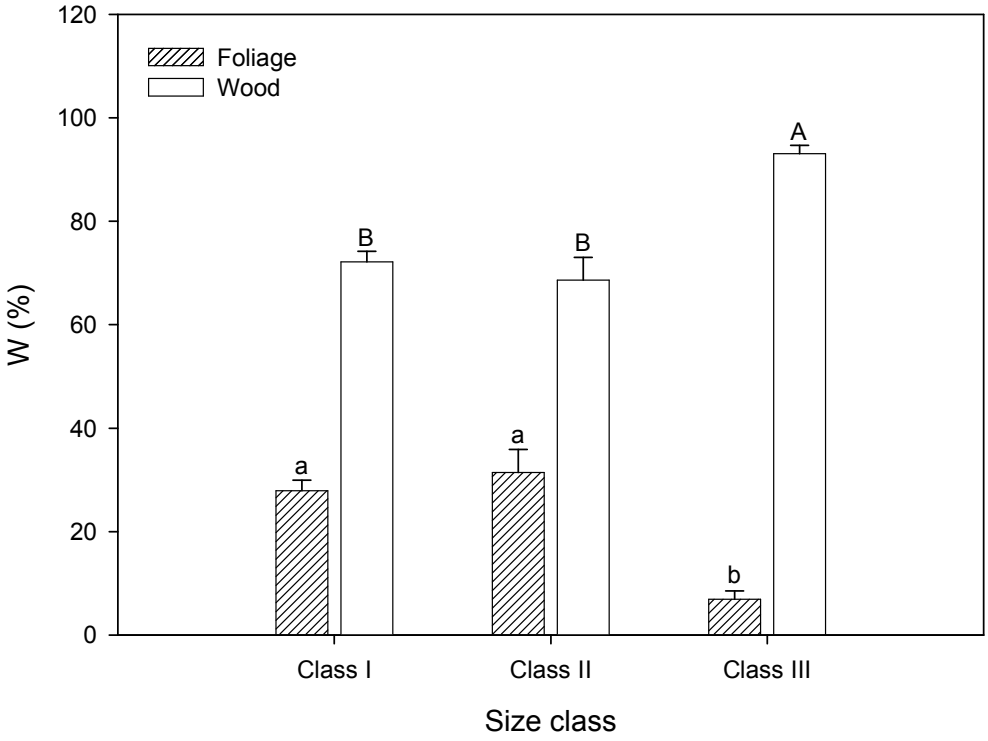


**Fig. 3.1.** Aboveground biomass distribution (W, %) by size classes. Vertical bars are mean ± S.E. (n = 6). For foliage and wood biomass, columns with the same letter are not significantly different at $P < 0.05$.

*3.4.2. Biomass regressions*

Based on the correlation coefficients (Table 3.3), $C_d$ showed the weakest relationship with W ($r = 0.035$, $P > 0.05$) and was not used on the construction of the best regression model. In general, $d_b$ alone or combined with $h_t$ had the highest correlation with W ($r = 0.891$ and $r = 0.950$, $P < 0.001$, respectively). Plant height ($h_t$), crown depth ($C_l$) and number of stems ($n_s$) were also significantly related to aboveground biomass but the relationships were weaker ($r = 0.521$, $r = 0.628$, and $r = 0.607$, respectively).

**Table 3.3.** Pearson´s correlation coefficients (r) between aboveground biomass (W) and morphological variables.

| | W | $h_t$ | $d_b$ | $C_d$ | $C_l$ | $n_s$ | $d^2h$ |
|---|---|---|---|---|---|---|---|
| W | 1 | 0.521* | 0.891*** | $0.035^{n.s.}$ | 0.628** | 0.607** | 0.950*** |
| $h_t$ | | 1 | $0.301^{n.s.}$ | $-0.456^{n.s}$ | 0.825*** | $-0.059^{n.s.}$ | 0.493* |
| $d_b$ | | | 1 | $0.228^{n.s.}$ | 0.497* | 0.677** | 0.884*** |
| $d_c$ | | | | 1 | $-0.220^{n.s.}$ | 0.527* | $-0.117^{n.s.}$ |
| $c_d$ | | | | | 1 | $0.117^{n.s.}$ | 0.643** |
| $n_s$ | | | | | | 1 | 0.488* |
| $d^2h$ | | | | | | | 1 |

W, aboveground biomass; $h_t$, plant height; $d_b$, diameter at the base; $C_d$, crown diameter; $C_l$, crown depth; $n_s$, number of stems; $d_b^2h$, $d_b$ squared x height. $^{n.s.}$ $P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

In the biomass regression models explored, none of the linear, exponential, logarithmic quadratic or cubic models tested provided consistent results for W and therefore only the best-fit equations for estimating W are presented in Fig. 3.2. The power function model with $d_b$, either alone or in combination with $h_t$, produced the highest coefficients of determination and could explain, with a high degree of significance, the various relationships with W ($R^2 > 0.841$). Comparing the two models, the power model with $d_b$, as the sole independent variable ($W = a + d_b^b$), had an associated error of about 15%. The other model, which included both $d_b$ and $h_t$ [$W = a + (d_b^2h)^b$] proved to be the one with the best fit for estimating W by increasing accuracy and reducing the associated error by 5%.

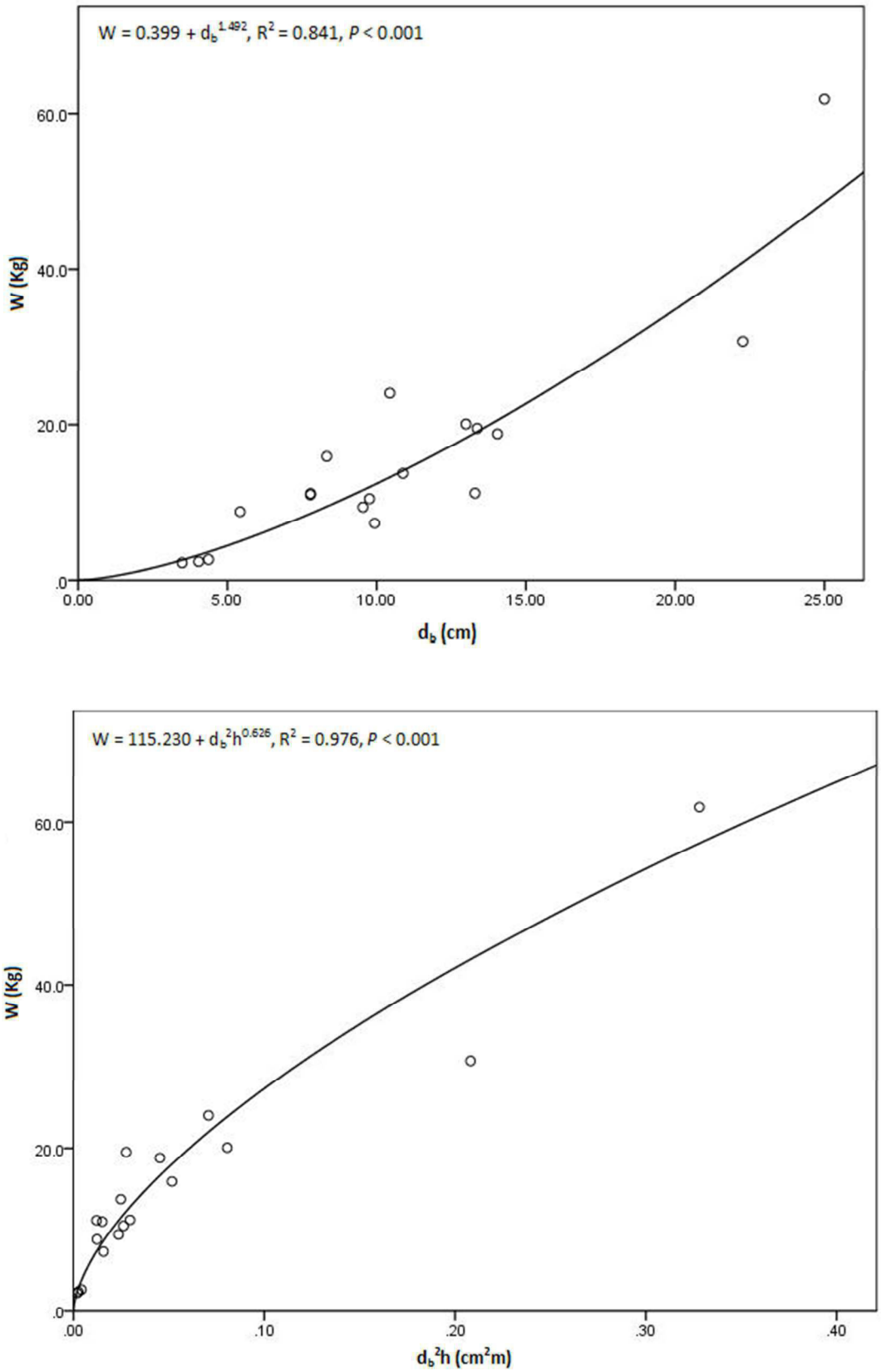


**Fig. 3.2.** The best power function models for estimating aboveground biomass (W, Kg) of *Acacia longifolia* plants based on $d_b$ (top) and $d_b^2h$ (bottom).

### 3.5. Discussion

As expected, aboveground biomass per plant (W) increased in line with the increase of plant size. This increment was closely related to the increase in plant diameter, as suggested by the highest significant correlation obtained between W and diameter at the base ($d_b$). Similar results have been reported for other *Acacia* species (Okello *et al*. 2001).

In this study, we also observed a different pattern of W allocation between the two components considered (foliage and wood) in the different size classes of plants. In general, the percentage contribution of wood increased from the smallest (70%) to the largest size class (93%) while foliage percentage decreased between the smallest (30%) and the largest size class (7%). The same pattern of biomass allocation was also observed in other *Acacia* species, such as *A. cyclops* and *A. saligna* (Milton and Siegfried 1981), *A. polyacantha* (Harmand *et al*. 2004), *A. rigidula* (Northup *et al*. 2005), and *A. mearnsii* (Barichello *et al*. 2005). These differences reflect changes in the crown structure and morphological characteristics of plants (Selaya *et al*. 2007). In this study, the plants from the smallest classes can be described as shrubs (1-4 m), with free space for developing their canopy and branches, which tend to grow towards the horizontal periphery, forming an extended crown that is well exposed to light and faces less competition for water and nutrients (Valladares and Niinemets 2007). The large plants, on the other hand, were small trees, with single or multi-stems. Their tops were usually flattened and the crown had many branches (some dead) and upright twigs, with few phyllodes, spreading within the upright part.

One of the main objectives of this study was to establish a practical and easy model, which could predict, with a high degree of accuracy, the aboveground biomass of *A. longifolia* plants. Based on the coefficients of correlation, the crown diameter ($C_d$) proved unsuitable for estimating W, most likely a result of the different branch heights and crown morphologies of these plants. This lack of a significant relationship between the $c_d$ and biomass was also found by Aleixo *et al*. (2008) for *Leucaena leucocephala*.

Diameter at the base ($d_b$) is a variable easily measured in the field, with high level of accuracy and one of the most commonly used variables to predict biomass (Haase and Haase 1995; Paton *et al*. 1998; Abdelkader *et al*. 2009). In this study, $d_b$ alone and in combination with $h_t$ described most of the variability in W. This is consistent with previous studies that cite $d_b$ and $d_b^2h$ as suitable variables for estimating biomass (Zeng *et al*. 2010).

Normally, most regression equations for estimating biomass are expressed in polynomial, power and combined variable model (Zianis *et al*. 2005). In this study, several regression equations were tested and the best results were found with the power function models. These models had significant parameters ($P < 0.001$) and predicted the aboveground biomass reasonably well, indicated by the small standard error ($S_{y,x} < 0.360$) and high coefficients of determination ($R^2 > 0.84$). The superiority of this model has been observed in other species of shrubs or trees (Smith and Brand 1983; Patón *et al*. 1998; Zeng *et al*. 2010), and in *Acacia* species (Vélez and del Valle 2007).

When both power models were compared, there was an increase in the predictive ability of biomass estimation when $d_b^2h$ was included as the independent variable. In fact, the power model with $d^2h$ presented the highest $R^2$ values and the lowest relative errors ($W = 115.230 + d_b^2h^{0.626}$, $R^2 = 0.926$, $P < 0.001$, $S_{y,x} = 0.246$) in comparison with the models using $d_b$ as independent variable ($W = 0.399 + d_b^{1.492}$, $R^2 = 0.841$, $P < 0.001$, $S_{y,x} = 0.360$). However, the time spent in obtaining reliable height data in the field and a good performance from power model based on $d_b$ only, suggests that it is appropriate and more practical to use models based solely in $d_b$. Patón *et al*. (1998) and Abdelkader *et al*. (2009) also noted useful results in estimating biomass with one variable.

The results of this study represent the first step towards the development of an effective and flexible method for determining the aboveground biomass of *A. longifolia*. Diameter at the base ($d_b$) proved to be the most important independent variable in all biomass equations and the combination with height, in the form $d_b^2h$, slightly improved the equations. The biomass equations presented here provide a useful tool for rapid and accurate estimation of aboveground biomass of *A. longifolia* in the coastal areas invaded by this species. Aboveground biomass estimation and its allocation in the different plant components will be helpful for the development of control strategies to promote the restoration of areas invaded by *A. longifolia*.

Although the specific application of the equations developed in this study is limited to *A. longifolia* plants, the models and procedures presented here have valuable applications for other invasive plants that grow in the same ecosystems, for example, *Acacia saligna*.

Finally, this study should be considered as part of an ongoing work, in which the model will be refined by adding more plants to the available data.

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**CHAPTER 4**

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# THE ACCLIMATION POTENTIAL OF *ACACIA LONGIFOLIA* TO WATER STRESS: IMPLICATIONS FOR INVASIVENESS

Maria Cristina Morais, Helena Freitas

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## 4. The acclimation potential of *Acacia longifolia* to water stress: Implications for invasiveness

### 4.1. Abstract

The ability of an invasive species to establish and spread to new areas may depend on its ability to tolerate a broad range of environmental conditions. Due to climate change, increasing occurrences of extreme events such as droughts are expected in the Mediterranean region and invasive species may expand if they cope with water stress. Limited information is available on the responses of *Acacia longifolia*, one of the most aggressive plant species in Portuguese coastal sand dune ecosystems, to prolonged water stress. In this study, we exposed *A. longifolia* plants from two distinct populations, one from the wet (northern) and another from the dry (southern) climate regions of Portugal, to drought conditions, and monitored morphological, physiological and biochemical responses. One-month-old seedlings were submitted to three different water treatments which involved watering twice a week, every 7 days and every 10 days, respectively, for three months, under controlled conditions. Overall, the progressive drought stress significantly affected most of the growth parameters considered, except the root:shoot ratio. Water stress also increased the uptake of ions ($Ca^{2+}$, $Mg^{2+}$, $K^{+}$ and $Na^{+}$) and N concentration. On the contrary, the C/N ratio decreased under water stress conditions. Isotopic analysis did not reveal significant differences in $\delta^{13}C$ with water treatments but the same pattern was not observed in $\delta^{15}N$ values. Compared with the wet climate population, the dry climate population showed somewhat differing responses to water stress, indicating a genetic difference between populations. These results provide insights into limitations and opportunities for establishment of *A. longifolia* in a drought-prone scenario.

**Keywords**: *Acacia longifolia*, ion content, invasion, plant growth, stable isotopes, water stress

### 4.2. Introduction

Water availability is one the most important abiotic filters controlling plant distribution including the spread of invasive species (Thuiller *et al*. 2005). In the Mediterranean region,

invasive species are exposed to summer water shortages, and the effect of this environmental constraint on their attributes is crucial to the success or failure of invasion (Garcia-Serrano *et al*. 2009). Invasive plant species threaten native species and natural ecosystems by competing directly for resources and by altering ecosystem processes such as nutrient and hydrological cycles, fire frequency and/or intensity (Mack *et al*. 2010). The rate and magnitude of these changes may be aggravated by climate change (Dukes and Mooney 1999). The predicted reduction of precipitation in the coming decades in the Mediterranean region may increase the risk of water shortages (IPCC 2007) and the presence of invasive species may worsen this problem due to their water use ability (Crous *et al*. 2011).

In general, the success of invasive species under various environmental conditions can be linked to their ability to use limited resources more efficiently than native species (Funk and Vitousek 2007). A recent study (Crous *et al*. 2011) showed that when compared to the co-occurring natives, the invasive *Acacia mearnsii* exhibits high resistance to drought induced by xylem cavitation, which provides some advantages during periods of limited water conditions. In addition, it is hypothesised that invasive species have high phenotypic plasticity (Hulme 2008; Davidson *et al*. 2011), enabling them to acclimatise to a wide range of environmental conditions (Droste *et al*. 2010) and to expand into new areas (Mal and Lovett-Doust 2005). For example, Droste *et al*. (2010) demonstrated that the invasive grass *Microstegium vimineum* subjected to water restriction respond through plasticity in several ecophysiological traits (biomass production and SLA) to limit stress and this may be positively associated with its successful invasion. Understanding the factors that contribute to the success of invasive species in variable environments may facilitate the prediction of future invasions, determine the best strategies to control invasive species, and elucidate the impact of invasive species on native communities (Mack *et al*. 2010).

*Acacia longifolia* (Andrews) Willd., an evergreen shrub or small tree, is an invasive species that was introduced in Portugal at the beginning of the 20$^{th}$ century to stabilise dunes and curb sand movement (Marchante *et al*. 2011). This species is invasive in many Portuguese coastal sand dunes areas, but it can also grow along the sides of rivers and roads and on mountains slopes (Marchante *et al*. 2008), reflecting its ability to invade multiple habitats. *Acacia longifolia* is much more abundant in the northern regions of Portugal, which are characterised by higher precipitation and a less intensive drought period, than in the arid

south of the country (Werner *et al*. 2010). This present distribution may be influenced by differences in its ability to cope with water stress, and this ability to respond to various environmental conditions could determine its performance relative to native species and consequently its invasiveness potential.

In this study, we experimentally simulated drought conditions, as is predicted for the Mediterranean region, and assessed how these conditions would affect the growth and performance of *A. longifolia* from two distinct populations. We hypothesized that the responses to drought conditions could be different between the populations from different climate regions. To achieve this, morphological, physiological and biochemical parameters were studied in plants from the two populations, grown under controlled conditions and submitted to three different water treatments. Specifically, we addressed the following questions: (1) Are *A. longifolia* plants affected by different levels of water availability? (2) Do these two populations differ in their response to different levels of water availability? (3) Can these responses affect the invasiveness of *A. longifolia* under drought scenarios? The answers to these questions will provide us with a basis for understanding invasion mechanisms and the potential range expansion of *A. longifolia* in a drought-prone scenario.

## 4.3. Materials and methods

### *4.3.1. Plant material and growth conditions*

Seeds of *Acacia longifolia* (Andrews) Willd. were collected from two different coastal populations in Portugal, taking annual rainfall as an indicator of environmental variability. The two populations were selected from the wet and dry climate regions in Portugal, hereafter referred to as the North and South populations. The northern population (41°31'N, 8°47'W, 0-20 m a.s.l.) is located in the protected coastal area of the North Littoral Natural Park, near the municipality of Esposende in the north of Portugal. The weather station closest to this population, weather station of Viana do Castelo (41°42'N, 8°48'W) reports, for the period 1971-2000, a mean monthly temperature of 14.8°C, a mean maximum temperature of 38.6°C in June, and a mean minimum temperature of -4.0°C in December. The southern population (38°03'N 08°48'W, 0-47 m a.s.l.) is on the Sto. André and Sancha Lagoons Natural Reserve, a protected area located in the Alentejo coast (SW Portugal) close

to the town of Vila Nova de Santo André. According to the Sines weather station (37°57'N, 8°53'W, 1971-2000), this population presents an average mean air temperature of 15.8°C, a maximum of 37.1°C in July and a minimum temperature of 0.5°C in January. During 1971-2000, annual precipitation was 1470.2 mm and 511.0 mm for the northern and the southern population, respectively. Distributions of precipitation throughout the year are presented in Fig. 4.1 for the two considered populations.

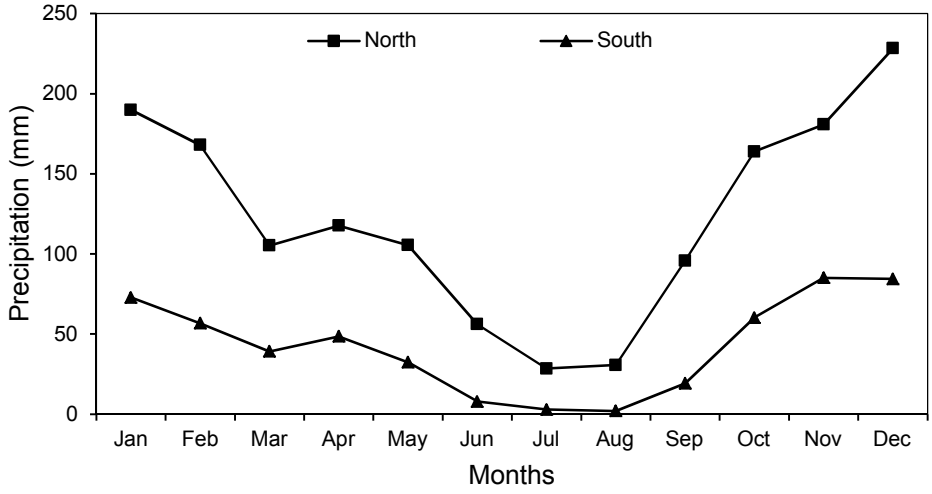


**Fig. 4.1.** Distribution of precipitation during the year at the two populations of *Acacia longifolia* used in this study. Climatic data were obtained from the meteorological station of Viana do Castelo and Sines, for the Northern and the Southern populations, respectively, provided by the Institute of Meteorology, Portugal for the period 1971-2000.

Seeds from both populations were collected during the summer of 2009 and stored in paper bags at room temperature prior to germination. The seeds were surface sterilised by sequential immersion in ethanol 96% for 30 s and commercial bleach at 4% for 2 min, followed by six washes in autoclaved water. They were then pre-treated by manual scarification using a scalpel. Seeds from both populations were placed in wet autoclaved sand in Petri dishes for germination under controlled conditions in a growth chamber, with a cycle of 16 h of light at 25°C, and 8 h of darkness at 18°C. After the emergence of the radicle and cotyledons, seedlings from both populations were transplanted into 9 cm x 10 cm plastic pots containing autoclaved sand and grown for 1 month in the same growth chamber. The

plants were watered twice a week before starting the water treatments. After this acclimation period, eighteen healthy seedlings of uniform height were chosen from each population and they were subjected to three different water treatments, namely: (1) seedlings watered twice a week (a well-watered treatment, WW, as a control), (2) seedlings watered weekly (a moderate-watered treatment, MW) and (3) seedlings watered every 10 days (a low-watered treatment, LW). All plot received 10 ml of tap water on each watering date. Each treatment (*i.e.* both the control and water stress experiments) involved 6 plants per population. Plants were allowed to grow in the same growth chamber conditions for three months during 2010/2011. The pots were covered with plastic film and aluminium foil to prevent evaporation from the surface of the soil and to minimise temperature increases inside the pots. All the pots were rotated weekly during the experimental period.

*4.3.2. Growth measurements*

Prior to starting the water treatments, six randomly chosen plants from each population were harvested to determine the average dry weight and leaf area of plants. At the end of the experiment, all plants were harvested, and shoots and roots were collected after removing roots from the substrate and washing them carefully. The plant height, shoot and root length and the presence of root nodules were recorded for each plant. The shoots and leaves were scanned with a HP scanjet 5370c at 300 dpi, and the leaf area was determined with the ImageJ-Image Processing and Analysis software in Java (available at <http://rsb.info.nih.gov/ij/index.html>). Dry weight of shoots and roots were determined after drying at 65°C for 48 h in oven. Based on the dry weight and/or leaf area data, the relative growth rate (RGR), as the rate of total dry weight increase per unit dry weight, the net assimilation rate (NAR), as the rate of increase in dry weight per unit of leaf area, the leaf area ratio (LAR), as the ratio of the total leaf area to the plant dry weight, specific leaf area (SLA), as the ratio of the total leaf area to the leaf dry weight, and the root:shoot ratio (WR:WS), given as the ratio of the weight of the roots to the weight of the shoots, were calculated. For statistical comparisons, RGR and NAR were calculated by using data from the initial (corresponding to the average dry weight and leaf area per population) and final harvests (corresponding to the final dry weight and leaf area per sample).

*4.3.3. Determination of ion concentrations*

Samples of dried shoots from each population were used to determine $Na^+$, $K^+$, $Ca^{2+}$ and $Mg^{2+}$, using an atomic absorption spectrophotometer after acid digestion (Perkin-Elmer Analyst 100). A suitable plant extract dilution was made, using distilled water. The total P of the shoots was determined according to the phosphomolybdate-ascorbic acid method (Murphy and Riley 1962). All measurements were taken in three replicates and the results expressed in mg $g^{-1}$ dry weight.

*4.3.4. Nitrogen content, carbon and nitrogen isotope composition*

Samples of shoots from each population and water treatment were oven-dried for 24 h at 80°C and ground to a fine powder in a ball mill. The carbon and nitrogen isotope ratio ($\delta^{13}C$ and $\delta^{15}N$, respectively), the C/N ratio and the total shoot N content, expressed as the total N per unit dry weight (%) were measured with ThermoFinnigan FlasdEA 1112 elemental analyser. This analyser was connected to Finnigan MAT-DELTA plus isotope ratio mass spectrometry using a ConFlo II interface, with an analytical precision of ± 0.2‰. All the measurements were taken in three replicates. All the analyses were carried out at the Servizos de Apoio á Investigación (SAI) at the University of A Coruña, in Spain.

*4.3.5. Statistical analysis*

Data were analysed using a two-way analysis of variance (ANOVA) with population and water treatments as the main factors. When the differences were significant ($P < 0.05$), a multiple comparison of means test (post-hoc Duncan's multiple range test) was carried out. All data sets satisfied the ANOVA assumptions of homogeneity of variance and normality of errors. Statistical analyses were performed using the PASW Statistics 18 software package.

**4.4. Results**

The results show that most of the growth parameters were significantly affected ($P < 0.001$) by the water treatments (Table 4.1, Fig. 4.2 and Fig. 4.3), except for the root:shoot ratio (WR:WS). Interestingly, among the different water treatments, the MW treatment produced

significant increases in plant height, shoot and root length, shoot and root dry weight and leaf area. On the contrary, the maximum reduction in these parameters was observed in the LW treatment, in which the plant height, shoot length and root length were approximately 19%, 5% and 34% lower, respectively, in comparison with the WW (control) treatment.

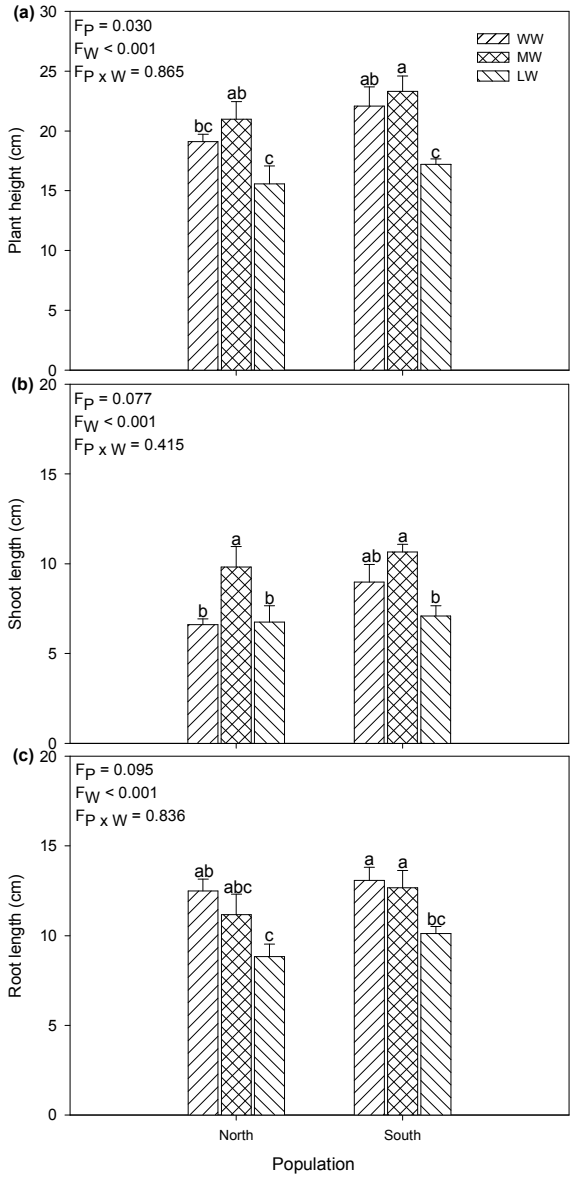


**Fig. 4.2.** a) Plant height, b) shoot length and c) root length of two populations of *Acacia longifolia* under water treatments (WW, well-watered; MS, moderate-watered; LW, low-watered). The *P*-values for watered treatments ($F_W$), population ($F_P$) and population x watered treatments ($F_{P \times W}$) are shown in the upper right of each panel. The bars with different letters are significant different from each other ($P < 0.05$). Values are means of six replicates ± S.E.

The different water treatments also led to a general decrease in all the biomass parameters (Fig. 4.3). The plants in the LW treatment produced less total, shoot and root biomass, averaging 53%, 62% and 39% respectively of the control.

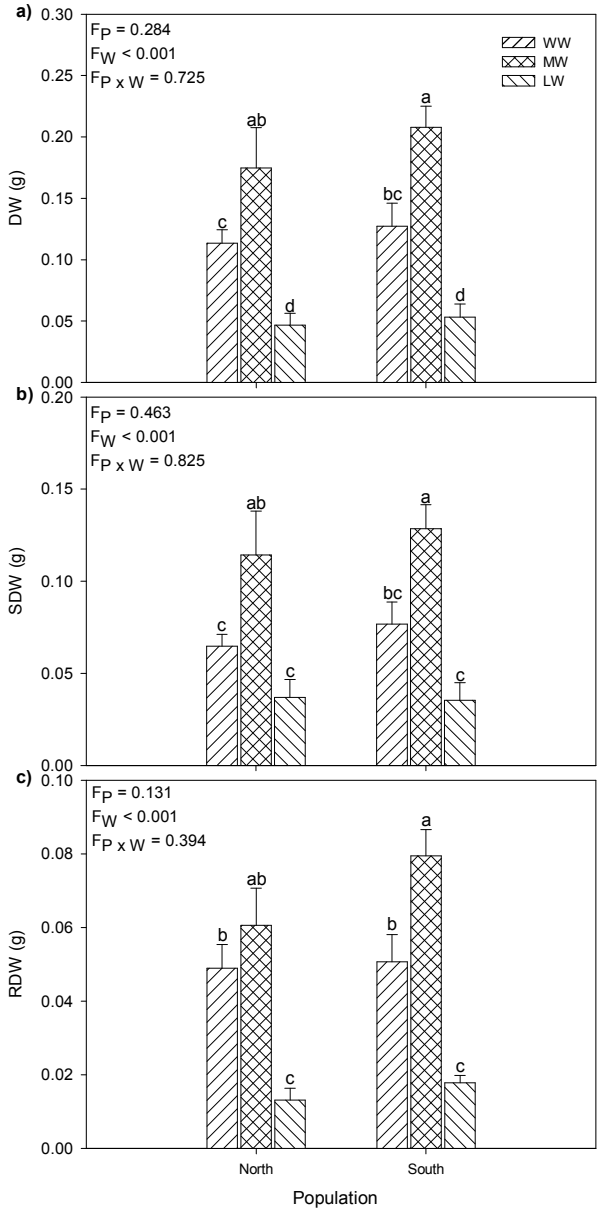


**Fig. 4.3.** a) Plant dry weight (DW), b) shoot dry weight (SDW) and c) root dry weight (RDW) of two populations of *Acacia longifolia* under water treatments (WW, well-watered; MS, moderate-watered; LW, low-watered). The *P*-values for watered treatments ($F_W$), population ($F_P$) and population x watered treatments ($F_{P \times W}$) are shown in the upper right of each panel. The bars with different letters are significant different from each other ($P < 0.05$). Values are means of six replicates ± S.E.

In terms of biomass partitioning, a parallel decrease in shoot and root dry weight resulted in no significant change in WR:WS ($P > 0.05$) amongst the different water treatments (Table 4.1). Although reducing the water availability had a negative effect on the relative growth rate (RGR), only the LW treatment was significantly different from the control, with a reduction of about 62%. As with the RGR, the NAR values decreased significantly with the water treatments, although only the LW treatment caused a significant reduction in NAR in comparison with the control (- 71%). The specific leaf area (SLA) and leaf area ratio (LAR) increased significantly in response to the water treatments. Under LW conditions, the *A. longifolia* plants showed a 120% and 80% increase in SLA and LAR respectively in relation to the control (Table 4.1). The two populations of *A. longifolia* differed significantly in their morphological traits, *i.e.* plant height, LAR and SLA under the different water treatments. The plants from the dry climate population (southern population) were significantly taller than the plants from the wet climate population (northern population) but showed lower LAR and SLA. The population x water treatments effect had only significant effect in LAR and SLA.

**Table 4.1.** Root:shoot ratio (WR:WS), RGR, leaf area (LA), LAR, SLA and NAR of two populations of *Acacia longifolia* under water treatments (WW, well-watered; MS, moderate-watered; LW, low-watered). The values (mean of six replicates ± S.E.) in the same column with different letters are significantly different from each other ($P < 0.05$).

| Population | Water treatment | WR:WS | RGR (mg $g^{-1}$ $day^{-1}$) | LA ($cm^2$) | LAR ($m^2$ $Kg^{-1}$) | SLA ($m^2$ $Kg^{-1}$) | NAR (g $m^2$ $day^{-1}$) |
|---|---|---|---|---|---|---|---|
| North | WW | 0.77 ± 0.09a | 0.016 ± 0.001a | 4.99 ± 0.69b | 44.24 ± 4.38b | 8.38 ± 0.61c | 3.32 ± 0.37a |
| | MW | 0.55 ± 0.05a | 0.020 ± 0.002a | 9.56 ± 1.62a | 55.75 ± 3.76b | 9.22 ± 0.77bc | 3.48 ± 0.40a |
| | LW | 0.47 ± 0.18a | 0.005 ± 0.003b | 5.66 ± 1.12b | 123.60 ± 13.89a | 18.06 ± 1.22a | 0.63 ± 0.48b |
| South | WW | 0.67 ± 0.06a | 0.016 ± 0.002a | 6.66 ± 0.95ab | 53.34 ± 4.51b | 9.57 ± 0.73bc | 2.85 ± 0.52a |
| | MW | 0.64 ± 0.06a | 0.022 ± 0.001a | 9.70 ± 1.12a | 46.44 ± 3.10b | 8.21 ± 0.33c | 4.07 ± 0.38a |
| | LW | 0.48 ± 0.16a | 0.006 ± 0.002b | 4.11 ± 1.23b | 70.57 ± 9.25b | 12.54 ± 0.53b | 0.97 ± 0.38b |
| Two-way ANOVA | | | | | | | |
| Population (P) | | 0.995 | 0.290 | 0.954 | 0.007 | 0.007 | 0.663 |
| Water treatment (W) | | 0.105 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| P x W | | 0.717 | 0.866 | 0.422 | <0.001 | <0.001 | 0.439 |

The $Ca^{2+}$, $Mg^{2+}$, $K^+$ and $Na^+$ content, expressed in mg $g^{-1}$ DW (Fig. 4.4), increased significantly as the water deficit increased. Compared to the control conditions, the greatest increases in these macronutrients concentrations were observed in the LW treatment (420%, 424%, 1600% and 3062% for $Ca^{2+}$, $Mg^{2+}$, $K^+$ and $Na^+$, respectively). As the changes in $Na^+$ and $K^+$ were similar, the $K^+$/ $Na^+$ was unaffected by the water treatments ($P < 0.05$).

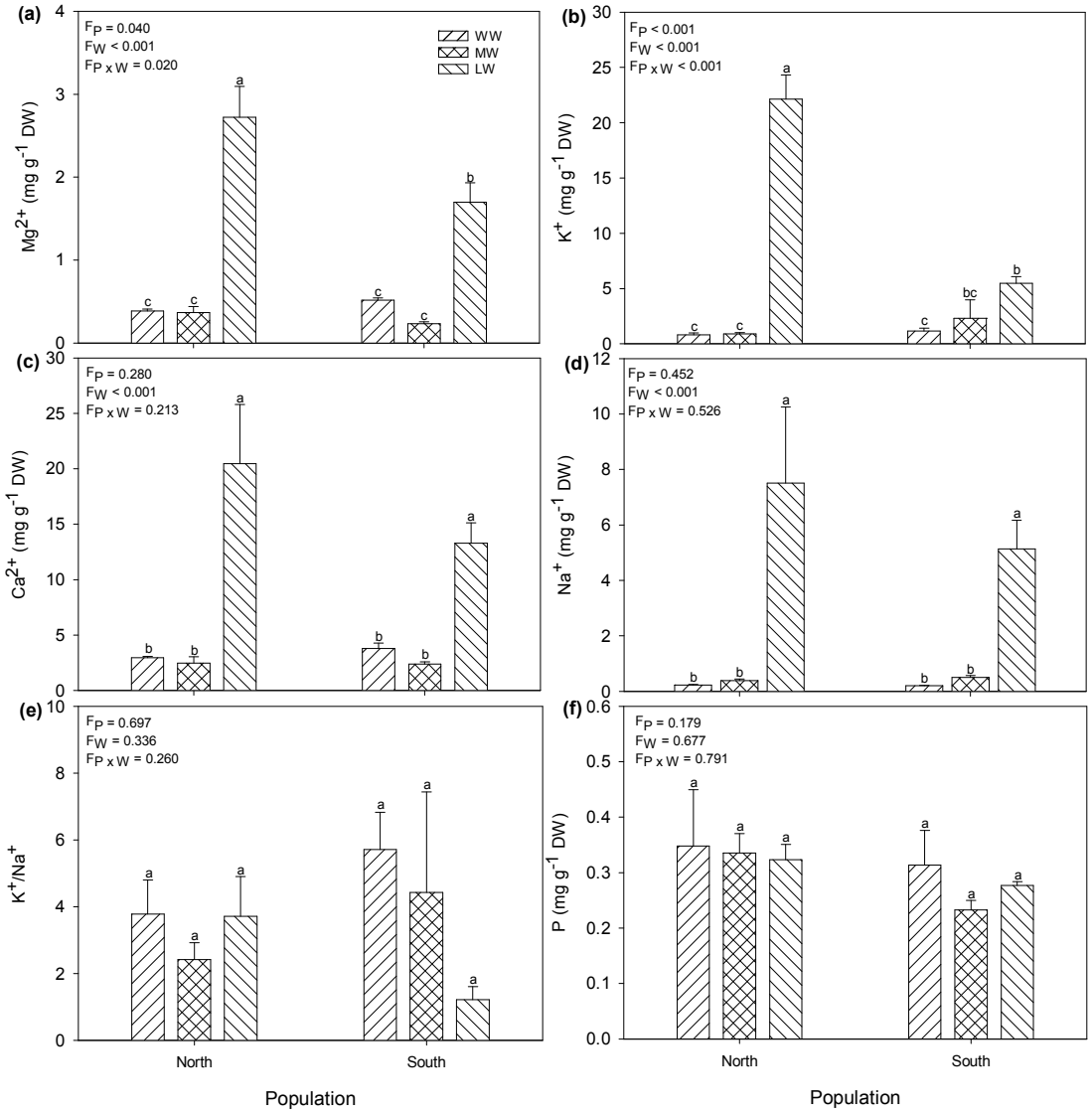


**Fig. 4.4.** a) $Mg^{2+}$, b) $K^+$, c) $Ca^{2+}$, d) $Na^{2+}$, e) $K^+/Na^+$, and f) P contents of two populations of *Acacia longifolia* under water treatments (WW, well-watered; MS, moderate-watered; LW, low-wateried). The *P*-values for watered treatments ($F_W$), population ($F_P$) and population x watered treatments ($F_{P \times W}$) are shown in the upper right of each panel. The bars with different letters are significant different from each other ($P < 0.05$). Values are means of three replicates ± S.E.

In response to the water treatments, the P content, expressed in mg $g^{-1}$ dry weight, tended to decrease but no differences were observed among the water treatments ($P < 0.05$). As the water stress continued, the nitrogen (N) concentration, expressed as percentage of dry weight, increased significantly (Table 4.2). The greatest differences in this respect were observed in the LW treatment, which showed 40% more N concentration, in comparison with the control. In contrast, the C/N ratio (Table 4.2) was negatively affected by water treatments. The highest ratio was found in the control treatment and the lowest under the LW treatment. In relation to the root nodules, no significant differences were found among water stress treatments, since we noted their presence in all treatments (data not shown). There were significant differences between the studied populations in the content of $Mg^{2+}$, $K^{+}$, C/N and N. Compared with the southern population, the northern population had lower C/N and higher $Mg^{2+}$, $K^{+}$ and N content. No population difference in the other ions was detected. Overall, the combined effect of population and water treatment was not significant except for $Mg^{2+}$, $K^{+}$, C/N and N.

Water stress also induced changes in isotope composition (Table 4.2). According to the two-way analysis of variance, the carbon isotope composition ($\delta^{13}C$) did not differ significantly among water treatments. The same was not found in the nitrogen isotope composition ($\delta^{15}N$), where greater differences under the MW treatment than the WW and LW treatments were observed. Values of $\delta^{15}N$ negative or around zero were obtained in all water treatments. No significant differences between populations were found in these two physiological traits and the interaction population x water treatments was only significant for the $\delta^{15}N$.

**Table 4.2.** C/N ratio, N concentration (%), carbon isotope composition ($\delta^{13}C$) and nitrogen isotope composition ($\delta^{15}N$) of two populations of *Acacia longifolia* under water treatments (WW, well-watered; MS, moderate-watered; LW, low-watered). The values (mean of three replicates ± S.E.) in the same column with different letters are significantly different from each other ($P < 0.05$).

| Population | Water treatment | C/N | N (%) | $\delta^{13}C$ | $\delta^{15}N$ |
|---|---|---|---|---|---|
| North | WW | 37.33 ± 8.53a | 1.25 ± 0.18b | -33.32 ± 0.47 | -2.33 ± 0.18b |
| | MW | 28.05 ± 2.57a | 1.63 ± 0.09b | -35.25 ± 0.76 | -2.52 ± 0.14b |
| | LW | 12.53 ± 0.63b | 3.42 ± 0.08a | -34.50 ± 0.43 | -0.65 ± 0.45a |
| South | WW | 34.82 ± 6.65a | 1.30 ± 0.16b | -35.73 ± 0.36 | -0.97 ± 0.29a |
| | MW | 30.03 ± 3.19a | 1.58 ± 0.12b | -35.45 ± 0.76 | -2.93 ± 0.13b |
| | LW | 30.24 ± 2.40a | 1.53 ± 0.08b | -34.28 ± 0.09 | -2.45 ± 0.06b |
| Two-way ANOVA | | | | | |
| Population (P) | | 0.027 | <0.001 | 0.091 | 0.183 |
| Water treatment (W) | | <0.001 | <0.001 | 0.192 | <0.001 |
| W x P | | 0.009 | <0.001 | 0.062 | <0.001 |

### 4.5. Discussion

Knowledge about invasive plant responses to water stress becomes relevant as most climate-change scenarios suggest an increase of droughts in the Mediterranean region in the next decades (IPCC 2007). In this study, *A. longifolia* plants were submitted to increasing water stress conditions and the results demonstrated that most of the studied morphological traits were negatively affected by low water availability. This finding is consistent with other studies conducted on *Acacia* species (Otieno *et al*. 2001; Kabir *et al*. 2006; Werner *et al*. 2010) and on *Indigofera* species (Hassen *et al*. 2007), which demonstrated that severe water stress had negative effects on their productivity and growth. Despite this, an increment in some morphological parameters (shoot length, dry weight, shoot and root dry weight and leaf area), in comparison with the control, was observed. The significant increase in these morphological parameters could be seen as an important adaptive mechanism of *A. longifolia* plants to drought of short duration, and

suggests that *A. longifolia* plants use their internal resources to promote growth until almost water from the soil is exhausted. As a result, *A. longifolia* plants continue to growth and maximize its productivity under this limiting water availability. Generally, when water does not restrict growth, plants invest a considerable fraction of assimilates to the photosynthetic organs, maximizing light interception and consequently, growth (Guo *et al*. 2010). However, under prolonged unfavourable water stress conditions the efficiency with which solar radiation biomass is used to accumulate biomass decreases, as a result of a reduction in photosynthetic capacity, causing a decline in growth (Ogbonnaya *et al*. 1998). The reduction in total dry weight of *A. longifolia* was due to reductions in both shoots and roots dry weight, as reported in a previous study with the same species (Werner *et al*. 2010). The decrease in biomass production could result from reduced formation of new leaves, increased leaf shedding (Patger *et al*. 2005) and reduced average leaf size and leaf area (Li *et al*. 2009). However, in this study, the reduction in biomass resulted from water stress effects on leaf growth, as indicated by the smaller average leaf size and leaf area in plants submitted to LW treatment, rather than leaf shedding, which was negligible in all treatments (data not shown). Under conditions of water deficiency, leaf growth is one of the first plant processes to be affected (Chaves *et al*. 2003) and was frequently observed as responses to water stress in many plants (Ogbonnaya *et al*. 1998; Osório *et al*. 1998; De Smedt *et al*. 2012). The reduction in leaf area can be considered as a dehydration-avoidance mechanism minimising water loss by transpiration (Liu *et al*. 2012) and hence conserving water during periods of drought (Chaves *et al*. 2003). In addition, as root growth depends on the supply of carbohydrates from parts of the plant above the ground (Ogbonnaya *et al*. 1998), a reduction in leaf area would be expected to reduce root growth.

Several studies have reported that plants will react to limited water availability with a relative increase in the capacity to uptake and minimise the loss of water via the roots, leading to an increased WR:WS (Otieno *et al*. 2001; Kabir *et al*. 2006; Lei *et al*. 2006; De Smedt *et al*. 2012). However, in our study, the WR:WS of *A. longifolia* plants remained similar in all water treatments, and this was due to the synchronous reduction in root and shoot dry weight. Thus, our data are consistent with the findings that *A. longifolia* plants fail to adjust this trait in response to drought (Werner *et al*. 2010). Similar findings have been reported by Garcia-Serrano *et al*. (2009) in three *Senecio* species, Otieno *et al*. (2001) in

*Acacia xanthophloea*, Ogbonnaya *et al*. (1998) in *Hibiscus cannabis*, Li *et al*. (2009) in *Sophora davidii*, and by Osório *et al*. (1998) in *Eucalyptus globulus*. Sobrado and Turner (1986) suggested that a similar degree of osmotic adjustment in root and shoot would help to explain the similar ratio which they found in water-stressed and unstressed *Helianthus annuus*.

At the whole-plant level, decreasing water availability substantially reduced the RGR of *A. longifolia*, which agrees with Otieno *et al*. (2001), who indicated that low RGR is a common plant response to unfavourable conditions (for example, low water availability). Such observation has also been reported for *Bromus pictus* when submitted to water stress (Rotundo *et al*. 2006). On the base of these results, the low RGR observed in *A. longifolia* plants could be an advantage in dry conditions, since the plants have a low demand for resources and therefore will not exhaust the limited soil water reserve, saving it for later growth (Sobrado and Turner 1986). The drop in RGR was mainly the result of a lower rate of carbon gain per unit leaf area, *i.e.* NAR (Lambers *et al*. 1998). This result supports the findings of Galmés *et al*. (2005), reporting that NAR is the most important determinant of RGR in woody perennial plants. The decrease in NAR could be related to stomatal closure under water stress conditions (Boutraa 2010) and suggests that water availability affects the net photosynthetic rate (Boughallab and Mhamdi 2011). Moreover, the lower NAR is counterbalanced by allocating more biomass into photosynthetic organs (high LAR) and by producing thinner or less dense leaves generating a high SLA. Similar LAR and SLA responses to water stress have been reported for *Artocarpus chaplasha* (Ferdousee *et a*l. 2011) and other *Acacia* species such as *A. auriculiformis* (Kabir *et al*. 2006) The significant increase in LAR by increasing SLA might be interpreted as a compensation mechanism for reduced assimilation (Anyia and Herzog 2004) and seems to be an important adaptation in terms of growth in *A. longifolia* plants in conditions in which water is limited.

The water stress treatments also affected the nutrient metabolism in *A. longifolia* plants. Exposing plants to water stress led to the uptake and accumulation of considerable amounts of inorganic ions such as $Ca^{2+}$, $Mg^{2+}$, $K^{+}$ and $Na^{+}$. A similar result has been documented for *Vitis vinifera* (Patakas *et al*. 2002) and *Atriplex halimus* (Martìnez *et al*. 2003). The increase in inorganic ions in response to water stress suggests that they are involved in osmotic adjustment in *A. longifolia*, which may allow these plants to continue to grow when water is

scarce, as it has been observed in *Ziziphus rotundifolia* (2001). Osmotic adjustment has been considered one of the crucial processes in plant adaptation to drought, as it is associated with maintaining turgor and metabolic activity (Arndt *et al*. 2001). In contrast, phosphorus did not appear to be involved in osmotic adjustment since it remained unchanged in all the water treatments. Besides these changes, the decline in water availability also had implications on the N content. There was generally an increasing trend of N accumulation in *A. longifolia* shoots and the increase was higher at the LW treatments. A comparable response has been reported by Farquhar *et al*. (2002) who found that plants predominantly growth-limited by water, allocate more N per unit leaf area in order to increase assimilation with a reduced total leaf area and stomatal conductance. The observed increase in N content of *A. longifolia* shoots could have mitigated the effects of drought on photosynthesis (Susiluoto and Berninger 2007) however seems not to be sufficient to maximise growth under water stress. A very different plant response was observed for the carbon and nitrogen utilization as indicated by the variation in the C/N ratio. This ratio decreased under water-stressed treatments and is mostly associated with a decrease in C content. This process of higher N content and lower C/N ratio under severe water stress is also accompanied by a slight increase in $\delta^{15}$N values, and by the presence of root nodules (data not shown). This reflects the N-fixing ability of *A. longifolia* plants, as documented in previous studies (Peperkorn *et al.* 2005; Werner *et al*. 2010), and could be considered as an important mechanism for plants under drought (Farquhar *et al*. 2002).

Higher water-use efficiency is mentioned as an important factor determining plant performance under water limited conditions (Amudha and Balasubramani 2011). As an indirect indicator of water-use efficiency, $\delta^{13}$C provides a useful measure of integrated carbon/water balance in plants over a long period of time (Farquhar *et al*. 1989). In this study, water treatments did not modify $\delta^{13}$C values which contrast with the findings of Osório *et al*. (1998) and Zhang *et al*. (2004), who reported a clear increase of $\delta^{13}$C under water stress. The lack of a significant $\delta^{13}$C response to water stress suggests that *A. longifolia* did not significantly alter its water-use efficiency in presence of drought and this may be related to the short period of time in which the plants were submitted to the water treatments. This conclusion is further supported by the observation that bulk leaf $\delta^{13}$C is unresponsive to short-term changes in environmental conditions (Keitel *et al*. 2003).

Variation in $\delta^{13}C$ with tissue type (O'Leary 1981), plant´s stage of development (Maftzner *et al.* 2003) and environmental conditions observed during the growth period (Marshall *et al*. 2007) has been reported previously. In our study, the $\delta^{13}C$ values integrated different plant organs (young leaves and stems) as well as material originated from seedlings and therefore the results cannot be directly compared with those found in the literature for the same species (Rascher *et al*. 2010).

This study also highlights some significant differences between wet and dry climate populations of *A. longifolia*, which reflect, at least partly, a genetic influence since the environmental variation within the growth chamber were negligible. This result is corroborated by previous studies in which many plants show genetic variability to particular conditions of water availability (Osório *et al*. 1998; Rotundo *et al*. 2006; Maatallah *et al*. 2010; De Smedt *et al*. 2012). Significant differences between populations were detected in plant height, LAR, SLA and accumulation of $Mg^{2+}$, $K^{+}$, N concentration and C/N ratio. The lack of population by water treatment interaction indicates that both populations presented the same tolerance to the water conditions imposed in this study. Since survival strategies are adaptations to the environmental conditions in which plants have evolved, the differences detected may explain specific adaptation mechanisms within the species under water stress (Zhang *et al*. 2004). In general, *A. longifolia* plants from the dry (southern) climate population showed less reduction of growth and exhibited higher C/N ratio when water stress increased. On the other hand, the wet (northern) climate population showed higher accumulation of some inorganic ions ($Mg^{2+}$ and $K^{+}$) and a higher SLA and LAR, which play an important role in minimising the adverse effects of water stress (Boughallab and Mhamdi 2011) and should enable the *A. longifolia* plants to maintain their growth and establish themselves more efficiently under water stress conditions.

The hypothesis that plants growing in dry climate regions can survive water deficits more effectively than those from wet climate (Lei *et al*. 2006) is not supported by our results since *A. longifolia* from the northern population responded most favourably to some of the observed traits. Despite the wide range of habitats where this invasive species is found, it is mainly reported in the northern regions of Portugal, where the precipitation is higher and the drought period is less intensive (Werner *et al*. 2010). By comparison, the southern population receives about one third less precipitation than the wet population and the

summer precipitation (June to August) is approximately 90% less at the southern population. The southern population is also subjected to periods of high temperature and the combination of these environmental constraints produced severe drought stress in plants. Thus, the severeness of such climatic conditions has detrimental effects on its performance. According to this and considering the predicted climate change scenario for the Mediterranean region, it is expected that severe drought conditions may limit the spread of this species and probably *A. longifolia* will not progress towards the drier regions in the southern parts of Portugal. Furthermore, if it occurs, *A. longifolia* will be present only in habitats with a moderate degree of water stress. Our results also suggest that the northern population may be more invasive than the southern population and may spread more rapidly.

In conclusion, our results indicate that *A. longifolia* plants were capable of surviving under water-stressed conditions independently of their origins since there was no mortality or significant leaf shedding during the period of this study. However, the effects were greatly dependent on the severity of water stress. A moderate water stress may not dramatically affect *A. longifolia* performance but the low growth and the reduction of the most physiological traits revealed the limitation of these plants under prolonged water stress. As a strategy to survive under these limited water conditions, *A. longifolia* plants show increase in LAR and SLA and, at the same time, accumulate more inorganic ions and promoted efficient nitrogen uptake. Moreover, its capacity to modify soil conditions, in part due to its great capacity to assimilate nitrogen, would help *A. longifolia* to colonise unfavourable habitats and, as a result, could have important implications on the native communities. From a study of *A. longifolia* and related natives, Peperkon *et al*. (2005) and Werner *et al*. (2010) and concluded that the natives have higher efficient drought adaptations when compared to the invasive species but this trait did not provide them a competitive advantage under limited water conditions. Because of this and based on our results it is expected that water stress conditions could limit the expansion of *A. longifolia* to the drier regions of the south of Portugal. However we recommend further studies on longer and in situ water stress experiments in order to confirm these results.

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**CHAPTER 5**

# DOES SALT STRESS INCREASE THE ABILITY OF THE EXOTIC LEGUME *ACACIA LONGIFOLIA* TO COMPETE WITH NATIVE LEGUMES IN SAND DUNE ECOSYSTEMS?

Maria Cristina Morais, Maria Rosaria Panuccio, Adele Muscolo, Helena Freitas

[Environmental and Experimental Botany (2012) 82: 74-79]

## 5. Does salt stress increase the ability of the exotic legume *Acacia longifolia* to compete with native legumes in sand dune ecosystems?

### 5.1. Abstract

Sand dune ecosystems are one of the areas most affected by the introduction of invasive species which represents a threat for biodiversity conservation. Their invasion patterns and spread may depend on their salinity tolerance, besides other factors. To test this hypothesis, we investigated the effects of salt stress on seed germination and on the activity of antioxidant enzymes (catalase, CAT; ascorbate peroxidase, APX; peroxidase, POX; and glutathione reductase, GR) in two legume species, an invasive, *Acacia longifolia* (Andrews.) Willd., and a native, *Ulex europaeus* (L.), very common in the sand dunes of the coast of Portugal. Salt stress was induced by adding NaCl at different concentrations, 0, 50, 100 and 200 mM, for 15 days. Results showed that the highest germination percentages were obtained in distilled water (control) and that, with increasing salt concentration, seed germination was delayed and decreased in both species. Inhibition of germination was higher in the native species: only 3% of seeds germinated at 100 mM and no seeds germinated at 200 mM NaCl. In the invasive species, the reduction was higher at 200 mM NaCl (16%). Considering the coefficient of germination velocity, a decrease in both species with increasing NaCl concentration was observed. The CAT and GR activities decreased in A. longifolia with increasing salinity. In turn, APX activity significantly increased as NaCl concentration increased while the POX activities declined at the highest NaCl concentration. On the other hand, at 50 mM NaCl lower activity of CAT and APX and higher GR and POX were found in *U. europaeus*. In both species, protein content increased as NaCl concentration increased. In addition, it seems that APX activities play an essential role in the scavenging reactive oxygen species (ROS). These results suggest that the seeds of the invasive legume *A. longifolia* are more tolerant to salinity than the native legume *U. europaeus*, and seem better equipped to handle the physiological stress of high salinity, which may contribute to its invasive ability in sand dunes.

**Keywords:** *Acacia longifolia*, antioxidant enzymes, germination, invasion ability, NaCl, *Ulex europaeus*

## 5.2. Introduction

Salinity is one of the environmental factors that have a critical influence on the germination of halophyte seeds and plant establishment (Katembe *et al*. 1998). Increasing salt concentration often causes osmotic/or specific ionic toxicity, which may reduce, retard (Khan and Gulzar 2003; Abari *et al*. 2011) or completely inhibit seed germination (Benabderrahim *et al*. 2011). High levels of salt also promote alterations in the integrity of cell membranes, inhibition of different enzymatic activities and photosynthesis (Sairam and Tyagi 2004). However, low salt concentration can stimulate germination in some species (Croser *et al*. 2001).

The effects on germination depend on the concentration of NaCl and on the species examined (Croser *et al*. 2001). It is well recognized that plant species differ in their sensitivity or tolerance to salts (Ashraf and Harris 2004). There are also evidences that the organs, tissues and cells of plants exhibit varying degrees of tolerance to salinity at different developmental stages (Abari *et al*. 2011).

One of the biochemical changes occurring when plants are subjected to biotic or abiotic stresses such as salinity, drought or extreme temperatures is the formation of reactive oxygen species (ROS) (Dionisio-Sese and Tobita 1998; Eyidogan and Oz 2007). ROS are a product of altered chloroplast and mitochondrial metabolism during stress (Manchanda and Garg 2008). The major sources of ROS are the superoxide radical ($O_2^-$), hydrogen peroxidase ($H_2O_2$), single oxygen ($O_2$) and the hydroxyl radical (OH) which are produced in all cellular compartments within a variety of processes. In general, they are produced during normal aerobic metabolism in plants (Noctor and Foyer 1998; Ashraf and Harris 2004; Kim *et al*. 2004; Sohn *et al*. 2005) and maintained at constant basal levels in healthy cells, but in stress situations in the absence of any protective mechanism they can seriously damage the normal metabolism of plants through oxidative damage to lipids, protein and nucleic acids (Meloni *et al*. 2003). They also severely impair seedling survival (Mitler 2002), damage photosynthetic components, inactivate enzymes and permeabilize membranes by causing lipid peroxidation (Meloni *et al*. 2003).

Adequate defense against such adversities requires efficient scavenging of these highly forms of oxygen (Noctor and Foyer 1998; Kim *et al*. 2004). Plants possess an effective antioxidant system (enzymatic and non-enzymatic) that can neutralize free radicals and may

reduce or even help some of potential damage they cause (Lin and Kao 2000; Sohn *et al*. 2005; Manchanda and Garg 2008).

Antioxidant molecules such as carotenoids, ascorbate, glutathione and tocopherols (Bandeoğlu *et al*. 2004) and antioxidant enzymes such as catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX) and glutathione reductase (GR) are the most important components in the scavenging system of these reactive molecules (Meloni *et al*. 2003; Jogeswar *et al*. 2006; Eyidogan and Oz 2007).

Earlier works have reported that plants under a variety of environmental stresses, including salt stress, respond by increasing the levels of antioxidant enzymes (Dionisio-Sese and Tobita 1998; Gao *et al*. 2008). In this situation, plants show considerable resistance to the oxidative damage caused by ROS (Dionisio-Sese and Tobita 1998; Parida *et al*. 2004). The correlation between antioxidant capacity and salt tolerance was demonstrated in a large number of plants, such as *Gossypium* cultivars (Meloni *et al*. 2003), *Echinochloa oryzicola* (Kim *et al*. 2004), and *Jatropha curcas* (Gao *et al*. 2008).

While a large volume of studies have investigated species interactions along salinity gradients, little information is available regarding the effect of salinity in invasion. It is expected that native halophytes will be more tolerant to high salinities than non-natives, but there are contradictory findings in the literature. For example, in coastal grasslands Kolb and Alpert (2003) found that the invasive species appeared to tolerate salinity better than the native ones they studied, whereas Kuhn and Zedler (1997) found a native, perennial salt marsh species to be more salt-tolerant than a non-native, invasive annual.

The purpose of this study was to evaluate the effect of different NaCl concentrations on the germination of a native legume, *Ulex europaeus*, and an invasive one, *Acacia longifolia*. Both species are present in Portuguese coastal sand dune habitats, where they are subjected to varying levels of substrate salinity and seawater spray. In coastal areas, *A. longifolia* is an aggressive invader and it is becoming a serious ecological problem, particularly by decreasing plant diversity (Marchante *et al*. 2003).

Comparison of these responses could be useful to identify the differences in ability of each plant to cope with salinity and to understand potential invasiveness in coastal habitats.

Further, it may be also valuable in developing restorative management of sand dune ecosystems.

The specific objectives of our study were to assess and compare the seed germination response of the two legume species under different NaCl concentrations and to further investigate the effects of salt stress on the activity of antioxidant enzymes associated with seed germination.

## 5.3. Material and methods

### *5.3.1. Germination study*

Seeds of *Acacia longifolia* and *Ulex europaeus* (Fabaceae) were collected at the Natural Reserve of Dunes of S. Jacinto, Portugal (40°39´N, 8° 44´W), and stored at room temperature until used. Prior to the germination study, seeds of *A. longifolia* were disinfected by sequential immersion in ethanol 96% for 30 s, commercial bleach at 4% for 2 minutes and six washes in autoclaved water. Thereafter, the seeds were pretreated by manual scarification with a scalpel. On the other hand, seeds of *U. europaeus* were soaked in concentrated sulphuric acid (36N) for 180 min and rinsed with autoclaved water. Both seeds were placed to germinate in Petri dishes containing two sheets of filter paper, moistened with 3 ml of distilled water (control) or NaCl solutions (50, 100 and 200 mM NaCl) in a growth chamber, with a cycle of 16 hours of light (250 $\mu Mm^{-2}s^{-2}$) at 24°C and a relative humidity of 70-80%. Petri dishes were sealed with parafilm to prevent evaporation. Treatments were replicated three times in a randomized block design and each replicate included 20 and 30 seeds of *A. longifolia* and *U. europaeus*, respectively.

The number of germinated seeds was recorded every two days up to 15 days, and the seeds were considered germinated when the radicle reached 2 mm. At the end of the germination period, the percentage of germinated seeds (the proportion of seeds in which the germination process reaches the end) and the coefficient of germination velocity, CVG, (Ranal and Santana 2006) were determined for both species. The seedlings were then used for enzyme determinations.

*5.3.2. Enzyme determinations*

For protein and enzyme assays, fresh shoot material (0.5 g) was ground using a chilled (4°C) mortar and pestle and then homogenized in 0.1 M phosphate buffer solution (pH 7.0) containing 100 mg soluble polyvinylpyrrolidone (PVPP) and 0.1 mM ethylenediamine tetraacetic acid (EDTA). The homogenate was centrifuged at 6000 x g at 4°C for 15 min and the resulting supernatant was used as enzyme source for protein content and assays of the activities of enzymes selected based on their different functional role: catalase (CAT, EC 1.11.1.6), peroxidase (POX, EC 1.11.1.7), gluthathione reductase (GR, EC 1.6.4.2), and ascorbate peroxidase (APX, EC 1.11.1.11). All spectrophotometric analyses were measured at 25°C on a Shimadzu (UV 1800 CE) spectrophotometer.

CAT activity was determined following the Beers and Sizer (1952) method, with minor modifications. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 40 µl enzyme extract and 5 µl $H_2O_2$. The decrease in $H_2O_2$ was monitored at 240 nm and calculated by using its extinction coefficient (ε) = 0.036 $mM^{-1}cm^{-1}$.

Total APX activity was measured by monitoring the decline in 290 nm as ascorbate was oxidized, for 90 s using the method of Amako *et al*. (1994). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 5 mM ascorbate, 40 µl enzyme extract and 100 µl $H_2O_2$. The molar extinction coefficient 2.8 $mM^{-1}cm^{-1}$ was used to calculate APX activity.

GR activity was estimated by following the oxidation of $NADPH_2$ at 340 nm (ε = 6.2 $mM^{-1}cm^{-1}$). The reaction mixture contained 0.1 M potassium phosphate buffer (pH 7.0), 20 mM GSSG, 2 mM $NADPH_2$, 350 µl $H_2O$ and 50 µl enzyme extract.

POX activity was measured on the basis of determination of guaiacol oxidation at 436 nm for 90s. The reaction mixture contained 0.1 M potassium phosphate buffer (pH 7.0), 20 µl guaiacol, 40 µl enzyme extract and 15 µl $H_2O_2$. POX activity was quantified by the amount of tetraguaiacol formed using its extinction coefficient (ε) = 25.5 $mM^{-1}cm^{-1}$.

For CAT, APX, GR and POX activities, the results were expressed as enzyme units (U) per mg soluble protein. One unit of enzyme was defined as the amount of enzyme necessary to decompose 1 µmol of substrate per min at 25°C.

Total protein content of the enzyme extracts was determined according to the method of Bradford (1976), using 80 µl $H_2O$, 20 µl enzyme extract and 5 ml Comassie blue solution. Absorbance by the reaction mixture was read at 595 nm.

### *5.3.3. Statistical analysis*

Comparisons of germination percentage, CVG, enzyme activities and protein content among different salinity treatments were done separately for each species. All data was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests at a 95% confidence level. When necessary, germination data were transformed (arcsine) before statistical analysis to ensure homogeneity of variance. All data collected were statistically analyzed using PASW Statistics 18 software.

## 5.4. Results

### *5.4.1. Seed germination under different salt treatments*

The percentage of germination of seeds of *A. longifolia* and *U. europaeus* (Fig. 5.1 and Fig. 5.2) was affected by increasing NaCl concentrations. The two species showed considerable differences in the timing of initial and final germination percentage.

The final germination percentage of the invasive legume (Fig. 5.1a) was approximately 90% in the control treatment (distilled water) and at 50 mM NaCl concentration. At 100 mM NaCl there was an initial delay in the germination rate, but on the seventh day, it recovered and it was similar to the control. *Acacia longifolia* was able to germinate under the highest concentration of NaCl (200 mM) with a delay and a reduction in germination of approximately 16% (Fig. 5.2), but no significant difference was noticed between this salt treatment and the control ($P > 0.05$). Salt treatments affected the germination of the native legume, *U. europaeus* (Fig. 5.1b and 5.2) more adversely than the invasive one. In general, seeds of *U. europaeus* began to germinate six days after sowing and the highest values of germination were recorded on distilled water (control). Increasing salinity concentration beyond 50 mM NaCl significantly ($P < 0.05$) inhibited germination. At 50 and 100 mM NaCl, the reduction of germination was 39 and 92%, respectively, as compared with control, and at the highest concentration of salt (200 mM) no germination occurred (Fig. 5.2).

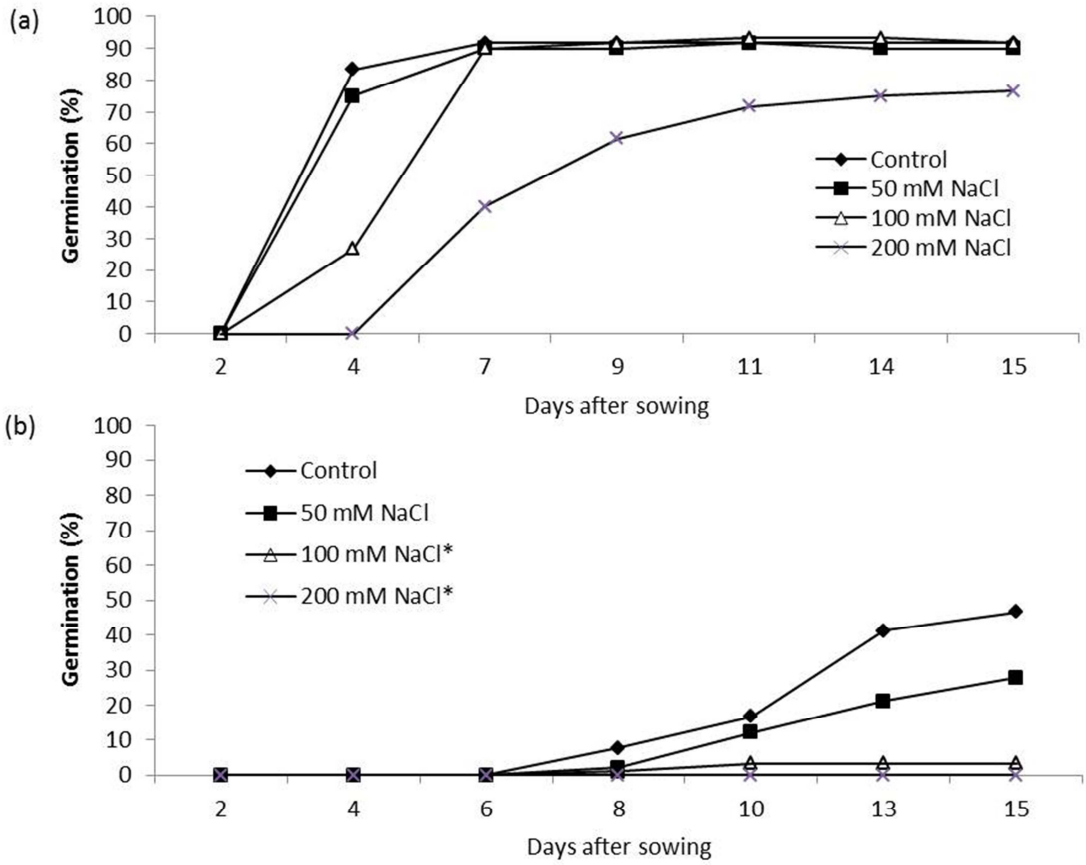


**Fig. 5.1.** Cumulative percentage of germination of *Acacia longifolia* (a) and *Ulex europaeus* (b) under different NaCl concentrations. Data represent mean ± S.E. of three replicates.

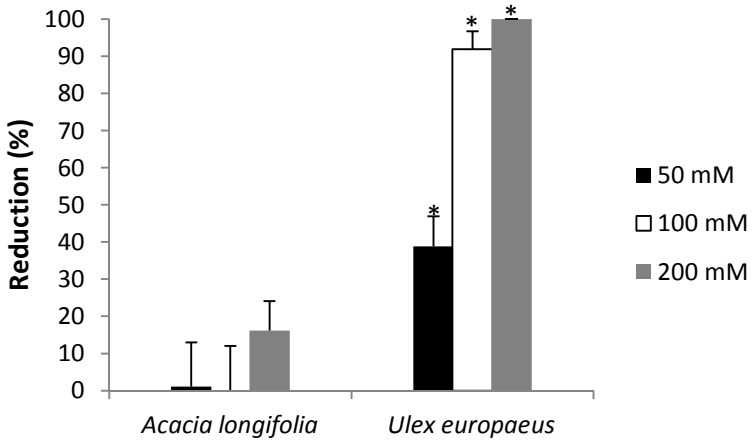


**Fig. 5.2.** Effect of NaCl concentrations on germination of *Acacia longifolia* and *Ulex europaeus* after 15 days of experiment. Results are mean ± S.E. of three replicates and expressed as reduction percentage of controls. Asterisks represent significant differences between the salt treatment and the control ($P < 0.05$).

As shown in Fig. 5.3, the CVG, as a measure of germination speed, decreased as the NaCl concentration increased and was higher in *A. longifolia*. For this species, concentrations up to 100 mM caused a significant ($P < 0.05$) reduction in CVG in comparison to the control treatment but for *U. europaeus* the CVG was not affected by the NaCl concentrations.

Based on the differences of germination percentage and CVG, *U. europaeus* was more sensitive to NaCl even at low concentrations; while *A. longifolia* seeds could adapt and grow up to high NaCl concentrations.

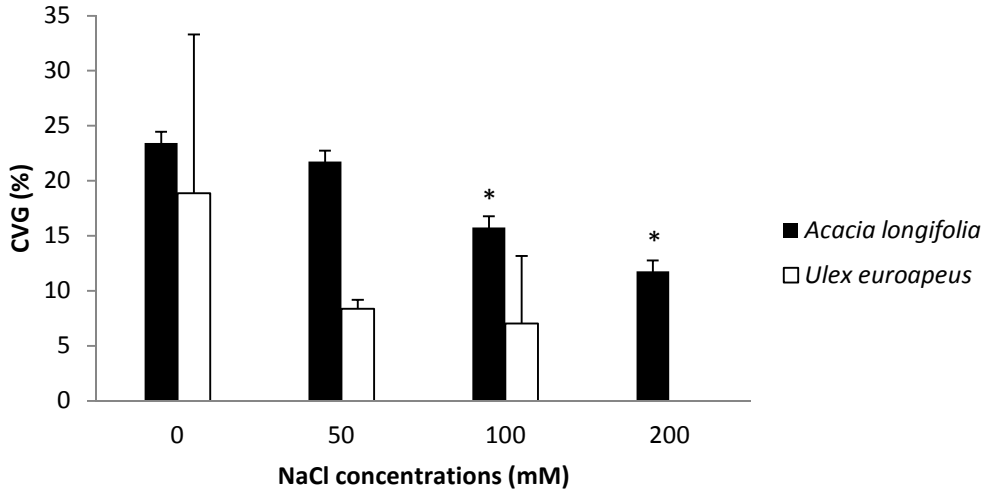


**Fig. 5.3.** Coefficient of germination velocity (%) of *Acacia longifolia* and *Ulex europaeus* under different NaCl concentrations. Results are mean ± S.E. of three replicates. Asterisks represent significant differences between the salt treatment and the control ($P = 0.05$).

*5.4.2. Effect of NaCl treatments on CAT, POX APX and GR activities*

The activities of CAT, APX, POX and GR in *A. longifolia* and *U. europaeus* under the effect of NaCl concentrations are given in Table 5.1. Due to the irregular germination of *U. europaeus* at 100 mM NaCl and the absence of germination at 200 mM NaCl, antioxidant enzyme activities were only determined for the control and 50 mM NaCl.

**Table 5.1.** Changes in the activities of CAT, APX, GR and POX enzymes (U/mg Protein) in *Acacia longifolia* and *Ulex europaeus* under different NaCl concentrations.

| Species | NaCl (mM) | CAT | APX | GR | POX |
|---|---|---|---|---|---|
| *Acacia longifolia* | 0 | 183.383 ± 46.864a | 0.355 ± 0.157b | 0.179 ± 0.028a | 0.059 ± 0.035ab |
| | 50 | 150.804 ± 28.295a | 0.404 ± 0.169b | 0.158 ± 0.031a | 0.081 ± 0.027a |
| | 100 | 142.676 ± 12.586a | 0.752 ± 0.180a | 0.117 ± 0.038ab | 0.067 ± 0.014ab |
| | 200 | 123.046 ± 60.085a | 0.589 ± 0.129ab | 0.091 ± 0.017b | 0.024 ± 0.013b |
| *Ulex europaeus* | 0 | 128.800 ± 4.368A | 0.185 ± 0.007A | 0.125 ± 0.009A | 0.672 ± 0.057A |
| | 50 | 101.298 ± 55.005A | 0.150 ± 0.038A | 0.163 ± 0.009A | 0.681 ± 0.133A |
| | 100 | - | - | - | - |
| | 200 | - | - | - | - |

Data represent mean values ± SD of analysis of 4 replicates for *Acacia longifolia* and of 2 replicates for *Ulex europaeus*. Means followed by the same letter are not significantly different at $P = 0.05$ within the same species.

CAT activity in both species decreased with increasing NaCl concentrations and for the same salt concentrations was higher in *A. longifolia*. As compared to control, CAT activity at 50 mM NaCl was decreased about 14% and 21% in *A. longifolia* and *U. europaeus*, respectively. However, this decrease was statistically insignificant. Salinity considerably ($P < 0.05$) increased the APX activity in *A. longifolia* as compared to that of the control. The maximum APX activity was obtained at 100 mM, which was ca. 144% higher than that of the control. In contrast, the activity of this enzyme slightly decreased in *U. europaeus* but at 50 mM NaCl was not significantly lower than those of the control. Under salinity, there was a gradual decrease in the activity of GR of *A. longifolia* which showed at 200 mM the strongest reduction (about 46% decrement). On the contrary, in *U. europaeus* a 30% increment occurred at 50 mM NaCl. With the increase in salinity to 50 and 100 mM NaCl, POX activity increased by 92% and 34% respectively, compared with control. At 200 mM NaCl, the activity of this enzyme was strongly reduced by about 36% compared with the control. In *U. europaeus*, POX activity remained essentially unchanged under 50 mM NaCl, compared to control.

*5.4.3. Effect of NaCl treatments on protein content*

A significant ($P < 0.05$) increase was seen in protein content of both species with increasing NaCl concentrations (Fig. 5.4). Moreover, protein content was higher in *U. europaeus* than in *A. longifolia* under the same salt concentrations. Overall, protein content in *A. longifolia* increased by 175% compared to the control. At 50 mM NaCl, the protein content was ca. 30% and 21% higher than that of the control in *A. longifolia* and *U. europaeus*, respectively.

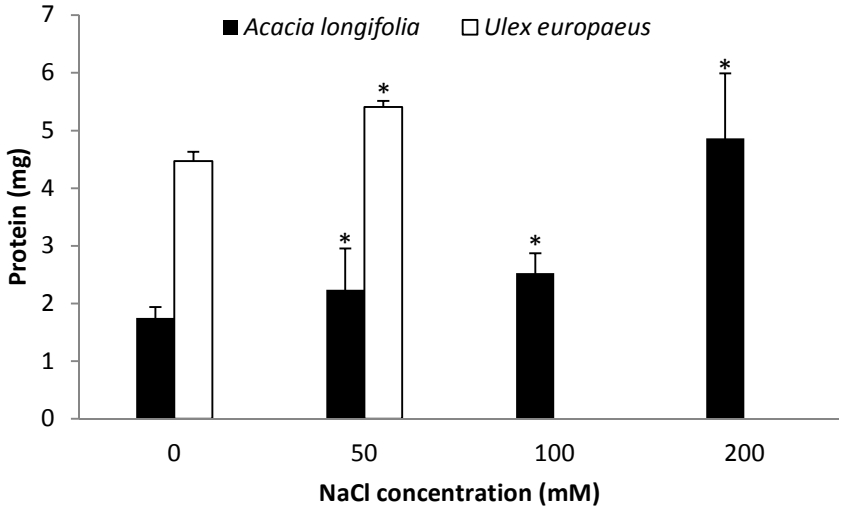


**Fig. 5.4.** Protein content (mg) in *Acacia longifolia* and *Ulex europaeus* seeds in response to different NaCl concentrations. Results are mean ± S.E. of three replicates. Asterisks represent significant differences between the salt treatment and the control ($P < 0.05$).

## 5.5. Discussion

The invasive legume *A. longifolia*, and the native legume U. europaeus, presented variable behavior in their germination under different NaCl concentrations. These results corroborate several other studies, showing that the effect of salinity on germination changes with species (Kuhn and Zedler 1997; Weber and D'Antonio 1999; Croser *et al*. 2001; Khan and Gulzar 2003). Moreover, *Acacia* species can differ in their tolerance to NaCl concentrations. For example, Abari *et al*. (2011) found in a laboratory experiment that *A. tortilis* and *A. nilotica* are salt tolerant and even survived at 300 mM NaCl.

*Acacia longifolia* seeds germinated well over the studied range of salt concentrations, with a slight decrease at the highest salt concentration tested (200 mM NaCl). This pattern has been described by numerous authors in other halophytic acacia species (Aref *et al*. 2004; Hardikar and Pandey 2008; Abari *et al*. 2011) and in other legumes (Taffouo *et al*. 2009; Benabderrahim *et al*. 2011). At the higher salt concentrations (100 and 200 mM NaCl) some delay in germination was observed but did not appreciably reduce the final germination percentage. Similar result was obtained by Kuhn and Zedler (1997) in *Salicornia subterminalis*.

On the other hand, the percentage of germination of *U. europaeus* seeds decreased progressively with increasing salinity. The reduction was about 39% for 50 mM, 92% for 100 mM NaCl and 100% for 200 mM NaCl. Although *U. europaeus* is considered a salt-tolerant species (Dagar and Singh 2007), salt concentration up to 100 mM NaCl was detrimental to seed germination. The sensitivity of plants to salinity may depend on their developmental stage (Abari *et al*. 2011). In this regard our results are in agreement with those of Ungar (1996), who found that the seeds and seedlings of several halophytes were less tolerant to salinity than growing plants because germination usually occurs in surface soils which accumulate soluble salts as a result of evaporation and capillary rise of water (Almansouri *et al*. 2001; Sidari *et al*. 2008).

The delay in seed germination or the decrease in germination percentage of the two studied species as a result of increased NaCl concentrations may cause some metabolic disorders. In fact, salinity may affect the germination either by creating an osmotic potential external to the seed that prevents water uptake (Fung *et al*. 1998; Hardikar and Pandey 2008; Abari *et al*. 2011), or by facilitating the intake of toxic ions which may change certain enzymatic or hormonal activities of the seed (Kaveh *et al*. 2011). Also Rehman *et al*. (1997), in ten *Acacia* species, and Katembe *et al*. (1998), in two *Atriplex* species, found that the adverse effects of NaCl on seed germination is a combination of an internal osmotic effect and an ion effect which may reduce or prevent seed germination.

The studied species also differed in the velocity of germination. In both species, the maximum coefficient was found in the control treatment and decreased with increasing NaCl concentrations. Considering all salt concentrations, *A. longifolia* presented the highest

coefficients which may prove that the tolerance of *A. longifolia* to NaCl is higher than that of *U. europaeus*.

The higher coefficients of velocity and shorter emergence times observed in *A. longifolia* can be related to its seed size. *Acacia longifolia* seeds are two to three times larger than those of *U. europaeus* and according to the literature (Croser *et al*. 2001), larger seeds may contain more solutes, which could be used to overcome osmotic effects of salts, and greater energy reserves making them less dependent on photosynthesis for early growth. As a consequence, seedlings from larger-seeded species should be able to establish themselves under a range of environmental conditions that cannot be exploited by smaller-seeded species (Easton and Kleindorfer 2009). Previous studies carried out with *Pinus banksiana* (Croser *et al*. 2001), as well as with *Atriplex* species (Katembe *et al*. 1998) also showed that larger seeds had greater success in overcoming osmotic constraints during the initial stages of germination.

Differences in salt tolerance among species can affect invasive potential directly (Kuhn and Zedler 1997) and can determine which species will successfully colonize a new area (Weber and D'Antonio 1999). Based on the results of germination percentage and on the velocity of germination, *A. longifolia* appears to be more salt tolerant than *U. europaeus* which might suggest a better establishment success of *Acacia* in soils with high NaCl concentrations.

Salt tolerance in plants is a complex phenomenon that involves morphological and developmental changes as well as physiological and biochemical processes (Muscolo *et al*. 2003). One consequence of high salinity is the generation of ROS, which have a negative oxidative stress effect on cellular structures and metabolism. Therefore, CAT, APX, GR and POX, as antioxidant enzymes, are involved in the scavenging of these species in the cells (Noctor and Foyer 1998). In the present study, the diverse response of these enzyme activities to different NaCl concentrations in *A. longifolia* and *U. europaeus* seeds suggests that oxidative stress could be an influential component of environmental stresses on germination. Moreover, according to the inconsistency of most of the enzymes activities in response to stress we could conclude that the mechanisms in scavenging $H_2O_2$ and subsequent tolerance to salinity were different.

In *A. longifolia*, the CAT activities were higher than in *U. europaeus* both in the control and under NaCl concentrations and decreased with salt concentrations. Even though no

statistical differences were found, this is a circumstantial evidence to support the hypothesis that salinity causes the formation of ROS (Dionisio-Sese and Tobita 1998; Eyidogan and Oz 2007). This could be due to the flux of superoxide radicals that are known to inhibit CAT biosynthesis (Kono and Fridovich 1982) and may be very important to the antioxidant enzymes related to the ascorbate-gluthatione cycle of the $H_2O_2$ scavenging metabolism such as APX and GR. This observation indicates that CAT activity is not implicated in the detoxification processes in seed germination and early seedling growth in salt- stress conditions and then is not correlated with differences in seed germination between the two species. However, its presence suggests that this enzyme may participate in protection against free superoxide radicals.

APX utilizes the reducing power of ascorbic acid to eliminate potentially harmful $H_2O_2$ (Asada 1992; Meloni *et al*. 2003). Our data show that elevated NaCl concentrations led to an increase in APX activity only in *A. longifolia*, suggesting its important role in controlling increase of $H_2O_2$ concentration in plant cells during the oxidative stress. This is in good agreement with previous results in two rice cultivars (Sohn *et al*. 2005) and some melon varieties and cultivars (Yasar *et al*. 2006) which indicate that activity of antioxidant enzymes such APX is the most useful way of scavenging $H_2O_2$, preserving seeds by oxidation. The inactivity of CAT in this case may be complemented by the activation of APX.

In the antioxidant defense system pathway, GR catalyses the NADPH-dependent reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) and it is involved in the maintenance of the high ratio of GSH/GSSG that is required for the regeneration of ascorbate (Meloni *et al*. 2003). Even though in some species salt tolerance was associated with increases in GR activities (Dionisio-Sese and Tobita 1998; Yasar *et al*. 2006), this was not completely observed in our study. In fact, GR activity showed some tendency to increase in seeds of *U. europaeus* and decreased in seeds of *A. longifolia* with increasing NaCl concentration. This decrease in GR activity in seeds of *A. longifolia* resulted in the accumulation of $H_2O_2$ and a higher lipid peroxidation level in seeds, showing that seeds of *A. longifolia* could suffer from severe toxicity of single oxygen and hydroxyl ions under salt stress conditions. This result is supported by findings of Wang *et al*. (2009) who reported significant reduction in GR activity in salt affected *Medicago sativa*.

POX activity has been used as a general indicator of stress by NaCl (Lee *et al*. 2001). It is an enzyme widespread in all cellular compartments (Hoque *et al*. 2007), where the function is to neutralize $H_2O_2$ using various electron donors. Our results show that the activity of POX in *U. europaeus* seeds did not change with NaCl concentrations. In contrast, in *A. longifolia* POX activity decreased only at the highest NaCl concentration (200 mM), suggesting that POX might not play the key role in reducing $H_2O_2$ in seeds of both species. A similar result was obtained by Wang *et al*. (2009) for two *Medicago* species under salinity conditions.

Protein synthesis has been considered as a possible primary target of salt toxicity (Gulen *et al*. 2006). In our study, NaCl treatment induced a protein content increase in seeds of the two species indicating that proteins may be involved in salt tolerance. This finding is consistent with the hypothesis that proteins accumulated under salt stress play an important role in osmotic adjustment (Ashraf and Harris 2004). According to Bavei *et al*. (2011), proteins may be synthesized *de novo* in response to salt stress, or may be present constitutively in low concentrations and increased when plants are exposed to salt stress. In agreement with the present results, protein content in seeds of *Vigna unguiculata* (Lobato *et al*. 2009) and in five *Sesamum indicum* cultivars (Gehlot *et al*. 2005) increased under NaCl stress.

Overall this study showed that the increase in NaCl concentration had an adverse effect on seed germination in both species. However, this effect was more prominent in *U. europaeus*, in which changes in salt concentration either delayed germination or inhibited it at high concentrations. The results of this study also presented some indications that the resistance to NaCl stress can be explained by the effective antioxidant capacity of the seeds. The observed increase in APX activity in *Acacia* seeds suggests that the mechanisms which protect the seeds against radicals that cause metabolic damage, inhibiting seed germination, are more efficient in this species than in the native one, *U. europaeus*.

In conclusion, the differences in salt tolerance and in defence against superoxide radicals between species suggest that, during germination, *A. longifolia* is better equipped to compete than *U. europaeus* in soils with high NaCl concentration.

Our study provides useful insights into the mechanisms involved in the invasive success of *Acacia longifolia* in coastal sand dune habitats. However, further studies including other enzymes and metabolites would provide a more comprehensive base for elucidating the

biochemical mechanisms that participate in salt tolerance during germination in these species.

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**CHAPTER 6**

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# SALT TOLERANCE TRAITS INCREASE THE INVASIVE SUCCESS OF *ACACIA LONGIFOLIA* IN PORTUGUESE COASTAL DUNES

Maria Cristina Morais, Maria Rosaria Panuccio, Adele Muscolo, Helena Freitas

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## 6. Salt tolerance traits increase the invasive success of *Acacia longifolia* in Portuguese coastal dunes

### 6.1. Abstract

Salt tolerance of two co-occurring legumes in coastal areas of Portugal, a native species - *Ulex europaeus*, and an invasive species - *Acacia longifolia*, was evaluated in relation to plant growth, ion content and antioxidant enzyme activities. Plants were submitted to four concentrations of NaCl (0, 50, 100 and 200 mM) for three months, under controlled conditions. The results showed that NaCl affects the growth of both species in different ways. Salt stress significantly reduced the plant height and the dry weight in *A. longifolia* whereas in *U. europaeus* the effect was not significant. Under salt stress, the root:shoot ratio (WR:WS) and root mass ratio (WR:WRS) increased as a result of increasing salinity in *A. longifolia* but the same was not observed in *U. europaeus*. In addition, salt stress caused a significant accumulation of $Na^+$, especially in U. europaeus, and a decrease in $K^+$ content and $K^+/Na^+$ ratio. The activities of antioxidant enzymes were higher in *A. longifolia* compared to *U. europaeus*. In *A. longifolia*, catalase (CAT, EC 1.11.1.6) and glutathione reductase (GR, EC 1.6.4.2.) activities increased significantly, while ascorbate peroxidase (APX, EC 1.11.1.11) and peroxidase (POX, EC 1.11.1.7) activities remained unchanged in comparison with the control. In *U. europaeus*, NaCl concentration significantly reduced APX activity but did not significantly affect CAT, GR and POX activities. Our results suggest that the invasive species copes better with salinity stress in part due to a higher rates of CAT and GR activities and a higher $K^+/Na^+$ ratio, which may represent an additional advantage when competing with native species in co-occurring salty habitats.

**Keywords:** *Acacia longifolia*, antioxidant enzymes, growth, invasion ability, ion content, salt tolerance, *Ulex europaeus*

### 6.2. Introduction

Sand dune ecosystems have a high conservation value worldwide, but they are highly threatened by exotic plant invasion (Maltez-Mouro *et al*. 2010). In Portugal, these ecosystems have been invaded by several exotic plant species, particularly *Acacia* species

(Marchante *et al*. 2003). *Acacia longifolia* (Andrews) Willd., introduced in Portugal at the beginning of the 20th century to stabilise dunes and curb sand movement (Marchante *et al*. 2011) is one of such species. *Acacia longifolia* is invasive in many coastal sand dunes, threatening native plant diversity, altering the functioning of the ecosystem (Marchante *et al*. 2008) and consequently preventing natural dune succession (Marchante *et al*. 2009).

The success of invasive species in establishing in non-native environments often depends on their ability to compete with native species for the essential resources of light, water and nutrients (Cordell *et al*. 2008; Morris *et al*. 2011). It is generally hypothesized that an increase in the availability of resources (Alpert *et al*. 2000; Kolb and Alpert 2003) or climate change leading to environmental constraints (*e.g.* salinity stress) tend to increase the risk of invasion by non-native plant species, which are better able to acquire limiting resources or to use resources more efficiently than native species (Cordell *et al*. 2008).

Salinity may play an important role in plant invasiveness. Up to now, few studies have been carried out in this area, and they reveal some contradictory findings. Kolb and Alpert (2003) found that high salinity significantly reduced the relative competitive ability of the native species, whereas Noe and Zedler (2001) pointed out that salinity was negatively associated with invasion by non-native plants in two salt marsh communities.

Salinity affects plants by creating numerous morphological, physiological, biochemical and molecular disturbances. In particular, high concentrations of salt limit plant growth (Levitt 1972; Fung *et al*. 1998; Meloni *et al*. 2004; Vicente *et al*. 2004; Sekmen *et al*. 2007; Amirjani 2010), damage photosynthesis, respiration (Marchner 1995), protein synthesis (Levitt 1972), nodule metabolism (Al-Shaharani and Shetta 2011), and enzymatic activities (Sairam and Tyagi 2004). The extent to which each parameter is affected depends on many factors, including the severity and duration of stress (Nawaz *et al*. 2010), the plant species (Levitt 1972; Greenway and Munns 1980; Croser *et al*. 2001) and genotypes (Fung *et al*. 1998), the composition of the saline solution (Läuchli and Grattan 2007) and the developmental growth stage (Levitt 1972).

To overcome salt stress, plants have evolved a wide range of mechanisms that help them to adapt to the osmotic and ionic stress caused by high salinity (Meloni *et al*. 2004; Sekmen *et al*. 2007). These mechanisms include osmotic adjustment, which is usually achieved either by the accumulation of compatible solutes or by uptake of inorganic ions (Meloni *et al*. 2004).

Variations in adaptive mechanisms are responsible for differences in the tolerance or resistance of plants to salt stress (Nasim *et al*. 2007). Plants under salt stress generally accumulate high intracellular concentrations of ions, mainly $Na^+$ and $Cl^-$ (Greenway and Munns 1980), which affect the cell uptake and homeostasis of many indispensable cations, especially $K^+$ and $Ca^{2+}$ (Vicente *et al.* 2004). Whereas $Na^+$ is detrimental to plant growth, $K^+$ is one of the essential elements required by the plant in large quantities (Mahajan and Tuteja 2005) due to its involvement in protein synthesis, enzyme activation, photosynthesis (Marchner 1995), stomatal movement and maintaining the osmotic balance (Mahajan and Tuteja 2005). Thus, under saline conditions a high $K^+/Na^+$ ratio in the cell is essential for normal functioning and also for improving resistance to salinity in plants (Greenway and Munns 1980).

The exposure of plants to salt stress can increase the production of reactive oxygen species (ROS) in plant tissues (Meloni *et al*. 2004; Sekmen *et al*. 2007). This can cause damage to cells and photosynthesis (Meloni *et al*. 2003) during environmental stress. To mitigate the oxidative damage triggered by ROS, plants have developed an efficient mechanism that includes low-molecular mass antioxidants as well as a cascade of antioxidant enzymes such as CAT, POX, APX and GR (Meloni *et al*. 2003), which detoxify the plant by scavenging oxygen radicals (Sekmen *et al*. 2007). The capacity to scavenge ROS and reduce their damaging effects on macromolecules appears to represent an important stress tolerance trait (Amor *et al*. 2007). A close positive correlation between antioxidant capacity and NaCl tolerance has been found in a wide variety of species, including coastal species such as *Beta maritima* (Bor *et al*. 2003), *Crithmum maritimum* (Amor *et al*. 2007), *Cakile maritima* (Amor *et al*. 2006) and *Plantago maritima* (Sekmen *et al*. 2007).

The occurrence and spread of invasive species in coastal plant communities may depend on their ability to withstand the effects of high salt which may vary at different stages of plant development (Weber and D'Antonio 1999). Previous studies (Morais *et al*. in preparation) showed that *A. longifolia* can germinate in the presence of high NaCl concentrations (200 mM) but very little information is available regarding the relative salt tolerance of this species at seedling stage. The aim of the present study is to compare the response to salt stress of an invasive legume (*Acacia longifolia*) and a native legume (*Ulex europaeus*). In the Portuguese coastal sand dunes, both species are often found in close proximity in the

secondary dunes, although *A. longifolia* occupies a larger area than the native *U. europaeus*. Specifically, we examined the effects of different NaCl concentrations on plant growth, ion content ($K^+$, $Na^+$) and antioxidant enzymes activities in both species in order to better understand the mechanisms relevant in salt tolerance. We predicted that differential ability to cope with salt stress could be related to the invasion success of *A. longifolia* in coastal salty habitats.

## 6.3. Material and Methods

### *6.3.1. Growth conditions*

*Acacia longifolia* (Andrews) Willd. and U*lex europaeus* L. seeds were collected in São Jacinto Dunes Nature Reserve, Portugal (40°39'N, 8°44'W) in 2009, and stored in paper bags at room temperature before being used. Seeds were obtained from a large number of adult plants of both species to ensure broad representation of the gene pool.

*Acacia longifolia* seeds were surface sterilized by sequential immersion in ethanol 96% for 30 s, commercial bleach at 4% for 2 minutes and six washes in autoclaved water. After that, seeds were pretreated by manual scarification with a scalpel. Seeds from *U. europaeus* were soaked in concentrated sulphuric acid (36N) for 180 min and rinsed with autoclaved water. Both seeds were placed to germinate in Petri dishes containing wet autoclaved sand in a growth chamber, with a cycle of 16 hours of light at 25°C, and 8 hours of darkness at 18°C. After the radicle and cotyledons appeared, sixty seedlings of each species were transferred to individual plastic pots 9 cm in diameter x 10 cm high, previously filled with autoclaved sand. The seedlings of each species were divided into four groups that were subjected to four salinity treatments (0, 50, 100 and 200 mM), created by adding NaCl to a commercial 6:3:6 NPK fertiliser. Each treatment (control and salt stress) consisted of fifteen plants per species. Plants were allowed to grow, with regular watering, indoor at laboratory conditions under natural photoperiod and irradiance, for three months during 2010/2011. The pots were covered with plastic film and aluminium foil to prevent evaporation from the surface of the soil and to minimise temperature increases inside the pots.

*6.3.2. Plant biomass and height*

At the end of the experiment, six plants of each species per treatment were randomly selected, harvested, gently removed from the substrate and the following parameters were measured: total height, root length, shoot and root dry weight (after drying at 65°C for 48 h). The root:shoot ratio (WR:WS) and root mass ratio (WR:WRS), given as the ratio of the weight of the roots to the weight of the plant, were calculated on the basis of the dry weight (DW).

*6.3.3. Shoot $Na^+$ and $K^+$*

Three samples of dried shoots from each treatment were used to determine $Na^+$ and $K^+$, using an atomic absorption spectrophotometer after acid digestion (Model AAnalyst 100, Perkin-Elmer). A suitable plant extract dilution was made, using distilled water. The results are expressed in mg $g^{-1}$ DW.

*6.3.4. Enzyme extractions and assays*

For protein and enzyme extractions, fresh shoot material (0.5 g) was ground using a chilled (4°C) mortar and pestle and then homogenized in a 0.1 M phosphate buffer solution (pH 7.0) containing 100 mg polyvinylpolypyrrolidone (PVPP) and 0.1 mM ethylenediaminetetraacetic acid (EDTA). The homogenate was centrifuged at 6 000 × g at 4°C for 15 min and the resulting supernatant was used to determine the protein content and enzyme activity assays. The total protein content of the enzyme extracts was determined by the method of Bradford (1976), using 80 µl $H_2O$, 20 µl enzyme extract and 5 ml Comassie blue solution. Absorbance by the reaction mixture was read at 595 nm. All the spectrophotometric analyses were conducted at 25°C on a Shimadzu (UV 1800 CE) spectrophotometer.

CAT (EC 1.11.1.6) activity was measured following the Beers and Sizer (1952) method, with minor modifications. The reaction mixture contained 1 ml potassium phosphate buffer (50 mM, pH 7.0), 40 µl enzyme extract and 5 µl $H_2O_2$. The decrease in $H_2O_2$ was monitored at 240 nm and quantified by its molar extinction coefficient (0.036 $mM^{-1}$ $cm^{-1}$).

Total APX (EC 1.11.1.11) activity was measured by monitoring the decline in 290 nm as ascorbate was oxidized, for 90 s using the method described by Amako (1994). The reaction

mixture contained 1 ml potassium phosphate buffer (50 mM, pH 7.0), 5 mM ascorbate, 100 µl $H_2O_2$ and 40 µl enzyme extract. Enzyme activity was quantified using the molar extinction coefficient for ascorbate (2.8 $mM^{-1}$ $cm^{-1}$).

GR (EC 1.6.4.2) activity was determined by measuring the rate of $NADPH_2$ oxidation as the decrease in absorbance at 340 nm (ε = 6.2 $mM^{-1}$ $cm^{-1}$). The reaction mixture contained 0.5 ml potassium phosphate buffer (0.1 M, pH 7.0), 20 mM oxidized gluthatione (GSSG), 2 mM $NADPH_2$, 350 µl $H_2O$ and 50 µl enzyme extract.

POX (EC 1.11.1.7) activity was estimated following the change in absorbance at 436 nm for 90s with 20 µl guaiacol, 40 µl enzyme extract and 15 µl $H_2O_2$ in 1 ml potassium phosphate buffer (0.1 M, pH 7.0). POX activity was quantified by the amount of tetraguaiacol formed, using its extinction coefficient (ε) = 25.5 $mM^{-1}$ $cm^{-1}$.

For CAT, APX, GR and POX activities the results were expressed as enzyme units (U) per mg soluble protein. One unit of enzyme was defined as the amount of enzyme necessary to decompose 1 µmol of substrate per min at 25°C.

### *6.3.5. Statistical analysis*

Data were statistically processed using PASW Statistics 18 software. Comparisons of growth, ion content and antioxidant enzyme activities among salt concentrations were done separately for each species. All data were analyzed by one-way analysis of variance (ANOVA) and the mean differences were compared using Duncan's multiple range tests. In all cases, *P* values < 0.05 were considered significant.

## 6.4. Results

### *6.4.1. Effect of NaCl on growth parameters*

The growth of *A. longifolia* and *U. europaeus* plants differed in response to NaCl concentrations (Table 6.1 and 6.2). In general, salt concentrations were negatively correlated with plant height, root length and dry weight (DW) and positively correlated with WR:WS and WR:WRS. None of these correlations were statistically significant in *U. europaeus* (Table 6.2). Salinity caused a significant reduction ($P < 0.05$) in the height of *A. longifolia* plants. In fact, increasing salt concentration to 50, 100 and 200 mM resulted in a reduction of plant

height of 11%, 17% and 30% in comparison with the control plants. The plant height of *U. europaeus* was not affected by salt concentrations but reduction of about 16% was found at 200 mM NaCl, in comparison with the control plants.

**Table 6.1.** Effect of NaCl concentrations on plant height (cm), root length (cm), DW (g/plant), WR:WS and WR:WRS of *A. longifolia* and *U. europaeus* plants. Values are mean ± S.E. of six replicates.

| | | NaCl (mM) | | | |
|---|---|---|---|---|---|
| Species | Parameters | 0 | 50 | 100 | 200 |
| *A. longifolia* | Plant height | 20.6 ± 0.98a | 18.4 ± 1.82ab | 16.8 ± 1.26ab | 14.4 ± 0.99b |
| | Root length | 7.1 ± 0.80a | 7.7 ± 0.45a | 7.1 ± 0.74a | 6.1 ± 0.65a |
| | DW | 0.053 ± 0.009a | 0.045 ± 0.015a | 0.027 ± 0.003ab | 0.014 ± 0.002b |
| | WR:WS | 0.125 ± 0.015b | 0.241 ± 0.057a | 0.278 ± 0.021a | 0.261 ± 0.035a |
| | WR:WRS | 0.111 ± 0.012b | 0.186 ± 0.035a | 0.216 ± 0.013a | 0.203 ± 0.219a |
| *U. europaeus* | Plant height | 8.8 ± 2.02a | 9.9 ± 2.26a | 9.2 ± 0.98a | 6.6 ± 1.42a |
| | Root length | 4.7 ± 1.11a | 5.3 ± 0.37a | 4.5 ± 1.04a | 3.6 ± 0.95a |
| | DW | 0.018 ± 0.005a | 0.018 ± 0.005a | 0.015 ± 0.005a | 0.011 ± 0.003a |
| | WR:WS | 0.117 ± 0.016a | 0.123 ± 0.036a | 0.248 ± 0.098a | 0.140 ± 0.038a |
| | WR:WRS | 0.104 ± 0.013a | 0.106 ± 0.029a | 0.148 ± 0.053a | 0.118 ± 0.029a |

The same letter on each line indicates no significant difference at $P < 0.05$.

Root length of both species did not show a significant effect of salt concentrations ($P > 0.05$) but salt concentrations up to 100 mM tended to reduce root length in *U. europaeus*, whereas in *A. longifolia* the reduction was only observed at 200 mM NaCl in comparison with the control. At the highest salt concentration (200 mM), root length was more reduced in the native plants (-21%) than in the invasive ones (-11%). In fact, under this salt concentration *A. longifolia* had very long, thin roots with few root hairs, whereas the *U. europaeus* roots were very short and had numerous root hairs (data not shown). In terms of biomass production per plant, the increase in NaCl concentrations significantly reduced the dry weight of the *A. longifolia* plants ($P < 0.01$), with the maximum reduction at 200 mM NaCl (-71%). On the contrary, no significant difference in biomass was detected in *U. europaeus*.

**Table 6.2.** Pearson´s correlation coefficient (r) between salt concentration and growth, ion content and antioxidant enzyme activities in *A. longifolia* and U. *europaeus*

| Parameter | *A. longifolia* | *U. europaeus* |
|---|---|---|
| Plant height | -0.610** | -0.197 |
| Root length | -0.235 | -0.130 |
| DW | -0.593** | -0.240 |
| $W_R$:$W_S$ | 0.497** | 0.160 |
| $W_R$:$W_{RS}$ | 0.533** | 0.117 |
| $Na^+$ | 0.842*** | 0.963*** |
| $K^+$ | -0.826*** | -0.693* |
| $K^+/Na^+$ | -0.711** | -0.751** |
| CAT | 0.724** | 0.424 |
| APX | 0.393 | -0.637* |
| GR | 0.550* | -0.024 |
| POX | -0.183 | -0.131 |

Significant at *$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

In order to assess the effect of NaCl concentrations on root development, the WR:WS and WR:WRS were calculated (Table 6.1). Under the effect of NaCl concentrations the proportion of dry weight allocated to roots increased significantly in *A. longifolia* ($P < 0.05$) in comparison with the control. Conversely, in *U. europaeus* no apparent impact of salt concentration was found. In both species, the highest increase of WR:WS and WR:WRS was obtained at 100 mM. Overall, the increase in WR:WS and WR:WRS in the invasive species, *A. longifolia*, was proportionately higher than in the native species ($P < 0.05$).

### *6.4.2. Effect of NaCl on $Na^+$, $K^+$ and $K^+/Na^+$*

The ion content ($K^+$ and $Na^+$) in *A. longifolia* and *U. europaeus* shoots in the presence of NaCl is shown in Figure 6.1. In general, $Na^+$ was positively correlated with NaCl concentrations, with r values of 0.84 and 0.96 ($P < 0.001$) for *A. longifolia* and *U. europaeus*, respectively (Table 6.2). Both species accumulated significantly more $Na^+$ ($P < 0.05$) in response to increases in NaCl concentration, with the maximum value reached at 200 mM NaCl. It was also evident that *U. europaeus* accumulated more $Na^+$ than *A. longifolia*. The percentage of $Na^+$ distribution in *A. longifolia* was 190% and 414% of the control at 50 and 100 mM NaCl,

respectively, and 765% at 200 mM NaCl. On the other hand, in *U. europaeus* the percentage of $Na^+$ distribution was 334%, 525%, and 868% of the control at 50, 100 and 200 mM NaCl, respectively.

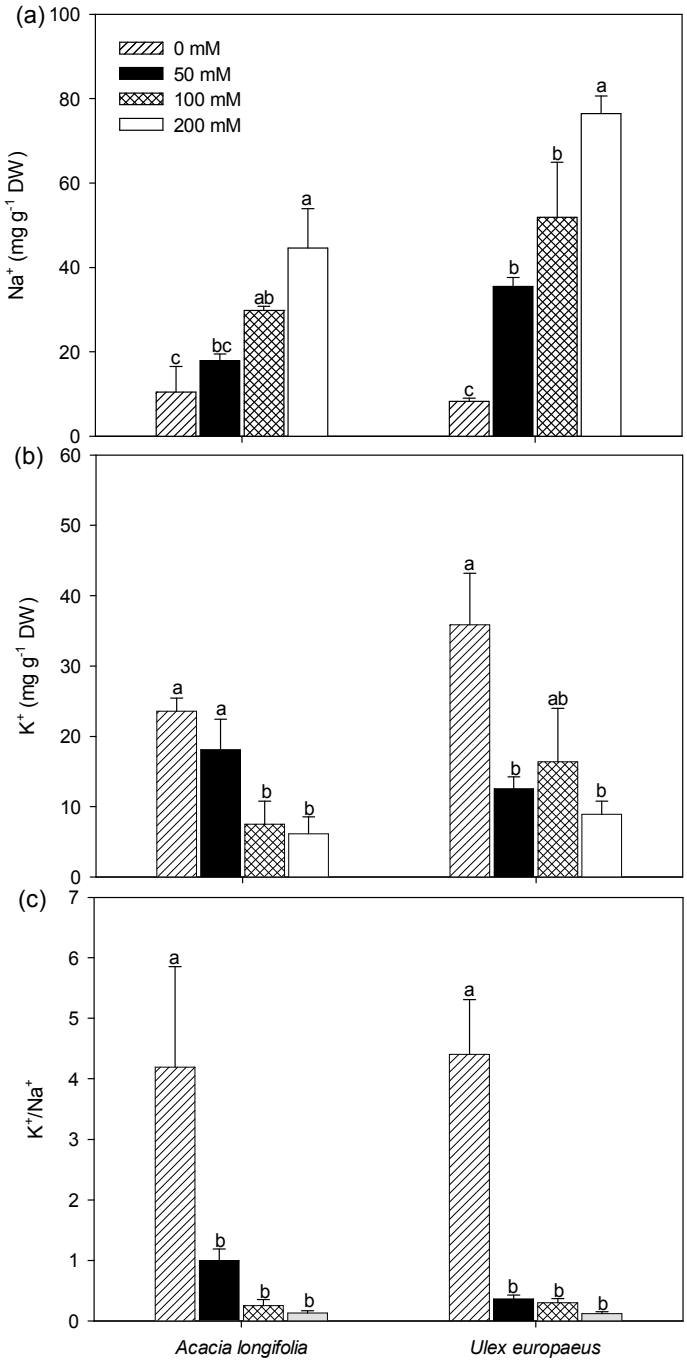


**Fig. 6.1.** Effect of NaCl concentration on (a) $Na^+$, (b) $K^+$ and (c) $K^+/Na^+$ in shoots of *A. longifolia* and *U. europaeus*. Vertical bars indicate mean ± S.E. of three replicates and for each species. Values sharing a common letter are not significantly different at $P < 0.05$.

Salinity significantly affected the concentration of $K^+$ in the shoots (Table 6.2), which decreased in line with salinity (r = - 0.83, $P < 0.001$ and - 0.69, $P < 0.05$, for *A. longifolia* and *U. europaeus*, respectively). Considering each species separately, a marked decrease ($P < 0.05$) of $K^+$ concentration in both species was observed as the NaCl concentration increased. Although the rate of $K^+$ reduction was not the same in the two species, under the higher salt concentration (200 mM NaCl) both species showed the same reduction percentage (-75%). As a consequence of the changes in $Na^+$ and $K^+$ concentrations, the $K^+/Na^+$ ratio significantly decreased in both species ($P < 0.05$) in response to an increase in the concentration of NaCl. The reduction was proportionately higher in *U. europaeus* than *A. longifolia*, particularly under 200 mM NaCl (-96%). Similarly, there was a significant negative relationship between the $K^+/Na^+$ ratio and NaCl concentration, with r values of 0.71 and 0.75 ($P < 0.005$) for *A. longifolia* and *U. europaeus*, respectively (Table 6.2).

### *6.4.3. Effect of NaCl on CAT, POX APX and GR activities*

The differences in the antioxidant enzyme (CAT, APX, GR and POX) activities of *A. longifolia* and *U. europaeus* in the presence of different NaCl concentrations are shown in Table 6.3. The results indicate that *A. longifolia* has higher levels of antioxidant enzyme activities in comparison with the native species.

CAT activity increased with increasing NaCl concentrations and was more pronounced in A. longifolia plants (r = 0.724, $P < 0.01$) (Table 6.2). At 200 mM NaCl, the invasive species showed values of activity that were 275 times higher than the control, whereas with a low NaCl concentration (50 mM) the increase was not as significant. In *U. europaeus*, CAT activity increased slightly with the NaCl concentrations and at 200 mM NaCl was about 4 times higher than the control but it was not statistically different in relation to the control (Table 6.3).

**Table 6.3**. Effect of NaCl concentration on the activities of CAT, APX, GR and POX activities (U/mg Protein) in shoots of *A. longifolia* and *U. europaeus*. Values are mean ± S.E. of three replicates.

| Parameter | Species | NaCl (mM) | | | |
|---|---|---|---|---|---|
| | | 0 | 50 | 100 | 200 |
| CAT | *A. longifolia* | 4.311 ± 1.952b | 93.630 ± 6.497b | 284.337 ± 155.738ab | 1187.146 ± 691.222a |
| | *U. europaeus* | 70.541 ± 45.489a | 141.952 ± 51.809a | 144.377 ± 94.773a | 273.330 ± 251.072a |
| APX | *A. longifolia* | 1.067 ± 0.418a | 0.916 ± 0.289a | 1.619 ± 0.463a | 1.605 ± 0.734a |
| | *U. europaeus* | 0.167 ± 0.039a | 0.201564 ± 0.029a | 0.097 ± 0.059a | 0.069 ± 0.039a |
| GR | *A. longifolia* | 0.389 ± 0.326a | 0.592 ± 0.539a | 0.636 ± 0.589a | 1.629 ± 0.635a |
| | *U. europaeus* | 0.083 ± 0.066a | 0.0152 ± 0.040a | 0.161 ± 0.022a | 0.080 ± 0.053a |
| POX | *A. longifolia* | 0.604 ± 0.402a | 0.390 ± 0.108a | 0.826 ± 0.620a | 0.170 ± 0.092a |
| | *U. europaeus* | 0.173 ± 0.055a | 0.168 ± 0.017a | 0.189 ± 0.004a | 0.144 ± 0.087a |

The same letter on each line indicates no significant difference at $P < 0.05$.

In *A. longifolia*, APX activity tended to increase as the NaCl concentration increased (r = 0.393, $P > 0.05$), whereas in *U. europaeus* a significant reduction (r = -0.637, $P < 0.05$) was observed with increasing concentrations of NaCl (Table 2). In *A. longifolia*, the 100 and 200 mM NaCl concentrations caused a 52% and 50% increase in total APX activity respectively, whereas the 50 mM NaCl reduced the activity of this enzyme by 14%. In *U. europaeus*, APX increased (20%) at 50 mM NaCl and decreased (-42% and -59%) at 100 and 200 mM NaCl, respectively. There was a higher rate of APX activity in *A. longifolia* than in *U. europaeus* under all salt concentrations.

The pattern for GR activity in *A. longifolia* was similar to CAT activity, *i.e.* it rose in line with the increase in NaCl concentrations (r = 0.550, $P < 0.05$) but the same was not observed in *U. europaeus*. GR in *U. europaeus* increased from control to 100 mM NaCl and slightly decreased (-4%) at 200 mM NaCl with no significant differences among salt treatments. Under the same NaCl concentrations, *A. longifolia* showed a higher rate of GR activity than *U. europaeus*.

In general, POX activity followed the same pattern in both species, *i.e.* tended to decrease with increasing concentrations of NaCl (Table 6.2). In both species, a decrease was observed from control to 50 mM NaCl and from 100 mM to 200 mM NaCl, with the highest value

reached at 100 mM NaCl. Comparing the two species, POX activity was higher in the *A. longifolia* under all NaCl concentrations.

### 6.5. Discussion

In this study, two legume species, one invasive, *A. longifolia*, and one native, *U. europaeus*, were submitted to increasing NaCl concentrations (0, 50, 100 and 200 mM NaCl), and clear differences were observed in growth parameters, concentration of $Na^+$ and $K^+$ and activities of antioxidant enzymes that are involved in the detoxification of ROS.

In general, plant height, root length and dry weight decreased with an increase in the NaCl concentration. However, the response of each species differed with respect to the measured parameter. In terms of plant height, increasing NaCl concentration gradually decreased the height of *A. longifolia* especially at higher concentrations (200 mM). *Ulex europaeus* grew well up to 100 mM, but growth was also inhibited at 200 mM NaCl. Reduction of plant height at high salt concentrations confirms the results of Al-Shaharani and Shetta (2011) in two *Acacia* species, *A. ehrenbergiana* and *A. tortilis*, as well as Amirjani (2010) in *Glycine max*. In both species, root length tended to decrease with increasing salt stress, but low salt concentration (50 mM NaCl) resulted in a small increase of this parameter. This suggests that root length of both species is less affected by increasing NaCl concentrations. Similar findings have earlier been reported by Amor *et al*. (2007) in *Crithmum maritimum*.

The increase in NaCl concentrations also reduced the dry weight of the two legume species, mainly in the invasive species. This finding has been reported in some *Acacia* species such as *A. holosericea* (Yokota 2003), *A. senegal* (Hardikar and Pandey 2008) and *A. ampliceps* (Yokota 2003; Marcar and Crawford 2011) and in several other plant species (Meloni *et al.* 2004; Yildiztugay *et al*. 2011). It is important to note that salinity also modified the biomass distribution in organs. The results for the dry weight of shoots and roots in response to increasing salinity, determined as the WR:WS and WR:WRS ratios, suggest that shoots were more severely affected by increasing NaCl concentrations than roots. Concurring with this study, Croser *et al*. (2001) in three conifers species, Amirjani (2010) in soybean as well as Berrichi *et al*. (2010) in jojoba showed that shoot growth was inhibited more than root growth in response to NaCl concentration. An increase in the WR:WS indicates that a plant

growing under less favourable conditions tends to allocate a large proportion of its photosynthate to storage organs, which are often subterranean (1996). This seems to be an adaptation to salinity, resulting in more efficient water and nutrient uptake under saline stress (1985) which is favourable for root expansion (2007). In this study, *A. longifolia* also showed higher WR:WS for all NaCl concentrations suggesting that its root system was expanding rather fast which may help the plant to adapt to salty habitats.

Reduced plant growth under salinity is probably the result of osmotic and/or ionic effects of salt (Greenway and Munns 1980; Marschner 1995; Hardikar and Pandey 2008; Nawaz *et al*. 2010). Salinity also causes ionic imbalances that may lead to potassium deficiency (Nawaz *et al*. 2010). In this study, NaCl caused an accumulation of $Na^+$ mainly in the native species. Conversely, an increase in the NaCl concentration was accompanied by a decrease in $K^+$ concentration. The same results have been reported for *Plantago crassifolia* (Vicente *et al*. 2004), *Acacia senegal* (Hardikar and Pandey 2008) and *Gypsophila oblanceolata* (Sekmen *et al*. 2012). This may be explained by the competitive interaction between $Na^+$ and $K^+$ and the inhibition of $K^+$ uptake caused by high levels of $Na^+$ (Hardikar and Pandey 2008; Nawaz *et al*. 2010; Sekmen *et al*. 2012) which reduce the capacity for osmotic adjustment and turgor maintenance and adversely affect metabolic functions (Greenway and Munns 1980). Regulation of $K^+$ uptake, prevention of $Na^+$ entry and efflux of $Na^+$ from cell are the strategies commonly used by plants to maintain desirable $K^+/Na^+$ ratio in the cytosol (Kim *et al*. 2004). The $K^+/Na^+$ ratio is considered one of the key selection criteria for salt tolerance (Amor *et al*. 2007). In the present study, the differential accumulation of $Na^+$ and $K^+$ under salt stress caused a significant reduction in the $K^+/Na^+$ ratio with increasing NaCl in both species, but mainly in the native species. This suggests that *A. longifolia* has more capacity to regulate $K^+$ transport during salt stress, which may be an important component of its salt tolerance.

The distinct $Na^+$ accumulation might have caused the different response in antioxidant enzyme activities involved in the oxygen metabolism during salt stress (Kim *et al*. 2004). Excess concentrations of ions associated with salinity can cause enzyme inhibition and therefore alter metabolism and/or physiological functions (Flowers *et al*. 1992). In general, osmotic and ionic stresses caused by salinity enhance the production of ROS in plants (Koca *et al*. 2007). In particular, it is known that $H_2O_2$ is a strong inhibitor of the Calvin cycle and for this reason must be eliminated by conversion to $H_2O$ in reactions involving CAT, APX, GR and

POX (Sorkheh *et al*. 2012). An increase in the activity of these enzymes could lead to an increase in antioxidative protection and a decrease in oxidative damage (Kim *et al*. 2004).

Our results showed that *A. longifolia* presented high activity levels of these enzymes in comparison with *U. europaeus*, which suggests that *Acacia* plants are more capable to scavenge the ROS generated by salt stress. On the other hand, it was evident that NaCl stress reduced APX activity and did not significantly affect CAT, GR and POX activities of *U. europaeus*. The reduction in APX activity could diminish the ability of these plants to cope with harmful concentrations of $H_2O_2$ and thus may become more sensitive to salt stress.

In the present study, CAT activity seems to have an important role in the mechanism of salt tolerance in *A. longifolia*. Compared to control, CAT activity showed a significant increase under saline concentrations, which might reflect its high capacity for decomposition of $H_2O_2$. Sekmen *et al*. (2007), Yildiztugay *et al*. (2011) and Sekmen et al. (2012) also observed salt-induced activity of CAT in *Plantago maritime*, *Centaurea tuzgoluensis* and *Gypsophila oblanceolata*, respectively. Besides CAT, GR is another important $H_2O_2$ detoxifying enzyme. The salt-induced enhancement of GR activity in *A. longifolia* plants also indicates that this enzyme may play a crucial role in defence mechanisms against oxidative stress, possibly in co-operation with CAT, by controlling the appropriate concentrations of salt. These results are consistent with those of Hernández *et al*. (2000) and Meloni *et al*. (2003). These authors have suggested that the salt tolerance character is related to increased GR activity in salt-tolerant cultivars. APX activity also increased as the NaCl concentration increase but the rate of increment was lower and not significant when compared to CAT and GR. From this point of view, it may be estimated that APX activity is not sufficient to prevent ROS production. In addition, unchanged POX activity as NaCl increased indicates that this enzyme is not crucial in removing $H_2O_2$ and does not play an important role in imparting tolerance against NaCl stress. A fall in POX activity due to salt stress was also demonstrated in *Calendula officinalis* (Chaparzadeh *et al*. 2004).

In conclusion, the differences in the root growth, CAT and GR activities, $K^+/Na^+$ ratio in the two sand dune legumes indicate that the mechanisms which protect the plant against salt-generated oxidative stress are more efficient in *A. longifolia* than in *U. europaeus*. A lower toxic ion accumulation associated with a higher capacity for oxygen radical scavenging can be considered as an important strategy for *A. longifolia* to grow under high NaCl

concentrations. These ecophysiological traits, combined with its competitive grow strategy in very dense monospecific stands may facilitate its capacity to spread and invade a wide range of habitats, displacing native species such as *U. europaeus*.

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# CHAPTER 7

## FINAL REMARKS

## 7. Final remarks

This chapter provides a general discussion of the research work that was undertaken, highlighting the most important conclusions of the thesis, and also plans for future research.

### 7.1. General discussion

*Acacia longifolia* is an invasive species that threatens the conservation of natural ecosystems in many regions of Portugal, especially coastal areas. Invasion of these ecosystems by *A. longifolia* has resulted in the development of woodlands where this species is dominant (Marchante *et al*. 2008b), along with profound impacts on the diversity of the native vegetation (Marchante *et al*. 2003), chemical and microbial properties of the soil (Marchante *et al*. 2008b) and the functioning of the ecosystem (Marchante *et al*. 2008a). The high growth rate (Peperkorn *et al*. 2005), vast and persistent seed bank (Marchante *et al*. 2010), great ability to establish interactions with soil mutualists, especially with symbiotic nitrogen-fixing bacteria (Rodríguez-Echeverría *et al*. 2009), and the ability to fix nitrogen (Rodríguez-Echeverría *et al*. 2007), are some of the attributes that are often responsible for its profound impacts on the invaded ecosystems (chapter 1). Climate change will definitely introduce changes into these ecosystems by disturbing the dynamic equilibrium (Thuiller *et al*. 2007), which may aggravate the impacts caused by invasive species. The IPCC (2007) predicts an increase in mean temperatures, a decrease in precipitation as well as changes in salinity over the next century in the Mediterranean region. *Acacia longifolia* will have to grow in a hotter, drier and, in some cases, more saline environments. Such changes are likely to have considerable biological effects on the species (Thuiller *et al*. 2007) but, very little is known about the consequences of these new conditions for the growth and establishment of *A. longifolia*. Within this context, this research attempts to determine the role of environmental stresses (drought and salt) on the growth of *A. longifolia*. According to the experiments, *A. longifolia* plants can survive under water-stressed conditions (chapter 4) and possesses important salt resistance mechanisms (chapter 5 and 6), which provide them with an additional advantage when competing with native species under limited environmental stress. Consequently, this may facilitate their capacity to spread and invade a wide range of habitats, displacing native vegetation. In addition, *A. longifolia* plants are able to maintain their reproductive capacity even in the presence of environmental constraints (chapter 1),

which provides them with an advantage in terms of responding to climate change. Therefore, the main challenge is predict the spread of *A. longifolia* and, on the other hand, to apply effective control strategies to reduce the impact of *A. longifolia* on ecosystems. Prevention is by far the most cost-effective and successful means of managing invasive plants (Pyšek and Richardson 2010) and depends on identifying the plant traits that facilitate invasive success (Davies and Sheley 2007). The success of a control strategy also depends largely on the attributes of the invasive species (van Wilgen *et al*. 2001) that influence its growth and spread over a particular area. Therefore, an understanding of its phenology and variations according to climate factors (chapter 2), as well as quantification of the productivity of this species (chapter 3) represents a valuable tool for management decisions since it enables managers to determine the best control strategies and the optimal timing for their application.

One of the major challenges facing ecological studies today is to understand the impact of climate change in the development of invasive species. Many studies have documented the fact that climate change can increase opportunities for the establishment of invasive species (Dukes and Mooney 1999; Clarke 2007) of those already present, or others that do not display invasive behaviour at present but which reveal it when facing new climatic conditions. In either situation, successful invasion is associated with a wide variety of traits that give the plants a competitive edge over the native species in the community into which they are introduced (Cadotte and Lovett-Doust 2002). Traits associated with flowering and reproductive biology (Pyšek and Hulme 2005) and with ecological tolerance (Reynolds and Cooper 2010) are significantly related to successful plant invasion. Integrated phenological observations might therefore be the key to understanding the effects of climate change on the biological traits of invasive species.

The phenological pattern of *A. longifolia* was studied aiming at describing the vegetative and reproductive phenophases and their association with climatic parameters (chapter 2). In general, the phenological activity of *A. longifolia* extends throughout the year with reproductive phenophases occurring in the following sequence: summer to winter: appearance and growth of flower buds in axils of phyllodes; winter to early spring: flowering; spring to summer: fruiting. Previous studies have indicated that native species usually flower in spring in the Mediterranean region (Castro-Díez and Montserrat-Martí 1998) and

therefore the early flowering of invasive species represents an important advantage in terms of their success in this region and is often considered an important trait associated with invasiveness (Pyšek and Richardson 2007). This study also indicates that there is a geographical variation in the distribution of the reproductive phenophases. In general, the reproductive phenophases in *A. longifolia* populations from the northern part of Portugal, which is characterized by a wet climate, tend to appear about one month later than those in populations from the dry climate region, reflecting not only genetic variation but also year-to-year climatic variability as observed by Fenner (1998). Among the climatic factors, temperature and precipitation seem to be the most influential factor for the earliest display of flowering and development of the fruiting phenophase, respectively. Since the occurrence of periods of water stress is expected to intensify in the future climate change for the Mediterranean region (IPCC 2007), it will have serious implications for the reproductive success of this species and could affect its presence in the drier regions of the south of Portugal. This finding is in line with the results of the experiment presented in chapter 4, in which *A. longifolia* from the more extreme populations studied in the previous experiment, *i.e.* from the wet (northern) and dry (southern) climate regions of Portugal, were submitted to short and long periods of water scarcity for a period of three months.

In this experiment there was some evidence that *A. longifolia* plants present higher tolerance to short periods of water stress, revealing an increase in some morphological parameters. Unlike other studies which reported a high tolerance to water stress related to greater root development and consequently a high root:shoot ratio (Funk and Vitousek 2007; Davidson *et al*. 2011), this invasive species revealed a lack of plasticity in this trait, as previously observed by Werner *et al*. (2010) in a water stress study. In this experiment the higher tolerance to short periods of water stress was related to the increase in plant height, shoot height, dry weight, shoot dry weight and leaf area that can be seen as an important adaptive mechanism of *A. longifolia,* promoting plant growth until almost all the water in the soil is exhausted. However, the same result was not observed under prolonged periods of water stress to which *A. longifolia* plants seem to be less tolerant, and this finding was more evident in plants from the drier regions of the south of Portugal. Although the *A. longifolia* plants from the southern population showed less reduction in growth in comparison with the northern population, they also showed a lower accumulation of some inorganic ions

($Mg^{2+}$ and $K^{+}$) and a lower SLA and LAR, which are considered to play an important role in minimising the adverse effects of water stress (Boughalleb and Mhamdi 2011). As a result, *A. longifolia* plants from the northern population are better able to acclimatise to severe water stress conditions than plants from regions with a drier climate.

This result does not concur with the general hypothesis that plants growing in dry climate regions can survive water deficits more effectively than those in wet climate (Lei *et al*. 2006). Given that the climatic conditions in the southern population are more harmful to plant development (less precipitation, periods of drought stress more intense and higher temperatures), it is expected that these different environmental constraints will produce severe drought stress in plants. Based on these results, the severe drought conditions predicted for the Mediterranean region could limit the expansion of *A. longifolia* and this invasive species would probably have greater difficulty to spread towards the drier regions in the southern parts of Portugal, due to the probability of prolonged and severe drought events.

Another possible effect of climate change is salinization (IPCC 2007), which can influence the distribution of *A. longifolia* and its invasive success (chapter 5 and 6). In these experiments, the seed germination and seedlings of *A. longifolia* and a native species, *Ulex europaeus*, which is very common in the sand dunes of the coast of Portugal where the spread of *A. longifolia* is more evident, were evaluated in order to determine their capacity to acclimatise to this new condition. Therefore, effects of salinity (0, 50, 100 and 200 mM NaCl) on germination and changes in the activities of antioxidant enzymes were determined (chapter 5). The results showed that *A. longifolia* and *U. europaeus* seeds behaved differently in terms of germination. Overall, seeds germination was delayed and reduced with an increase in salinity. *A. longifolia* seeds germinated well under salinity, with a slight decrease in the presence of a high concentration of salt (200 mM NaCl). On the other hand, seeds of *U. europaeus* were less tolerant to salt, since they completely failed to germinate under the highest salt concentration. Moreover, the results of this experiment also presented some indications that *A. longifolia* seeds seem better equipped to handle the physiological stress, presenting a high APX enzyme activity. This finding can be considered an efficient antioxidant mechanism which protects the *A. longifolia* seeds against radicals that cause metabolic damage and inhibit seed germination.

Using both species, their growth, ion content and antioxidant enzymes activities responses to the same NaCl concentrations were also investigated (chapter 6). Generally, the obtained results confirm that salinity is an important environmental constraint for the survival of plants (Sekmen *et al*. 2012) by creating numerous morphological, physiological and biochemical disturbances (Levitt 1972). Given the results of this experiment, there were significant differences in growth parameters, concentration of $Na^+$ and $K^+$ and activities of antioxidant enzymes between the two species with increasing NaCl concentrations. In light of this, it is likely that *A. longifolia* used a wide variety of mechanisms to tolerate salt stress, such as a higher allocation of assimilates to roots and a lower toxic ion accumulation associated with a higher capacity for oxygen radical scavenging. Thus, it is possible to consider *A. longifolia* more capable of growing under higher salt concentrations in comparison to the native species, *U. europaeus*. The ability of *A. longifolia* to tolerate salt will play an increasingly important role in the long-term persistence of these plant populations in coastal environments and could have noticeable implications on the native plant communities. Based on these findings, it could be expected that *A. longifolia* can spread along the coast, displacing native species such as *U. europaeus*.

Taken as a whole, the results of these experiments showed that *A. longifolia* plants were capable of surviving under the stressful climatic conditions and, therefore, it will be more adaptive to extreme environmental conditions predicted to occur in a future climate change scenario. In this scenario, the ability of *A. longifolia* to tolerate such stressed conditions can be considered an important factor in the invasiveness of this species and may facilitate its long-term establishment. Moreover, their capacity to modify soil nutrient composition (Marchante *et al*. 2009), nodulate profusely (Rodríguez-Echeverría *et al*. 2009), fix nitrogen (Rodríguez-Echeverría *et al*. 2007) combined with competitive grow strategy in very dense monospecific stands (Marchante *et al*. 2003) can have important implications for ecosystem productivity by altering the biomass storage and dynamics.

Although this thesis has not addressed management issues, the above-mentioned results will lead to more enlightened perspective for effective control of this invasive species and its invaded areas. In this context, knowledge of biomass production of this species (chapter 3) plays a key role in the design of such strategies. The results of the biomass experiment showed that it is possible to determine the biomass production accurately for *A. longifolia*

by using allometric equations with certain morphological measures easily obtained in the field. The models and procedures presented in the experiment represent a necessary baseline for implementation in future *A. longifolia* control strategies, with the view to increase effectiveness and to reduce adverse impacts of its presence.

### 7.2. Conclusion

The results reported in this thesis provide a better understanding of the biology of *A. longifolia* and clarify how climate change affects its invasion dynamics, which is useful in the design of control strategies aimed at controlling its impacts on native species and the environment. In general, the main conclusions are the following:

1. The phenological pattern of *A. longifolia* in Portugal generally follows a geographical variation. Temperature, precipitation and irradiance are the major driving forces behind its phenology. This suggests that climate change can alter the current distribution of *A. longifolia* (chapter 2).
2. The biomass quantification of *A. longifolia* can be determined accurately by morphological parameters, namely: diameter at base and plant height, which represents a useful approach of calculating the production of areas invaded by this species (chapter 3).
3. Regardless of their origins, *A. longifolia* seedlings, can withstand water stress conditions, although this response is greatly dependent on the severity of the drought. Generally, *A. longifolia* seedlings are less resistant to prolonged drought periods (chapter 4).
4. The drought response of *A. longifolia* seedlings reflects a genetic influence and can limit the expansion of *A. longifolia* in the more arid environments of the south of Portugal (chapter 4).
5. *Acacia longifolia* seeds and seedlings are more tolerant to salinity in comparison to the native species, *U. europaeus* (chapter 5 and 6). The better performance of *A. longifolia* in relation to the native species is explained by its highly efficient antioxidant system.

Despite the usefulness of these results in the control of *A. longifolia*, they are an excellent starting point from which several areas of research could be developed towards a full understanding of the mechanisms underlying its invasion success, such as:

a) The application of phenological data to the development of remote sensing and GIS applications for monitoring the distribution of this invasive species across large spatial areas.
b) The construction of models based on phenological data that may help to predict the consequences of increasing temperature on the distribution of *A. longifolia*.
c) Variations in *A. longifolia* aboveground biomass over time. For this purpose, measurements should be taken in different seasons. Such information will open up new opportunities for designing the best control strategies for this invasive species.
d) Competitive interaction between *A. longifolia* and native species in salinity experiments. Such knowledge might enable us to get more insights into the factors that drive *A. longifolia* invasion.
e) The effects of drought and temperature interaction on the morphological, physiological and biochemical attributes of *A. longifolia*. Different developmental stages should be considered in these experiments. Future investigation in this area will extend knowledge of the behaviour of *A. longifolia* to the different unfavourable conditions predicted for the Mediterranean region in the coming decades.

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