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DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Reproductive Biology of Australian acacias in Portugal

Marta Cardoso Lopes Correia

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Reproductive Biology of Australian acacias in Portugal

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia, ramo de especialização Ecologia Aplicada, realizada sob a orientação científica da Professora Doutora Cristina Nabais (Universidade de Coimbra), da Doutora Susana Rodríguez-Echeverría (Centro de Ecologia Funcional, Universidade de Coimbra) e da Doutora Sílvia Castro (Centro de Ecologia Funcional, Universidade de Coimbra).

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" Contar os dias pelos dedos e encontrar a mão cheia."

José Saramago in Cadernos de Lanzarote

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Resumo

O tipo de sistema reprodutivo das plantas e as suas características reprodutivas desempenham um papel chave no processo de invasão por plantas exóticas. Uma reprodução bem-sucedida é fundamental para o estabelecimento de populações viáveis e capazes de se expandirem. Foi teorizado que as plantas auto-compatíveis têm vantagem no estabelecimento de populações em novas áreas porque a reprodução é menos restrita, quer pelo tamanho da população quer pela disponibilidade de polinizadores. As acácias australianas estão entre as plantas invasoras mais difundidas, sendo conhecidos e bem estudados os seus impactos negativos que provocam uma alteração na estrutura e funcionamento dos ecossistemas. Estas espécies podem provocar a homogeneização ecológica e uma redução da biodiversidade. São por isso excelentes modelos para o estudo das invasões biológicas e podem ajudar a explorar os determinantes e as dinâmicas da invasão. As acácias australianas são geralmente consideradas como as plantas mais problemáticas e invasoras em Portugal. Tendo em conta a área ocupada e o impacto causado sobre os ecossistemas nativos as mais agressivas são: a *Acacia dealbata*, *A. longifolia*, *A. melanoxylon* e *A. saligna*. Sendo a reprodução um mecanismo essencial para o estabelecimento das espécies exóticas, existe um total desconhecimento sobre a biologia da reprodução destas espécies nas áreas invadidas. Na área de distribuição natural, estas espécies são auto-incompatíveis e têm uma preferência clara pela polinização cruzada.

Neste estudo, as características florais, o sistema reprodutivo e a performance da descendência (sementes e plântulas) foram caracterizados em populações naturais na área invadida para as quatro espécies de acácia. Diferentes tratamentos de polinização envolvendo a exclusão dos polinizadores, a polinização suplementar, e autofecundação obrigatória, foram realizados para avaliar a auto-incompatibilidade e a limitação de pólen. A produção de frutos e sementes, o peso das sementes e a sua capacidade de germinação, e o crescimento das plântulas foram avaliados para os diferentes tratamentos. Os resultados deste trabalho mostram que as diferentes espécies de *Acacia* têm diferentes investimentos na produção de unidades reprodutivas (flores) e diferente sucesso reprodutivo natural. A *A. dealbata* apresentou um maior investimento na produção massiva de flores e um maior sucesso reprodutivo natural. Este resultado pode explicar, parcialmente, o facto de esta ser a mais agressiva de todas as espécies invasoras estudadas em Portugal. Uma estratégia reprodutiva diferente, a andromonoicia, foi encontrada para a *A. melanoxylon,* contrastando com as outras espécies que são na sua maioria hermafroditas. Todas as espécies revelaram ser parcialmente auto-compatíveis, embora haja uma grande variabilidade entre os diferentes indivíduos. O sistema reprodutivo destas espécies é caracterizada por um baixo vingamento do fruto e, consequentemente, um grande desperdício dos recursos investidos na produção de flores. A produção de sementes pode ser limitada pela disponibilidade de recursos e factores ambientais. *A. dealbata* e *A. longifolia* mostraram sofrer de limitação de pólen. A origem do pólen pode afectar o sucesso da descendência, causando uma menor viabilidade para a descendência obtida por autofertlização em *A. dealbata* e *A. melanoxylon*. No entanto, para *A. saligna*, a espécie mais auto-compatível, verificou-se que a descendência produzida por autofertilização tem o mesmo vigor que a obtida nos tratamento de polinização cruzada. Apesar do sucesso reprodutivo baixo, as diferentes espécies de acácias obtêm uma grande produção de sementes. Assim, as acácias australianas mostram uma baixa eficiência na utilização dos recursos, mas uma reprodução eficiente capaz de formar um prolífico banco de sementes.

O conhecimento da biologia reprodutiva de acácias australianas invasoras pode contribuir para o seu controlo eficaz. Estudos, como os de previsão dos impactos da introdução de novas espécies e os de avaliação dos danos causados por espécies invasoras devem considerar o seu sistema reprodutivo.

Palavras-chave: Biologia das invasões; Sistema reprodutivo; Acácias Australianas invasoras; Limitação de pólen; Sucesso reprodutivo.

Abstract

Reproductive traits play a key role in the invasion by exotic plants because successful reproduction is fundamental for the establishment of self-replacing populations. It has been theorized that self-compatible plants have an advantage for a successful establishment in a new range because reproduction is less constrained by population size and pollinator availability.

Australian *Acacia*s are among the most widespread invasive plants and have negative impacts in ecosystems structure and functioning, triggering ecological homogenization and reducing biodiversity. Thus, they are excellent models to study the biological invasions and explore the determinants of invasiveness.

In Portugal, Australian *Acacia*s can be considered as the most problematic and widespread invasive plants, considering the area occupied, aggressiveness and impact on native ecosystems and among them are *Acacia dealbata*, *Acacia longifolia, Acacia melanoxylon* and *Acacia saligna*. Even thought reproductive success is an essential factor in the colonization of new areas and long-term establishment of viable populations, no information, on any aspects of their reproductive biology was available in Portugal. In the native range, these species are mostly self-incompatible and have a clear tendency for outcrossing.

In this study, floral traits, breeding system and reproductive outcome were characterized in natural populations from the invaded range for the four *Acacia* species. Hand pollination experiments, involving pollinator exclusion, supplementary pollination, and obligate selfing were carried to assess self-incompatibility and pollen limitation. Fruit and seed set, seed mass and germinability, and seedling growth were evaluated for self- and cross-pollination treatments.

The results of this work show that the different *Acacia* species have different investments in the production of reproductive units (flowers) and in natural reproductive success. The massive flower production and the highest natural reproductive success of *A. dealbata* can partially explain why it is the most aggressive invader of all the studied species in Portugal. A different reproductive strategy, andromonoecy, was found in *A. melanoxylon*, contrasting with the other species that are mostly hermaphroditic. All species revealed to be partially self-compatible, although there is a high variability between individual trees.

The reproductive system of these species is characterized by a low fruit set and, consequently, a great sacrifice of floral resources. Seed production is likely to be limited by resources availability and environmental factors. *A. dealbata* and *A. longifolia* suffered from pollen limitation. The origin of pollen may affect offspring success with self-progeny having lower viability in *A. dealbata* and *A. melanoxylon*. However, *A. saligna,* the most self-compatible species, has a self-progeny as fit as the outcross-progeny.

Despite their low reproductive success, they achieved a great production of seeds due to their massive flower production. Hence, Australian *Acacia*s showed a low efficiency in the use of resources but a successful reproduction capable of providing a prolific seed bank.

The knowledge of the reproductive biology of invasive Australian *Acacia*s is fundamental to help in their effective control and should be included in screening protocols for predicting invasiveness.

Keywords: Biological invasions, Breeding system, Invasive Australian *Acacia*s, Pollen limitation; Reproductive success

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1. Introduction

Biological Invasions

Biological invasions can be defined as the processes by which species, with no historical record in an area, mostly through human-assisted introductions breach biogeographic barriers, establish new populations and extend their range (Richardson *et al.,* 2000). The established self-perpetuating populations become integrated into native communities and in many cases disrupt their functioning (Richardson *et al.,* 2000). Invasion by exotic species occur in all taxonomic groups and can affect all types of ecosystems (Elton, 1958; Vitousek, 2001; Perrings *et al.,* 2010). Although biological invasions can occur naturally through the arrival of propagules to a new region, the rate at which they are currently happening is clearly the result of human activities (Lodge, 1993; Rejmánek, 1996; Ewel *et al.,* 1991; Cronk and Fuller, 1995). The current rate of human trade and travel has accelerated the exchange of species among different regions, while human disturbances make ecosystems more susceptible to invasion by alien species (Richardson *et al.,* 2004).

Charles Elton was the first to recognize biological invasions as a problem that could lead to a worldwide biological homogenization (Elton, 1958). Since then, alien species have been recognised as one of the most important threat to biodiversity at the global level after habitat loss (Millenium Ecosystem Assessment 2005; Pysek and Richardson, 2010). Also, invasive species have negative effects on socioeconomic, cultural, and human health aspects by affecting all four categories of ecosystem services: supporting (i.e., alteration of succession patterns and soil and nutrient cycling), provisioning (i.e., threats to native species, alteration of genetic resources), regulating (i.e., changes in pollination services and fire regimes, vectors of diseases) and cultural services (i.e., effects on ecotourism, changes in perception of landscape) (Millennium Ecosystem Assessment, 2005; Pysek and Richardson, 2010; Vilá *et al.,* 2010). In many parts of the world, integrated strategies to reduce current and future impacts of biological invasions are currently being implemented (Pysek and Richardson, 2010). In Europe, the research project DAISIE (Delivering Alien Invasive Species Inventories for Europe) funded by the European Union in 2005 was the first international attempt to create an inventory of alien species that threaten European terrestrial, freshwater, and marine environments (Hulme *et al.,* 2009). The European Environment Agency has also been working together with the European member countries towards the development of common regulations to prevent and mitigate biological invasions.

From another perspective, biological invasions are large-scale natural experiments that provide challenging opportunities in ecological research. Thus, Invasion Ecology, or the study of the ecology of biological invasions, is a growing scientific discipline that aims at a) explaining how exotic species become invasive in new geographical areas and the impact they have in the invaded ecosystems and at b) developing early-detection and control tools for invasive species.

Among invasive organisms, vascular plants are the most intensively studied taxonomic group in Invasion Ecology (Pysek *et al.,* 2008). Several studies have shown that exotic plants that become invaders can cause profound changes in ecosystem structure and dynamics and lead to the displacement of native species (Yelenik *et al.,* 2004; Callaway *et al.,* 2005; Hierro *et al.,* 2005). The management and prevention of problematic introduced plant species can be improved by a better understanding of the intrinsic plant traits and the extrinsic factors that are associated with invasiveness at various scales (Richardson and Pyšek, 2006).

Richardson *et al.,* (2000) defined three key steps in the invasion process: introduction, naturalization and invasion. **Alien species** (synonyms: exotic plants or non-native plants) are **introduced**, intentionally or accidentally, in a new area as a result of human activity. Some alien species may establish and reproduce occasionally in this new area; however, most of them need repeated introductions to persist because they cannot form self-replacing populations (at this stage they are called **casual alien species**). When alien species gain the ability to reproduce consistently and sustain populations over many life cycles without direct human action in natural or semi-natural ecosystems, they are considered **naturalized.** A naturalized species can remain stable during a variable time until some change or disturbance rapidly stimulates an increase in their distribution range. The alien species reaching this phase are considered **invasive**. This state is characterized by the ability to recruit reproductive offspring, often in large numbers and at considerable distances from the introduction site and by the potential to spread over a considerable area

The progress of invasion depends not only on a specific combination of characteristics of the introduced species and of the ecosystem invaded, but also on disturbances affecting the transition between the different phases (Cohlen, 2002; Devinand Beisel, 2007). Any disturbance, natural or anthropogenic, which creates empty niches or leads to the introduction of essential mutualists, might help an exotic species to progress to the next phase. In summary, to become invasive, an organism needs to overcome a series of barriers, namely: geographical barriers, environmental barriers (abiotic and biotic) at the site of introduction, reproductive barriers, dispersal barriers, environmental barrier(s) in human-modified or alien-dominated vegetation, and finally environmental barriers in natural or semi-natural vegetation (Figure 1A).

Only a small percentage of introduced exotic species become invasive. Roughly, it is assumed that only 10 % of introduced species will become naturalized and only 10 % of those will become invasive (Pysek and Richardson, 2008). Despite of the clear classification of the invasion process defined by Richardson *et al.,* (2000), the process is rather complex and several other authors have proposed different key phases (e.g., arrival, establishment, dispersion and stabilization, Ricklefs, 2005, Davis, 2009, Reise *et al.,* 2006; introduction, naturalization, facilitation, increased distribution and stabilization, Marchante, 2001; introduction, establishment, naturalization, dispersal, population distribution and dispersal, Henderson *et al.,* 2006).

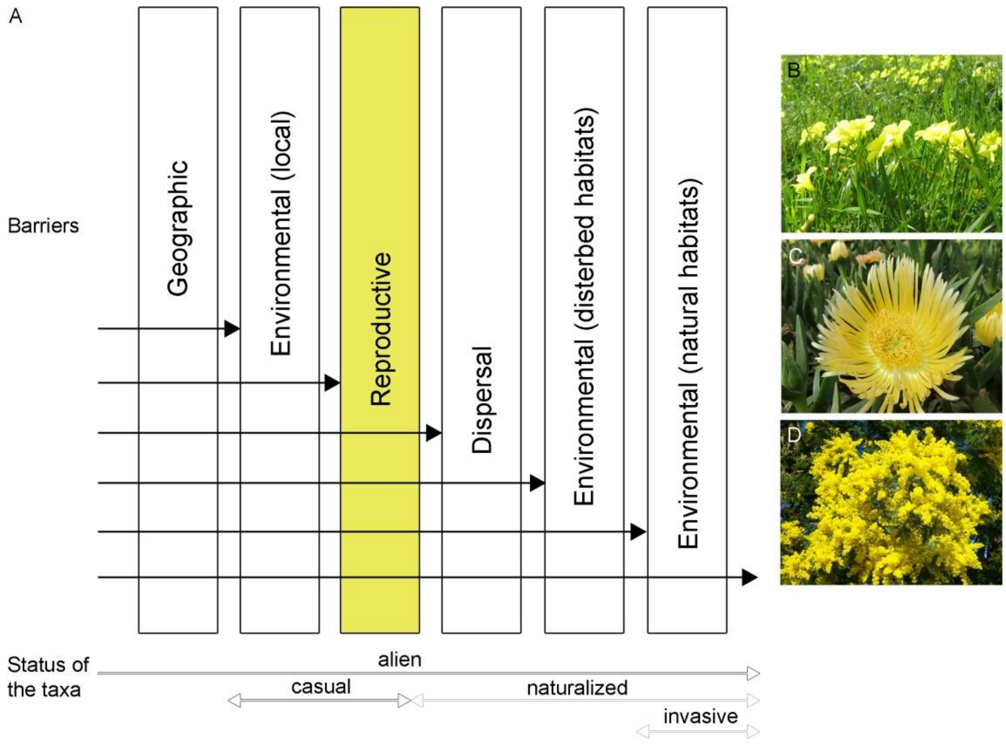

Figure 1. A: Schematic representation of the barriers that a species has to cross to become invasive after initial introduction. Reproductive barriers are highlighted because they are the subject of this study (adapted from Richardson et al., 2000). Figure **B, C** and **D:** Example of Invasive plants in Portugal (*Oxalis pes-caprae*, *Carpobrotus edulis* and *Acacia dealbata* respective.

Some trends in plant traits have been found for invasive plants. Plant traits related to seedling emergence, growth form, growth rate, breeding system, dispersal and environmental tolerance are important in predicting whether a species will become invasive (Thuiller et al., 2006; Pyšek and Richardson, 2007; van Kleunen and Johnson, 2007). Invasive species generally have a high sexual reproductive capacity, ability to reproduce asexually, rapid growth from seed to sexual maturity, a great dispersal and colonization efficiency, a high tolerance to environmental heterogeneity and disturbances, a high adaptation to environmental stress (phenotypic plasticity) and a greater competitive capacity than native species (Sakai et al, 2001; Vilá and Weiner, 2004, Werner et al., 2009). Several studies have also shown that invasive species have a larger ability to explore the resources in the receiving community as compared to native species (Holway, 1999; Sakai et al., 2001). On the other hand, ecosystem susceptibility to invasion is influenced by resource availability, climate similarity between source and target regions, availability of mutualistic symbionts (Crawley, 1987; Davis et al., 2000; Thuiller et al., 2005), and absence of herbivores and pathogens that control the invasive species in its native range (Richardson et al., 2000b; Lockwood et al., 2005).

Invasive plants: the reality in Portugal

"Portugal has the reputation of being particularly "rich" in aggressive alien plants and that reputation is fully confirmed. From *Eucalyptus* to *Carpobrotus*, many naturalized exotics work together in putting the country's rich native flora at risk" (Greuter, 2002).

Nowadays, alien species represent more than 15 % of the Portuguese vascular flora, which includes a total of ca. 3200 species and subspecies (Franco, 1971, 1984, 1994, 1998; Almeida, 1999). They were intentionally introduced for food, gardening, forestry, sand stabilization or industrial purposes (Almeida and Freitas, 2001). The environmental problem posed by alien species was recognized in 1999 by Portuguese legislation (*dec. - lei 565/99*). With this legislation the government provided a list of the exotic species introduced, identified the invasive species, and forbidden the introduction of new exotic species unless proven not to be harmful. Among the 550 species of exotic plants introduced in Portugal and currently considered as invasive or sub-spontaneous, about 400 were listed in Portuguese legislation and 30 were considered invasive. In Figure 2 B, C and D three invasive plants from different families (Oxalidaceae, Azoiaceae and Leguminosae respectively) are represented

Among all the introduced species and considering the area occupied, aggressiveness and impact on the native ecosystems, Australian *Acacia*s are considered the most problematic and widespread invasive plant species in Portugal (Almeida and Freitas, 2006).

In Portugal, there are 14 *Acacia* species currently recorded, 13 of which are Australian species (*A. baileyana* F. Muell.; *A. cultriformis* A. Cunn. ex G. Don; *A. cyclops* A. Cunn. ex G. Don fil.; *A. dealbata* Link; *A. decurrens* (J.C. Wendl.) Willd.; *A. longifolia* (Andrews) Willd. *A. mearnsii* De Wild. *A. melanoxylon* R. Br.; *A. pycnantha* Bentham; *A. retinodes* Schlecht.; *A. saligna* (Labill.) H.L. Wendl.; *A. sophorae* (Labill.) R. Br.; *A. verticillata* (L' Hér.) Willd.) and one is African species (*A. karoo* Hayne) (Marchante, 2001; Almeida and Freitas, 2006). Six of these species are classified as invasive by the Portuguese law (Ministério do Ambiente, 1999).

Australian *Acacia*s

The genus *Acacia* belongs to the family *Leguminosae,* sub-family *Mimosoideae*, and includes more than 1,350 bush and tree species (Maslin *et al.,* 2003). *Acacia* is a cosmopolitan genus distributed in the Australia-Pacific region, throughout the south of Asia, Africa and in North and South America. The genus occupies vast areas of these regions and can be found in a wide range of different habitats, from coastal to subalpine regions, and from high rainfall to arid inland areas, growing in tropical, subtropical and warm temperate regions (Maslin and Macdonald, 2004). Australian *Acacia*s include 1,012 species native to Australia, which were previously grouped in *Acacia* subgenus *Phyllodineae*.

1.3.1. Invasion process by Australian Acacias

In the last 250 years numerous species of *Acacia* have been introduced throughout the world, mostly for forestry or ornamental purposes, and several of them have become invasive in several countries like South Africa (Roux, 1961; Witkowski, 1991; Yelenik *et al.,* 2004), Portugal (Marchante, 2001) or Spain (Díaz *et al.,* 2007). As other nitrogen fixing legumes, *Acacia*s are particularly successful and invasive plants in Mediterranean climate and nutrient-poor ecosystems (Stock *et al.,* 1995). Their fast germination and seedling growth also contributes to their colonizing success (Ralp, 2003). Australian *Acacia*s were shown to have severe impacts on the invaded ecosystems due to a high production of litter, fixation of nitrogen, high germinability of seeds following a fire, allelopathic potential, high water consumption and high biomass yield and density (Levine *et al.,* 2003; Lorenzo *et al.,* 2010). Several studies have already shown that the invasion of ecosystems by *Acacia*s leads to significant changes in species richness, community structure, nutrient cycling, ecosystem productivity, food webs, mutualistic interactions, fire regimes and water availability (Levine *et al.,* 2003; Marchante, 2001; Marchante *et al.,* 2003; Marchante *et al.,* 2008; Rodriguez-Echeverria, 2010, Rodriguez-Echeverria *et al.,* 2012).

The worldwide exchange of Australian *Acacia*s has created an opportunity to explore how evolutionary, ecological and historical factors interact to affect the distribution and invasiveness of this group of plants. Therefore, it is currently considered a **model system** in Invasion Ecology (Richardson *et al.,* 2011). There are 23 Australian *Acacia* species that have become invasive in many parts of the world (Richardson and Rejmánek, 2011). Some invasive *Acacia* species are classified as "transformers" (Richardson *et al.,* 2000b) because they can change the structure and functioning of ecosystems over large areas altering important ecosystem properties such as nutrient content and cycling or fire regimes (Richardson and van Wilgen, 2004). In addition, after *Acacia*s are established and widespread, their eradication is considered to be virtually impossible due to the massive long-lived seed banks that they produce (Richardson and Kluge, 2008; Wilson *et al.,* 2011).

In spite of their fast expansion and ecological impacts in the invaded areas, little is known about their invasive dynamics. Enemy release might partially explain its success, but *Acacia*s are involved in many other biotic interactions that are essential for the colonization of new areas and long-term establishment of viable populations. For example, belowground mutualisms (with mycorrhizal fungi and symbiotic nitrogen-fixing bacteria) are crucial in the expansion of Australian *Acacia*s in new areas (Rodriguez-Echeverria *et al.,* 2009, 2012). Other factors such as, life-history traits, genetic variability, propagule pressure, repeated number of introductions and human usage have also been suggested to explain the invasive success of Australian *Acacia*s (Castro-Díez *et al.,* 2011; Gallagher *et al.,* 2011).

1.3.2. Reproduction and invasiveness

All above mentioned factors refer to the establishment and growth of the exotic plant in a new area; however, in order to establish self-replacing populations, exotic species also need to reproduce successfully in the new areas. Therefore, reproductive characteristics and reproductive success are crucial steps in invasion (Thuiller *et al.,* 2006; Pyšek and Richardson, 2007; Figure 1A). Still, despite of its importance, reproductive biology has been examined in detail in only a limited number of *Acacia* species belonging to subgenus *Phyllodineae* and mostly in their native geographic range (Kenrick, 2003; but see Gibson, 2012). The species studied so far show similar general floral characteristics although differences between species can also be found (Kenrick, 2003; Kenrick and Knox, 1989a; Sedgley, 1989) at flower head size, structure and grouping, polyad size, number of anthers, degree of self-compatibility and andromonoecy.

A suit of characters expected in successful invasive species have been proposed (Gibson *et al.,* 2011):

1. high attractiveness to available flower visitors and floral morphologies allowing pollination by many different organisms;

2. production of very large numbers of long-lived flowers allowing seed-set even when visitation rates are low; and/or ability to self-pollinate or reproduce vegetatively;

3. floral induction cues match those triggering flowering in native species and emergence of native flower visitors.

Among the above mentioned characters, there are several reproductive traits shared by Australian *Acacia*s that may contribute to their invasiveness: massive and long-lasting floral displays, generalist pollination syndromes, precocious production of a large number of long-lived and highly viable seeds resulting in massive seed banks, seed dispersal adaptations and a positive response to disturbance (e.g., resprouting ability or mass germination) (Milton and Hall, 1981). Nevertheless, many of these morphological traits are shared by both invasive and not-invasive *Acacia* species (Stone *et al.,* 2003) and, therefore, the contribution of these characteristics to *Acacia* invasiveness is still not clear.

1.3.3. Incompatibility and invasiveness

Self-incompatible species depend entirely on pollinator services and availability of mating partners to reproduce sexually, while self-compatible species have the ability to self-pollinate (autonomously or not) and ensure seed production when there is scarce or inefficient pollinators and/or limited mate availability (Eckert *et al.,* 2006). Consequently, species with the ability to self-fertilize are theoretically expected to be more invasive than self-incompatible species. The capacity to produce seeds after self-fertilization, even at a low rate, is especially important in the early stages of naturalization and invasion, because it reduces the need for pollinators and compatible plants (Baker, 1955; Davis *et al.,* 2004).

The availed information suggests that invasive taxa tend to have higher levels of self-compatibility. Despite the lack of data about *Acacia* species, the ability to self-fertilize can be one of the factors involved with their invasiveness (Gibson *et al.,* 2011). In spite of this, the extent of self-incompatibility is not well studied for most *Acacia*s and most studies have been done only in their native areas (Gibson *et al.,* 2011), revealing the need to test these hypothesis in population from the invaded ranges.

Sexual reproductive biology

Seed production is essential for the establishment of self-sustaining populations and subsequent naturalization of introduced species. However, seed production depends on pollination ecology and breeding system of the plants introduced and on environmental conditions of the recipient area (Richardson *et al.,* 2000). Thus, floral traits linked with the functioning of the flower and (in)dependence of pollinator, as well as with pollinator attraction will determine the final reproductive success of the plant.

1.4.1. Floral morphology and phenology of *Acacia*

Individual flowers of *Acacia* have a similar and simple structural organization, being adapted for generalist pollination by animals (Bernhardt 1989). Arroyo (1981) considered that the basic unit of reproduction in Mimosoidadeae was the flower head because the individual flowers are minute, numerous and grouped in the compact structure represented by the flower head (Figure 2). *Acacia* species can have globose or spicate flower heads, sessile or pedunculate, arranged singly, paired, several in a leaf axil, or in a racemes or panicles of heads. The number of flowers per flower head and the number of stamens per flower vary widely within and among species (Tyrbirk, 1989, 1993; Sedgley *et al.,* 1992; Kenrick, 2003).

Acacia have compound pollen grains called polyads. The number of pollen grains incorporated into each polyad varies depending on the species (4, 8, 16 or 32), but 16-grain polyads seem to be most common (Kenrick and Knox 1982; Kenrick 2003). It has been proposed that the number of pollen grains composing a polyad has evolved to achieve fertilization of all ovules from a single flower with a single pollination event (Kenrick and Knox 1982), minimizing the cost of pollen production (Cruden 1977; Kenrick and Knox 1989; Tybirck 1989 and Jørgensen 1994). The ovary contains 5-15 ovules in most Australian species (Kenrick, 2003).

Australian *Acacia*s have relatively long-lived individual flowers and flower heads. Individual flowers are open over a series of days and the flower head can last for up to 2 weeks (e.g., 8-15 days in *A. dealbata*, 4-8 days in *A. mearnsii*, 5-9 days in *A. melanoxylon,* 5-8 days in *A. paradoxa* and 6-10 days in *A. pycnantha*; Stone *et al.,* 2003). Thus, as referred above, *Acacia*s are characterized by massive and long-lasting floral displays. Flowering is often asynchronous within a single flower head and within a single tree (Stone *et al.,* 2003). Environmental conditions were shown to affect the number of flower heads in bloom (Sedgley, 1985; Gaol and Fox, 2000) and increased rainfall has been associated with higher inflorescence production (Broadhurst and Young, 2006).

Most Australian *Acacia*s flower in massive displays from late winter to mid spring and have long-lived inflorescences (Bernhardt, 1989; Costermans, 2007). In Mediterranean climate regions where they are invasive they flower earlier than most native species (Henderson, 2001; Godoy *et al.,* 2009). Data from invaded areas in Galicia (NW Spain) reveal that *A. dealbata* populations have longer flowering phases (10–22 days to flower heads). Field observations in Portugal show that flower duration of *A. dealbata* in Portugal is similar to the Galicia populations. *A. melanoxylon* populations also had a higher longevity of flower heads and flowers than the Australia populations (up to 25 days).

1.4.2. Floral biology and reproductive system of *Acacia*

Several reproductive strategies have been described in *Acacia* species. Separation of male and female stages in time (dichogamy) is widespread in Australian *Acacia*s and has been proposed as a mechanism to reduce self-pollination (Stone *et al.,* 2003). Within *Acacia* genus, Australian *Acacia* species are consistent in having strictly protogynous flowers where the stigma is receptive before anther dehiscence (Kenrick, 2003; Sedgley and Harbard, 1993). Records from *A. dealbata* in Australia show that female and male phase have the same duration (1-8 days). The *A. melanoxylon flowers* have a female phase of 3 to 5 days and male phase of 2 to 4 days. *A. dealbata* population in an invaded area (Galicia) has longer flowering female and male phases from 3 up to 15 days (Lorenzo *et al.,* 2010).

Another reproductive strategy reported in several species of *Acacia* is andromonoecy. In these species, flower heads can bear male and/or hermaphrodite flowers (Kenrick, 2003; George *et al.,* 2009). Andromonoecy is believed to have evolved from hermaphroditism and to be a possible first step in the evolution of monoecy, androdioecy or dioecy (Primack *et al.,* 1980; Bertin *et al.,* 1980). This sexual system is often associated with resource allocation to a flexible reproductive function, male or female, depending on the available resources (Miller *et al.,* 2007, and references therein). Andromonoecy has been described in several species such as *A. caesia* (Raju *et al.,* 2006), *A. macrantha* (Zapata *et al.,* 1978) and *A. mangium* (Sedgley *et al.,* 1992).

In addition to dicogamy and andromonoecy, different breeding systems have been observed in *Acacia*. The breeding system of Australian *Acacia*s varies from highly self-incompatible up to complete self-compatible species (Moffet, 1956; Bernhard *et al.,* 1984; Kenrick and Knox, 1989; Morgan *et al.,* 2002). Self-incompatibility has been widely reported for many species of the subgenus *Phyllodineae* and there are some evidences suggesting that the self-incompatibility in *Acacia* could be the result of post-zygotic lethal genes (Kenrick, 2003). High outcrossing rates have been detected in several species (e.g., *A. anfractuosa,* Coates *et al.,* 2006; *A. auriculiformis,* Moran *et al.,* 1989a; *A. crassicarpa,* Moran *et al.,* 1989a; and in some populations of *A. mangium,* Butcher *et al.,* 1999), so pollinators play an important role in the reproduction of these species (Bernhardt, 1989; Moncur *et al.,* 1985; Stone *et al.,* 2003). However partial self-compatibility is also relatively common in Australian *Acacia* species (Philp and Sherry, 1946; Moffett and Nixon, 1974). Interestingly, from the six species where self-compatibility was detected, five are invasive (*A. dealbata, A. decurrens, A. mearnsii, A. paradoxa, A. saligna*; Gibson *et al.,* 2011, and references therein).

Pollination of *Acacia*

Pollination followed by successful seed production are crucial aspects for plant invasion, however, they remain unstudied for most *Acacia* species (Stone *et al.,* 2003). Polyads are not suited to wind transport but are an efficient way of dispersal via pollinators (Kenrick, 2003; Kenrick and Knox, 1982). The open structure of the *Acacia* inflorescence with external anthers and pollen as reward makes flower exploitation accessible to a wide diversity of visitors.

The stamens were shown to be a powerful visual and olfactory advertisement to attract pollinators (Tyrbirk, 1993; Kenrick, 2003). Floral scent, an insect attractant, is located in the anthers and associated structures (Tybirk, 1993). The primary reward offered by Australian *Acacia* flowers is pollen (Bernhardt, 1989) and, thus, they are visited by pollen-collector pollinator and very rarely by nectar-feeding insects (Gibson *et al.,* 2012). In some *Acacia* species nectar is produced in small quantities at the base of the corolla tube, accessible only to specific insects. However, Australian *Acacia*s do not produce floral nectar; they can produce extra-floral nectar only as a reward that can attract insect and bird pollinators (Knox *et al.,* 1985; Vanstone and Panton, 1988: Kenrick, 2003). Stone *et al.,* (2003) divided *Acacia* floral visitors into the following three trophic groups: specialist pollen and flower feeders (bees, beetles, many flies), specialist nectar feeders (birds, butterflies and Bombylidae flies), and opportunist foragers (flies, ants and wasps). Despite the varied array of floral visitors it is possible that only a subset of them is effective pollinators (Stone *et al.,* 2003).

A consequence of the simple morphology of the flowers of Australian *Acacia*s is that flower access is unrestricted and, therefore, a wide variety of insects, native in the invaded area, could visit the flowers and become involved in pollination in the new areas where *Acacia*s are introduced. Information on the *Acacia* floral visitors and their efficiency is currently being studied in the invaded range (Portugal).

Seed biology of *Acacia*

Acacia species produce large quantities of hard-coated, heat-tolerant and long-lived seeds. The seeds have long dormancy (Milton, 1981; Marchante and Marchante, 2005; Richardson and Kluge, 2008) being able to form extensive and persistent soil seed banks (Richardson and Kluge, 2008). Finally, their germination is stimulated by heat and/or smoke (Milton, 1981; Marchante and Marchante, 2005; Richardson and Kluge, 2008). All these characteristics have been described to be fundamental to the invasion success and persistence of Australian *Acacia*s in the new ranges (Milton and Hall, 1981; Richardson and Kluge, 2008).

Seed dispersal of Australian *Acacia*s in the native range is mediated by animals: the elaiosomes attract ants and the red arils attract birds (O'Dowd and Gill, 1986; Orians and Milewski, 2007). In Portugal, *A. longifolia* and *A. dealbata*, are dispersed by ants (Marchante *et al.,* 2010) and seeds of *A. melanoxylon* and *A. dealbata* are occasionally seen in bird depositions (R. Heleno, personal communication). Long-distance dispersion can also be carried on by humans (cars and construction of roads) and by water courses (Richardson and Kluge, 2008).

Disturbance seems to be important for the germination of invasive Australian *Acacia* seeds (Gibson *et al.,* 2011). Fire and chemical scarification via ingestion by an appropriate dispersal agent are two critical stimuli for germination (Glyphis *et al.,* 1981; Fraser, 1990; Richardson and Kluge, 2008), breaking physical dormancy of the hard and water impermeable seed coat. High seed viability appears to be fundamental to their ability to invade (Richardson and Kluge, 2008; Marchante *et al.,* 2010).

A study in recently invaded soils by *A. longifolia* in Portugal shows low seed germinability (< 12%) but high viability of the surviving seeds (> 85%) (Marchante *et al.,*2010). Interestingly, a considerable number of seeds is lost due to early germination, granivory or decay in new areas but soil seed banks in invaded areas can contain up to 1500 seeds of *A. longifolia* per square-meter, make it difficult to control the invader once it becomes established (Marchante *et al.,* 2010).

Study species

For this study, the four most widespread species of Australian *Acacia*s growing in Portugal were used as study system. These species were, in decreasing order of aggressiveness: *A. dealbata*, *A. longifolia*, *A. saligna* and *A. melanoxylon* (Figure 2). These species were intentionally introduced in Portugal during the first half of the 20th century for forestry, soil stabilization and gardening purposes (Castroviejo *et al.,* 1999), being currently invasive in Portugal. *Acacia dealbata* and *A. melanoxylon* grow in mountain ranges and roadsides, being the former the most aggressive invader of inland Portugal. *Acacia longifolia* and *A. saligna* grow mainly in coastal sand dunes, being the former more abundant in the central and northern coast and the latter occurring predominantly in south Lisbon. The breeding system for the selected species is presented in Table I and concerns mostly the native range, with no information available for invasive populations.

A. dealbata Link *A. longifolia* (Andrews) Willd.

A. melanoxylon R. Br. *A. saligna* (Labill.) H.L. Wendl.

Figure 2. The most aggressive invasive Australian *Acacia* species in Portugal. Details of flower heads, a globose or spicate (*A. longifolia*) group of flowers. Details of leaves (*A. dealbata*) and phylodes.

Table I. The breeding system of the selected invasive *Acacia* species. Information mostly referent to the native range, with no data available for invasive populations.

Species	Range	Incompatibility	Breeding system	Reference
A. dealbata	Australia	Partially self-incompatible	-	Moffett and Nixon, 1974, cited in Kenrick, 2003
		Self-compatible	-	Gibson, 2012
		Self-incompatible	-	Broadhurst *et al.,* 2008
A. longifolia	Australia	no available information		Gibson, 2012
A. melanoxylon	Australia	-	Predominately out-crosser	Muona *et al.,* 1990;
A. saligna	Australia	-	Predominately out-crosser	Millar *et al.,* 2008
		-	Mixed mating, predominantly out-crosser	George *et al.,* 2008
	South Africa	Partially self-compatible	-	Gibson, 2012

Objectives

This thesis aims at obtaining information on the reproductive biology of the four most invasive Australian *Acacia* species in Portugal (*A. dealbata, A. longifolia, A. saligna* and *A. melanoxylon*) by characterizing floral traits and evaluating how the breeding system affects the sexual reproductive outcome of natural populations in the invasive range.

In theory, self-fertilizing plants have an advantage for the successful establishment in a new range because reproduction is less constraint by population size and pollinator availability, and thus, are expected to be more invasive than outcrossing plants. In the native range, the selected Australian *Acacia* species are mostly self-incompatible and have a clear tendency for outcrossing.

Thus, the main question is whether invasive populations maintain the same levels of self-incompatibility or have evolved mechanisms to increase autogamy rates as a mechanism of reproductive assurance. It is hypothesized that the invasive *Acacia*s are capable of some level of self-compatibility or autogamy and that they have a better reproductive performance in the invaded area in comparison with the native range.

In addition, since Australian *Acacia*s are pollinated by generalist insects in the native range, it is hypothesised that they will readily establish new interactions in the invaded range and thus will not suffer from pollen limitation. The impacts of pollen source were assessed in seed production, seed mass and germinability and seedling growth in seeds obtained from self and outcross pollinations.

2. Materials and Methods

2.1. Plant species

The four *Acacia* species (family Leguminosae, subfamily Mimosoideae, subgenus *Phyllodineae* following Maslin *et al.,* 2003) selected for this study were *Acacia dealbata* Link*, A. longifolia* (Andr.) Willd, *A. melanoxylon* R.Br. and *A. saligna* (Labill.) H. Wendl. A general description of each species is provided below (Figure 3 to 6).

2.1.1. ***Acacia* dealbata Link** (Silver wattle, acácia-mimosa)

Acacia dealbata is native to southeastern Australia and is especially widespread in Victoria and Eastern Tasmania, but it also occurs in New South Wales (Maslin, 2001; May and Attiwill, 2003). This species occurs in areas with rainfall over 500 mm, usually at altitudes between 350-1000 m (May and Attiwill, 2003). It also occurs naturally in New South Wales and eastern Tasmania (Maslin, 2001).

This species can be a tree reaching up to 30 m in height, or a shrub on drier sites. Leaves are greyish-green and segmented; leaf axis has glands only at the insertion of the pinnae. Flower heads are spherical with 5-6 mm in diameter and pale yellow. The flowering phenology in both native and invaded ranges is provided in Table II. Legume is compressed, barely constricted between the brown seeds (Walsh and Entwisle 1996). *Acacia dealbata* reaches sexual maturity within four to five years (Gowers 1990), and its seeds can persist in soil for around 50 years (Earl et al 2001).

Figure 3. *Acacia dealbata*: A. Tree; B. A flower branch used in supplementary treatment in the hand pollination experiment (green mark) and details of the bipinnate leaves and globose flower heads in large racemose inflorescences; C. Pods (flattened) and black seeds

Acacia dealbata was introduced in Europe in the 19th century as an ornamental plant (Sheppard *et al.,* 2006) and it became a problematic invasive species in Portugal (Almeida and Freitas, 2006), northwest Spain (Carballeira and Reigosa, 1999), France and Italy (Sheppard *et al.,* 2006). In Portugal, *A. dealbata* is present throughout mainland (Paiva, 1999) growing mostly in riparian zones, water courses and sunny edges of pinewoods or on south and west-facing slopes, where the plants form dense stands that strangle the natural vegetation (Lorenzo *et al.,* 2010). *Acacia dealbata* often invades areas under intensive agricultural use (Aguiar *et al.,* 2001), and areas recently burned.

2.1.2. ***Acacia* longifolia (Andrews) Willd.** (Sydney golden wattle, acácia-de-espigas)

Acacia longifolia is native to southeastern Australia (Orchard, A.E. and Wilson, A.J.G. 2001), and generally occurs in areas with more than 550 mm of annual rainfall (Muyt 2001). It is found in riparian zones, scrub areas, grassland and woodland (Muyt 2001; Weber 2003).

Acacia longifolia is a bushy shrub or small tree (that can reach up to 8 m height; Costermans 1983), which may form dense patches (Weber 2003). This species has linear to elliptic phyllodes, with 2–4 prominent primary veins. Flower heads are spikes 2–5 cm long of pale to golden yellow flowers, solitary or twinned in the axil of phyllodes. The flowering phenology in both native and invaded ranges is provided in Table II. Pods are generally straight to curved. The seeds are elliptic, sometimes irregularly shaped (Maslin, 2001). *Acacia longifolia* produces huge amounts of seeds annually and reaches sexual maturity within two to three years (Muyt 2001

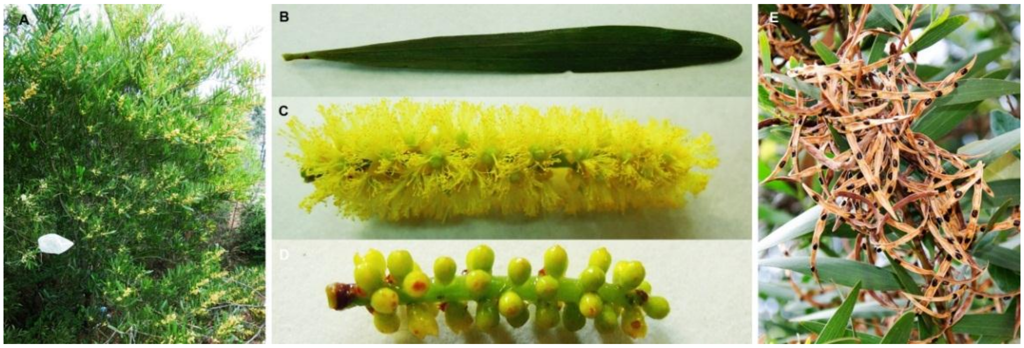

Figure 4. *Acacia longifolia*: A. Tree habit; it is visible a bag of nylon mesh used in hand pollination treatments to exclude insect interactions and to avoid open pollination; B. Phyllode; C. Spicate flower head with open flowers; D. Flower head with several small flower buds; E. Pods (straight to curved) containing seeds with a folded funicle.

This species is described as a rapidly growing shrub that can survive for over 50 years in its natural range, but in other areas commonly die within 25 years (Muyt 2001; Marchante *et al.,* 2004).*Acacia longifolia* was introduced in several areas to stabilize dunes and control erosion, and currently has the status of invader in Portugal (Marchante *et al.,* 2003), New Zealand (Parsons *et al.,* 1998) and South Africa (Cronk and Fuller 1995). In Portugal, *Acacia longifolia* is highly frequent in the north and central coastal sand dunes; however, it has been referenced across the entire Portuguese coast and also in some isolated inland locations, particularly in areas disturbed by fire (Marchante *et al.,* 2005). Their ability to fix nitrogen has enabled them to invade nutrient-poor environments (Rodríguez-Echeverría *et al.,* 2009). Several studies have demonstrated the negative impacts of *A. longifolia* in invaded ecosystems, namely, it leads to a decrease in native plant diversity (Marchante *et al.,* 2003) and significantly alters soil properties (Marchante *et al.,* 2008a,b, 2009) and water cycling (Rascher *et al.,* 2010).

2.1.3. ***Acacia* melanoxylon *R.Br.*** (Blackwood, acácia negra, acácia austrália)

Acacia melanoxylon is native to eastern Australia, occurring from the Atherton Tableland in northern Queensland to central Tasmania (Flora of Australia). It grows in a diversity of habitats from 0 up to 1000 m a.s.l., but prefers fertile soils in high rainfall areas (Farell and Ashton, 1978; Jennings, 2002), being intolerant to shade (Hopkins *et al.,*1977).

This species is either a tree 3-45 m high or a shrub 1.5-3m high. Phyllodes are narrowly elliptic and sometimes the bipinnate leaves persist on young plants. Phyllode morphology and development are strongly influenced by climatic conditions (Farrell and Ashton 1978). Flower heads are globular with 6 mm diameter and pale yellow flowers (Walsh and Entwisle 1996). Flowers are honey-scented (Gowers 1990). Not all trees within a population will flower every year, flowering phenology in both native and invaded ranges is provided in Table II. The plant lives for 15 up to 50 years, regularly producing large numbers of seeds. Pods are reddish-brown, narrower than leaves and slightly constricted or twisted. The small black seeds are almost encircled by a pinkish-red seed stalk (aril) (Henderson, 1995. In PIER, 2002) which is attractive to birds, the main dispersal vector of this species, and primates that ingest seeds with pods (Ruben Heleno, personal communication).

This species is cultivated as ornamental or for forestry mainly for fixing soils. It is a widespread invasive plant in Portugal, particularly after forest fires (Paiva, 1999). It is also invasive in South Africa where it invades forest edges or gaps, wooded kloofs, grasslands and watercourses (Henderson, 1995, in PIER, 2002).

Figure 5. *Acacia melanoxylon*: A. Tree habit; B. A flowering branch with several phyllodes and globose flower heads in different phases of flowering; C.; Pods (constricted or twisted) containing seeds with an aril.

2.1.4. ***Acacia*** **saligna (Labill.) H. Wendl.** (Blue-leafed Wattle, acácia)

Acacia saligna is native to southwestern Australia, occurring at low altitudes (from sea-level up to 300 m) and in various soil types, although it is particularly abundant on poor and calcareous sands (Midgely and Turnbull, 2003). In southwestern Australia it grows under a Mediterranean climate with annual rainfall between 300 and 1200 mm. *Acacia saligna* is capable of thriving on many soil types, including high pH sands and soils in sub-humid, semi-arid and arid temperate areas (Midgely and Turnbull, 2003).

Acacia saligna is a bushy shrub dividing near the base into several stems, resulting in a dense bush that may be wider than high, usually 2-5 m tall; however, sometimes it can form a small tree 5-9 m high (Midgely and Turnbull, 2003). *Acacia saligna* has phyllodes that can be 25 cm long. Flower heads are spherical with 10-15 mm in diameter bearing yellow flowers. The flowering phenology of the species in both native and invaded ranges is provided in Table II. Pods are narrow, usually 8-12 cm long and seeds are dark brown to black and shiny (Maslin 1974). This species reaches sexual maturity at two years old (Milton, 1980), and the plant has an average lifespan of 30 - 40 years (Milton and Hall, 1981; in Wood and Morris, 2007).

Acacia saligna has a long history of utilization across Australia and worldwide, becoming an aggressive invader in many regions of the world (Henderson, 2001; Nel *et al.,* 2004; Richardson and Rejmajnek, 2011). This species is planted in many temperate and semiarid countries for control of erosion and sand dune stabilization (Crompton 1992; Midgley and Turnbull 2003). It was introduced in Portugal for reforestation, for coastal dunes stabilization and for ornamental purposes (Marchante and Marchante, 2005, Gutierrez and Gil, 2010), and it is currently an invasive species, mainly in sandy soils in south Portugal (in Baixo Alentejo, Algarve, Beira Litoral and Estremadura provinces; Paiva, 1999).

Figure 6. *Acacia saligna* : A. Tree habit; B. Flowering branch with several phyllodes and globose flower heads; C. Pods (narrow) containing black seeds.

Table II. Flowering phenology of the studied *Acacia* species in Australia (in grey) and Portugal (in black). Based on data from Walsh and Entwisle, 1996, Castroviejo *et al.,*1999, and this thesis.

Species	**J**	**F**	**M**	**A**	**M**	**J**	**J**	**A**	**S**	**O**	**N**	**D**
A. dealbata	■	■	■	■			▒	▒	▒	▒		
A. longifolia		■	■	■	■	░	▒	▒	▒	▒	░	
A. melanoxylon			■	■	■			▒	▒	▒		
A. saligna			■	■	■			▒	▒	▒		

2.2. Study sites

This study was performed in two sites: Coimbra (40.20983ºN 8.40053ºW) for *A. dealbata, A. longifolia* and *A. melanoxylon*, and Tocha (40.31612ºN 8.81202ºW) for *A. saligna* (Figure 7). Both sites are located in central Portugal and are characterized by a meso-mediterranean climate.

- The study site in Coimbra is an urban woodland of approximately 45,000 m^2, dominated by native species like *Quercus suber, Arbutus unedo, Ulex europaeus and Pinus pinaster*. Based on a 30-year database (1971-2000), mean annual temperature in this area is 15.3 ºC and mean annual precipitation is 979 mm (Armas *et al.,* 2011).
- The study site in Tocha is a stabilized dune ecosystem where the vegetation is dominated by *Pinus pinaster, Corema album, Cistus salvifolius* and *Halimium halimifolium* and has approximately 55,000 m^2. Mean annual temperature in this area is 16 ºC and mean annual precipitation is 983 mm (period 1960-2008, F. Capelo, unpublished data).

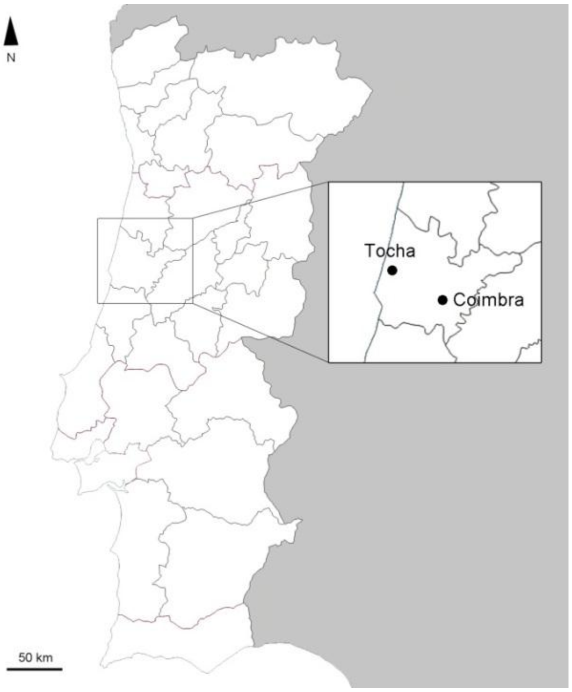

Figure 7. Location of the populations studied: Coimbra for *A. dealbata*, *A. longifolia* and *A. melanoxylon*, and Tocha for *A. saligna.*

In each site, at least 12 plants of each species were selected and marked (20 plants to *A. dealbata,* 12 to *A. longifolia*, 16 to *A. melanoxylon* and 12 to *A. saligna*) in 2011 and 2012. The selection was made just before the beginning of the flowering period: January for *A. dealbata* and *A. longifolia*, and February for *A. melanoxylon* and *A. saligna.* In both years, flowering was too prolific to allow accurate assessment of the number of inflorescences per plant. These plants were used to characterize the reproductive structures (section *1. Floral characterization*) and the reproductive system (section *2. Reproductive system*). Seeds produced by these trees were used in section 3. *Offspring performance*. Trees were selected at least 3-m apart to avoid sampling closely related individuals.

Data collection and analysis:

2.3. Floral characterization

To characterize the reproductive structures of these *Acacia*s, anther, pollen and ovule production per flower; proportion of male, hermaphrodite and female flowers per flower head; and floral display were assessed for each of the species studied. The following terminology for floral structures was used in this study: *flower head* refers to the globose or elongate (spicate) clusters of individual *flowers* (Figure 8, A and B respectively) that usually appear in groups in the phyllod/leaf axile (Figure 8, C and D); *flowering branch* was used to designate the apex of the branch with all the flower heads (Figure 8 E) (following Orchards and Wilson 2001).

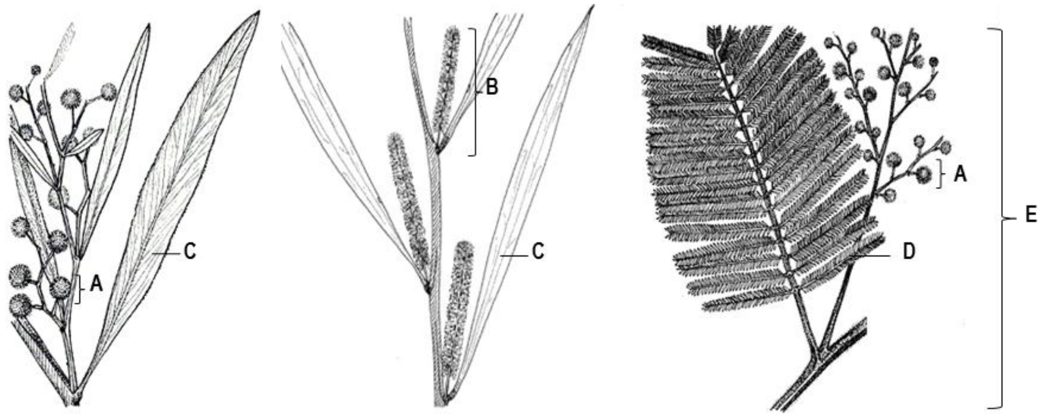

Figure 8. Flower heads (A, B), phyllodes (C), and leaves (D) and flowering branches (E) in the studied species, from left to right: *A. saligna/A. melanoxylon*, *A. longifolia* and *A. dealbata.*

2.3.2. Flower description

Open flower heads were collected from each inflorescence (20 flower heads per plant) and stored in 70% ethanol. Flower head development was also observed in the field to assess flower opening progression.

The number of anthers produced per flower was assessed in 10 flowers from distinct flower heads per plant using a dissecting microscope. The number of polyads produced per anther was also assessed for each species studied. One open flower head from five plants of each species was collected, left to dry at room temperature and stored in envelopes to assess the number of pollen grains per polyad.

In *A. dealbata*, *A. longifolia* and *A. saligna* the number of ovules per ovary was assessed in 10 mature pistils randomly selected per flower head and per plant. Flowers were dissected under a binocular microscope, pistils were removed and placed in 8 N sodium hydroxide for 48 h for tissues softening, washed in distilled water and subsequently transferred to 0.05 % aniline blue 0.1 N potassium phosphate for 48 h (Dafni 2005). Then, the pistils were placed in a drop of 50 % glycerin over a microscope slide and squashed with a coverslip. The number of ovules per ovary was counted using a Leika epifluorescent microscope equipped with a UV-2A filter cube (330–380 nm excitation). For *A. melanoxylon*, due to the variable morphology of the pistil, a more detailed screening of the ovules was made to correctly classify the flower as hermaphrodite or male (see below).

To assess the correlation between the number of pollen grains in a polyad and the number of ovules produced per flower, the ratio between them was calculated for each species. Total number of flowers per flower head and number of hermaphrodite and male flowers were assessed in five flower heads per plant using a dissecting microscope. In *A. dealbata*, *A. longifolia* and *A. saligna* hermaphrodite and male flowers were easily identified by the presence or absence of a well-developed pistil.

In *A. melanoxylon* a third type of inter-medium flowers having rudimentary pistils were present and examined in more detailed to determine if they were hermaphrodite or male (Figure 21, Appendix E) For this, up to 19 flowers per flower head from three floral heads per tree were softened in NaOH, stained in aniline blue and observed in the fluorescent microscope as described above. The number of ovules was counted and the flowers were classified as hermaphrodite when they had ovules or as male when the ovary was empty. The percentage of hermaphrodite flowers with small pistils and male flowers with rudimentary pistils was calculated for each tree. Because the number of ovules differed between hermaphrodite flowers with normal pistils and hermaphrodite flowers with small pistils, the percentage of each category was used to correct the number of ovules per flower head.

2.3.3. Floral display

To characterize the species floral display the number of flower heads per plant and species was assessed. The number of flower heads per flowering branch was counted in five flowering branches randomly chosen in each tree. The number of flowering branches was estimated for each tree by counting all flowering branches in one fourth of the canopy. The number of flower heads in each tree was then estimated by multiplying the total number of flowering branches by the mean number of flower heads per branch. The overall reproductive success was calculated for each tree and species by multiplying the mean number of hermaphrodite flowers per head, the estimated number of flowers heads produced per tree and the fruit set after open pollination (see details below in section 2. *Reproductive system*).

2.3.4. Statistical analysis

Descriptive statistics were calculated for flower characteristics (number of flowers per flower head, percentage of hermaphrodite flowers per flower head, number of anthers per flower and number of ovules per flower ovary) and are presented as the mean and standard error of the mean. Differences between species in floral characters were evaluated using a Generalized Linear Model (GLZ) with a gamma distribution and logit link function (including "tree" as a random factor). LSmeans were used to analyze differences between means. All the analyses were carried using the Glimmix procedure of SAS version 9.2 (SAS Institute Inc, Cary, North Carolina). A similar approach was used to check differences in the number of ovules in *A. melanoxylon* between hermaphrodite flowers with normal pistils and hermaphrodite flowers with rudimentary pistils.

Descriptive statistics were also calculated for flower display and natural reproductive success (Number of flower heads per flowering branch, estimated number of branches per plant and estimated overall reproductive success) and are presented as the mean and standard error of the mean for each species. Data were transformed (logarithmic, square root and logarithmic transformations respectively) to meet the assumptions of normality and homogeneity of variances. Univariate General Linear Models (GLM) were used to evaluate differences between species in the number of flower heads per flowering branch (species was used as fixed factor and tree was a random factor), followed by Tukey's test. Differences between *Acacia* species for estimated number of branches per plant and estimated overall reproductive success were analysed using a one-way ANOVA followed by Tukey´s test using one value per tree for each species). These analyses were carried out using SPSS version 19® (SPSS Inc, IBM).

2.4. Reproductive system

2.4.1. Hand-pollinations

To determine the reproductive system of the studied species, the effect of insect exclusion and pollen source on fruit production, seed set, and seed germination were investigated. Due to the small size of the flowers and to the tight flower heads, the emasculation procedure was not possible and thus, pollination treatments were undertaken without emasculation. Controlled hand pollination experiments were conducted in the field during the flowering seasons of 2011 and 2012.

The following treatments were applied to the selected trees per species:

(1) **open pollination**: 40 to 130 flower heads per plant were marked as control without manipulation.

(2) **supplementary pollination**: 40 to 140 flower heads per plant were left for open pollination and pollinated with xenogamous pollen; pollinations involved pollen from at least five unrelated trees that were at least 10 m apart from the treated tree;

(3) **spontaneous autogamy**: 20 to 110 flower heads per plant were bagged;

(4) **Self-pollination**: 20 to 85 flower heads per plant were bagged and pollinated with pollen collected from flower heads of the same plant; the flowers used as pollen donors were also bagged to avoid the presence of foreign pollen;

In those treatments that including bagging, the flower heads were covered with bags of fine nylon mesh prior to anthesis to exclude insect interactions and to avoid open pollination; the bags were maintained until fruit initiation. Flowers heads were followed daily and pollinations were initiated when the first flowers opened. Pollinations were conducted by gently rubbing the flower heads together every two days until all flowers were senescent to ensure that all flowers were pollinated, resulting in approximately six to twelve pollination events over approximately 15 days. At the end of the season (May for *A. dealbata* and *A. longifolia* and June/July for *A. melanoxylon* and *A. saligna*; Table II), all dry mature pods were collected for processing. In the laboratory, the number of pods per flower head, and plants and seeds per pod were counted for each species studied.

2.4.2. Reproductive outputs

For each treatment, fruit set, seed to ovule ratio and mean number of seeds per pod were calculated. **Fruit set** was calculated for each treatment and tree by dividing the total number of pods produced after a given pollination treatment by the total number of hermaphrodite flowers treated (obtained using the number of treated flower heads and the mean number of hermaphrodite flowers assessed in section 1.1 for each species). Mean fruit set was calculated for each species and pollination treatment. **Seed to ovule (S:O)** ratio was calculated for each treatment and tree by dividing the total number of seeds produced after a given pollination treatment by the total number of ovules available in the hermaphroditic flowers treated (estimated using the number of treated flower heads, the mean number of hermaphrodite flowers and the mean number of ovules produced per flower assessed in section 1.1 for each species). Mean S:O ratio was calculated for each species and pollination treatment. The **mean number of morphologically viable seeds produced per pod** was calculated for each treatment and tree by dividing the total number of seeds produced by the total number of pods obtained after a given pollination treatment.

The index of self-incompatibility (ISI), following Zapata and Arroyo (1978), and percentage of pollen limitation (PPL), following Jules and Ranthcke (1999), were calculated for each species as follows:

(1) **ISI** = Fruit set after self-pollination / Fruit set after cross-pollination; due to the difficulties in the emasculation procedure, the fruit set of supplementary pollination was used as a measure of cross-pollination; self-compatible species score > 1, partially self-incompatible species score < 1, complete self-incompatible species score < 0.2 (sensu Kenrick and Knox, 1989).

(2) **PPL** = [100 * (S:O ratio after supplementary pollination - S:O ratio after open pollination)] / S:O ratio after supplementary pollination.

2.4.3. Statistical analysis

Descriptive statistics were calculated for reproductive system (fruit set) and reproductive outputs (S:0 and mean number of seeds per pod) for each pollination treatment in each species, and are presented as the mean and standard error of the mean. Differences among pollination treatments in fruit set (FS), seed to ovule ratio (S:O) and seed production (mean number of seeds per pod and mean number of aborted seeds per pod) were carried out for each species using a GLZ with a gamma distribution and logit link function or a gaussian error and identity function (tree was used a random factor). LSmeans were used to analyze differences between means. All the analyses were carried using the Glimmix procedure of SAS version 9.2 (SAS Institute Inc, Cary, North Carolina).

2.5. Offspring performance

To assess the quality of the seeds and offspring performance for the different pollination regimes, seed weight, seed germination and seedling survival were assessed from seeds obtained in the hand pollination experiments.

5.5.1. Seed weight

Seeds collected from the four hand-pollination treatments were counted and seeds that appeared viable (no holes in testa, no discoloration) were weighted using a laboratory scale to obtain the mean seed weight of seeds produced by self or cross-pollination events.

5.5.2. Seed germination and seedling growth

A germination assay was executed to determine if there were differences in the germination rate and seedling early growth between seeds from self-pollinated flowers and seeds obtained by cross-pollination. All the pollination treatments were included. Seeds per treatment and per tree were separated to allow checking for interspecific variability. Up to 15 morphologically viable seeds for each treatment and species were placed to germinate. Seeds were accommodated on small containers with wet sand in a growth chamber (temperature 25ºC and a photoperiod of 12 hours). Seed germination was checked every 2 days during 45 days. A seed was considered germinated after radicle emergence, when radicle was 1-2 mm long). Germination percentages were calculated for each treatment and species. Afterwards, germinated seeds were planted into containers and seedlings were left to grow during one month (watered 1–3 times a week).

Final biomass was calculated for each species and treatment. Unfortunately, the seeds of *A. melanoxylon* from open pollination were lost during experimental manipulation and the experiment is currently being repeated, thus data on seed germination and seedling growth is missing for this treatment in the present thesis.

5.5.3. Statistical analysis

For offspring performance variables (seed weight, seed germination and seedling survival) descriptive statistics were calculated for each pollination treatment for each species, and are presented as the mean and standard error of the mean. Univariate Generalized Liner Model (GLM) analysis was used to test for the effect of each hand pollination treatment on seed weight for the four *Acacia* species studied (species was used as fixed factor and tree as a random factor), followed by Tukey's test. A square root transformation was applied to the original data for *A. dealbata*, *A. longifolia* and *A. melanoxylon* to meet the assumptions of normality and homogeneity of variances. These analyses were performed using SPSS version 19® (SPSS Inc, IBM).

A GLZ with a binomial distribution and logit link function was used to check for differences in seed germination between different treatments within each species. Tree was used as random factor. LSmeans were used to analyze differences between means. All the analyses were carried using the Glimmix procedure of SAS version 9.2 (SAS Institute Inc, Cary, North Carolina).

Univariate GLM analysis was used to test for the effect of each hand pollinations treatment on seedling weight for the four *Acacia* species studied (species was used as fixed factor and tree as a random factor), followed by Tukey's test. A logarithmic transformation was applied to the original data to meet the assumptions of normality and homogeneity of variance, except for *A. melanoxylon* data. Both spontaneous autogamy and self-pollination treatments had been removed from the statistically analysis due to a small sample size (n <5). The GLM analyses were performed using SPSS version 19 ® (SPSS Inc, IBM).

3. Results

3.1 Floral characterization

The characterization of flowers and flower heads of the four *Acacia* species studied is presented in Table III, Figure 9 and Appendix F (Table XVIII).

Table III Characterization of flowers and flower heads of the four *Acacia* species studied.

Floral characters	***A. dealbata***	***A. longifolia***	***A. melanoxylon***	***A. saligna***
Pollen grains per polyad	16 (50)	16 (50)	16 (50)	16 (50)
Polyads per anther	8 (10)	8 (10)	8 (10)	8 (10)
Stamens per flower	32.7 ± 0.58 (119)^{a}	92.3 ± 2.30 (109)^{d}	45.1 ± 0.71 (78)^{b}	70.3 ± 0.77 (95)^{c}
Stamens per flower head	872.6 ± 15.62 (119)	4632.7 ±115.21 (109)	1884.2 ±29.63 (78)	3443.0 ± 37.89 (95)
Polyads per flower head	6,984	37,048	15,088	27,560
Ovules per flower	13.0 ± 0.13 (97)	13.0 ± 0.15 (130)	13.1 ± 0.26 (168)	10.1 ± 0.13 (108)
	9 – 15^{a}	10 - 17^{a}	3 - 18^{a}	7 - 14^{b}
Ovules per flower head	329	634	165	485
Flower heads maturation	Basipetal	Acropetal	Random	Basipetal
Flowers per flower head	26.7 ± 0.73 (33)^{a}	50.2 ± 1.79 (33)^{c}	41.8 ± 1.11 (33)^{b}	49.0 ± 0.97 (60)^{c}
Hermaphrodite flowers per flower head (%)	94.9 ± 0.01 (33)^{a}	97.1 ± 0.01 (33)^{a}	31.2 ± 0.04 (33)^{b}	98.0 ± 0.01 (60)^{a}
Male flowers per flower head (%)	5.1 ± 0.01 (33)^{a}	2.92 ± 0.01(33)^{a}	68.8 ± 0.14 (33)^{b}	2.0 ± 0.01 (60)^{a}

Notes: values are given as means and standard error of the mean followed by sample size in parenthesis. Stamens per flower head are the product between the mean number of stamens per flower and mean number of flowers per flower head; polyads per flower head are the product between the number of polyads per anther and the number of anthers per flower head; ovules per flower head are the product between mean number of ovules per flower and mean number of hermaphrodite flowers per head. Flower head maturation refers to the flower opening direction within flower heads. Hermaphrodite and male flowers are given in percentage from the total number of flowers per flower head. Different letters reveal statistically significant differences at $P < 0.05$ among the species.

The number of pollen grains per polyad is constant to all species (16), as well as the number of polyads per anther (8) (Table III). The number of stamens per flower was significantly different among species ($P < 0.001$) with *A. longifolia* presenting the greatest number of stamens per flower (92.3 ± 2.30, n = 109) and *A. dealbata* the lowest (32.7 ± 0.58, n = 119) (Figure 9, Table III). *Acacia longifolia* had the biggest number of polyads per flower head followed by *A. saligna, A. melanoxylon* and *A. dealbata*, although no significant differences were found between species (Table III). The number of ovules per flower for each species was significantly different among species ($P < 0.001$) (Figure 9, Table III). *Acacia saligna* had the lowest number of ovules per flower (10.1 ± 0.13, n = 108) being different from the other three *Acacia* species, which had 13 ovules per flower (Figure 9, Table III).

In *A. melanoxylon* a third type of inter-medium flowers having rudimentary pistils was also found and examined in more detail to determine if they were hermaphrodite or male (Table IV; Figure 21 in Appendix E). Differences in the mean number of ovules in *A. melanoxylon* ($F_{1,156}$=23.71, P<0.001) were found between hermaphrodite flowers with normal pistils (14 ovules per flower) and hermaphrodite flowers with rudimentary pistils (nearly 11 ovules per flower) (Table IV).

Table IV. Characterization of flowers of *Acacia melanoxylon*: number of ovules per ovary in each flower type

Flower	**Hermaphrodite flowers**		**Male flowers**	
Pistil				
	normal	rudimentary		without pistil
No. per flower head	12.1 ± 4.78 (13.3%)	7.5 ± 0.46 (17.9%)	27.1 ± 1.62 (64.4%)	1.9 ± 0.34 (4.4%)
No. ovules per flower	14.0 ± 0.85 (118)^{b}	10.6±0.68(43)^{a}	0.0 ± 0.0 (155)	0.0

Notes: values are given as means and standard error of the mean followed by the percentage of each flower type per flower head (n=33) or sample size for the number (No.) of ovules in parenthesis

The number of flowers per flower head varied significantly ($P < 0.001$) between the four *Acacia* species (Table III). *Acacia saligna* and *A. longifolia* had more flowers per flower head (50 and 49, respectively) followed by *A. melanoxylon* (nearly 42 flowers per flower head) and by *A. dealbata* (nearly 27 flowers per flower head). Significant differences ($P < 0.001$) were found between species for the percentage of hermaphrodite flowers per flower head (Figure 9A). *Acacia saligna* and *A. longifolia* had more hermaphrodite flowers per flower head (approximately 98% of the total number of flowers per flower head) followed by *A. dealbata* (nearly 95%) and by *A. melanoxylon* that presented only 31% of hermaphroditic flowers per flower head.

In *A. melanoxylon*, hermaphroditic flowers with normal pistil accounted for 13% of the total number of flowers in the flower head, while flowers with rudimentary pistils represented almost 18% of the total number of flowers. About 65% of flowers with rudimentary pistils were male, and around 4% of the flowers were male without pistils (Table IV). There was a high intraspecific variability in flower characters within the four *Acacia* species, as it can be seen in Figure 9B (mean number of stamens per flower head) and Figure 9C (mean number of ovules per flower head).

For the four species studied, the number of pollen grains per polyad (16) was always bigger than the number of ovules per pistil (Figure 10). The majority of studied pistils had between 11 and 14 ovules. This number was even lower for *A. saligna*, with most pistils containing 9 to 11 ovules. Pistils with 16 ovules were only found in *A. longifolia* and *A. melanoxylon* but with a frequency lower than 5%. Despite this, the number of seeds per pod was rarely higher than 10 (Figure 10). The highest number of seeds per pod was obtained in *A. melanoxylon,* although with a frequency lower than 2%. All species, except *A. longifolia*, had a skewed distribution of the number of seeds per pod, with most pods containing 1-2 viable seeds.

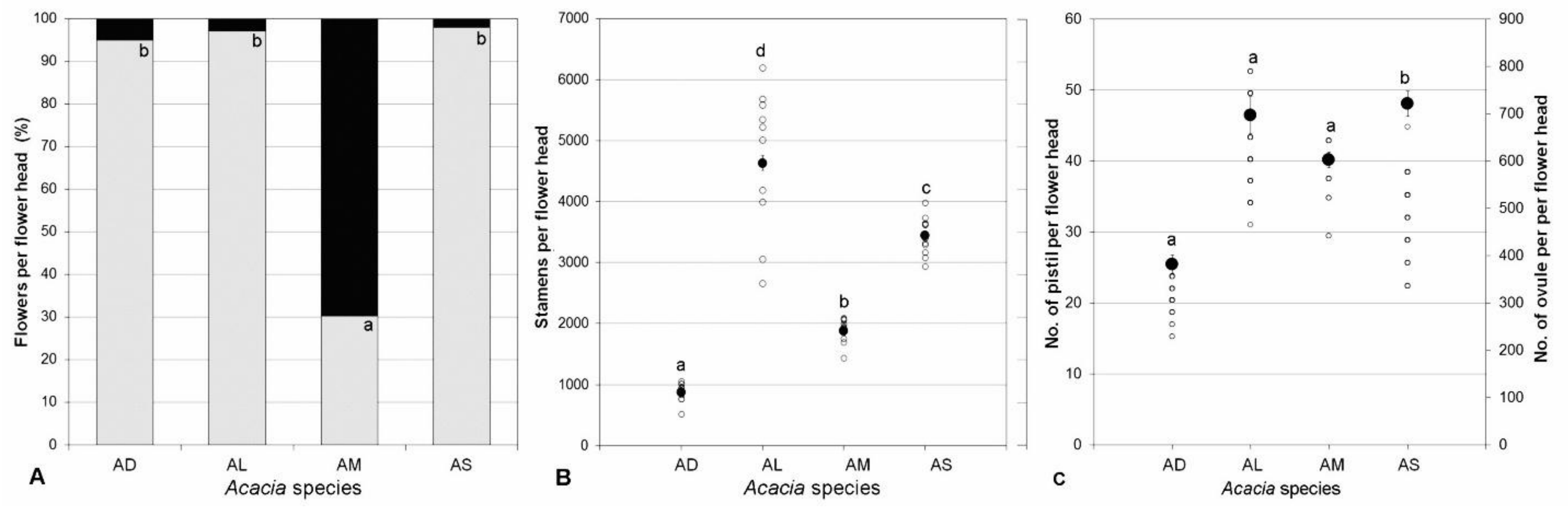

Figure 9. Characterization of the flower heads of the four *Acacia* species studied. A. Percentage of hermaphrodite (grey bars) and male flowers (black bars) within the flower head; B. Mean number of stamens per flower head per species (black circles) and per tree within species (open circles); C. Mean number of pistils per species (black circles) and ovules per flower head (open circles). Number of stamens per species and pistils per flower head are given as mean and standard error of the mean; number of ovules and stamens per flower head are raw data. *Acacia* species: **AD,** *A. dealbata*; **AL**, *A. longifolia*; **AM**, *A. melanoxylon*; **AS**, *A. saligna*. Different letters reveal statistically significant differences at $P < 0.05$ among treatments within species.

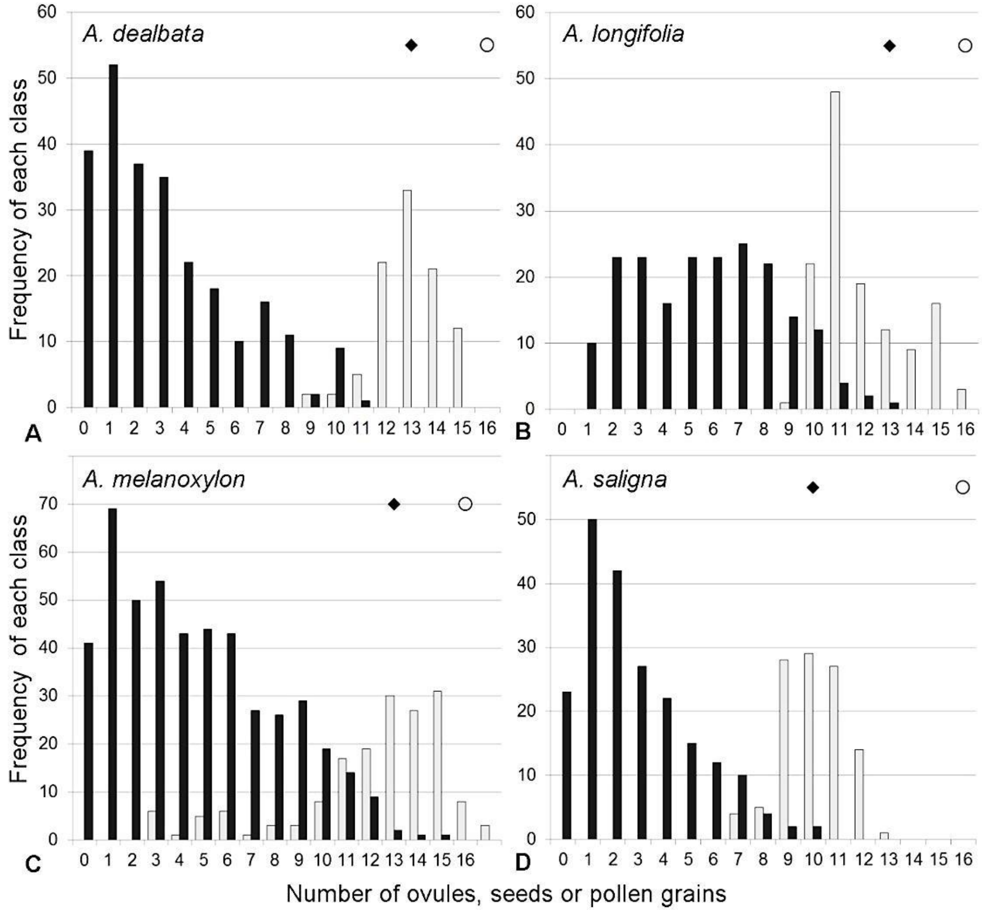

Figure 10. Histogram with the frequencies of the number of ovules per pistil (grey bars) and number of seeds per pod (black bars) obtained after open pollination for the four *Acacia* species studied; number of pollen grains per polyad (open circle) and mean number of ovules per pistil (black diamond) are also plotted. (A) *A. dealbata* (B*) A. longifolia* (C) *A. melanoxylon*; (D) *A. saligna*. Note different scales for different species.

3.2 Floral display

The results for the characterization of the four *Acacia* species studied for floral display and natural reproductive success are presented in Table V and Figure 11.The number of flower heads per flowering branch varied significantly between *Acacia* species (*P* < 0.001). *Acacia dealbata* presented the highest number of flower heads per flowering branch (272.3 ± 19.65) followed by *A. saligna* (33.2 ± 1.62) with a much lower value for this character (Table V). *Acacia longifolia* and *A. melanoxylon* had similar number of flower heads per flowering branch. There were also significant differences (*P* =

0.01) between *Acacia* species in the estimated number of branches per plant. *Acacia saligna* had significantly less branches per plant than *A. dealbata* and *A. longifolia*, despite of the variation observed among individual trees (Table V).

Table V - Characterization of the four *Acacia* species studied for floral display and natural reproductive success.

Floral display	***A. dealbata***	***A. longifolia***	***A. melanoxylon***	***A. saligna***
No. flower heads per flowering branch	272.3 ± 19.65 (58)^{c}	23.8 ± 1.06 (57)^{a}	24.9 ± 2.24 (76)^{a}	33.2 ± 1.62 (59)^{b}
Estimated no. of branches per plant	1135.3 ± 350.78 (12)^{cb}	1803.0 ± 452.09 (12)^{b}	656.4 ±121.2(14)^{ab}	449.2 ± 127.12(12)^{a}
Estimated overall reproductive success	67039.4 ± 11965.82 (18)^{c}	10443.1 ± 3005.09 (9)^{b}	5895.9 ± 1548.18 (16)^{bc}	4607.1 ± 1261.46 (12)^{a}

Notes: values are given as means and standard error of the mean followed by sample size in parenthesis. Different letters reveal statistically significant differences at $P < 0.05$ among species.

Significant differences between species were also found for the estimated overall reproductive success ($P < 0.001$; Table V, Figure 11). This parameter results from the multiplication of the mean number of hermaphrodite flowers per head, the estimated number of flowers heads produced per tree and the natural fruit set (produced after open pollination). The results showed that *A. dealbata* was the most successful species with significantly higher reproductive success than the other species ($P < 0.001$; Table V, Figure 11). There was a high intraspecific variability in the overall reproductive success for the four species with individual trees behaving very differently (Figure 11), a result that agrees with the observations made in the field.

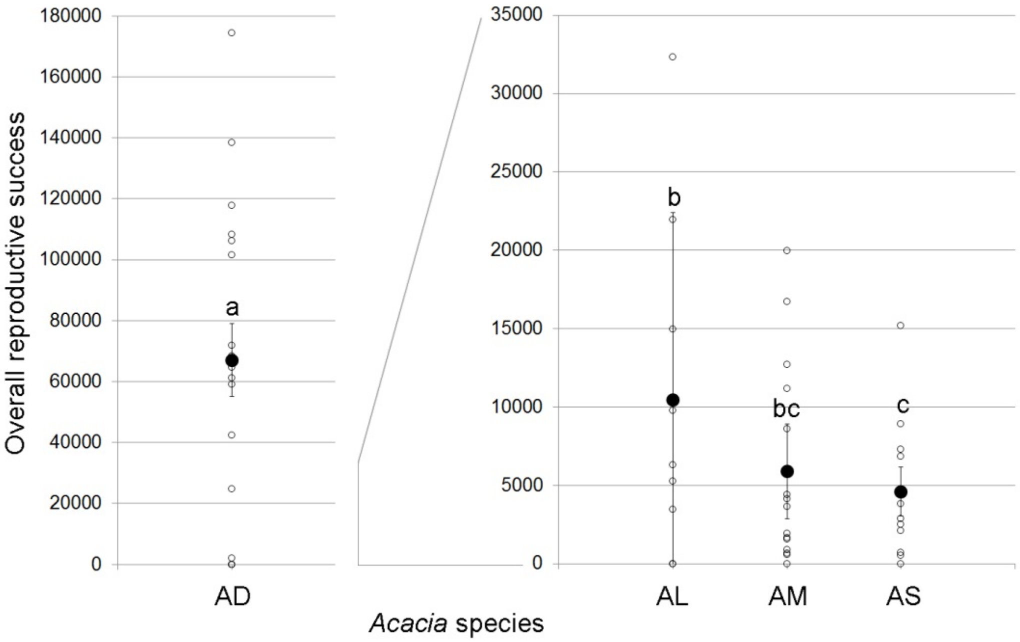

Figure 11. Overall reproductive success for the four *Acacia* species studied. Individual trees are represented by open circles and the mean for each species is given in closed circles. Values are given as mean and standard errors of the mean. Different letters reveal statistically significant differences at $P < 0.05$ between treatments within species.

3.3 Reproductive systems

The results of hand pollination experiments are presented as fruit set, seed to ovule ratio (S:O) and mean number of seeds per pod. These parameters are presented in Figures 12 to 14 respectively, and in Appendix A (Tables VII, VIII and IX, respectively). We found significant differences in fruit set between treatments for all the species (Table VII, Figure 12). The supplementary pollination treatment had a significantly higher value of fruit set in *A. dealbata, A. longifolia* and *A. saligna* (Figure 12 A-B, 4D and Table VII). Only in *A. melanoxylon* the supplementary pollination treatment was not significantly different from the open-pollination (Figure 12C). In all species the open-pollination and supplementary pollination treatments had higher level of fruit set than spontaneous selfing and self-pollination (Figure 12, Table VII). Only in *A. dealbata* the fruit set of the self-pollination treatment did not differ from the open pollination treatment.

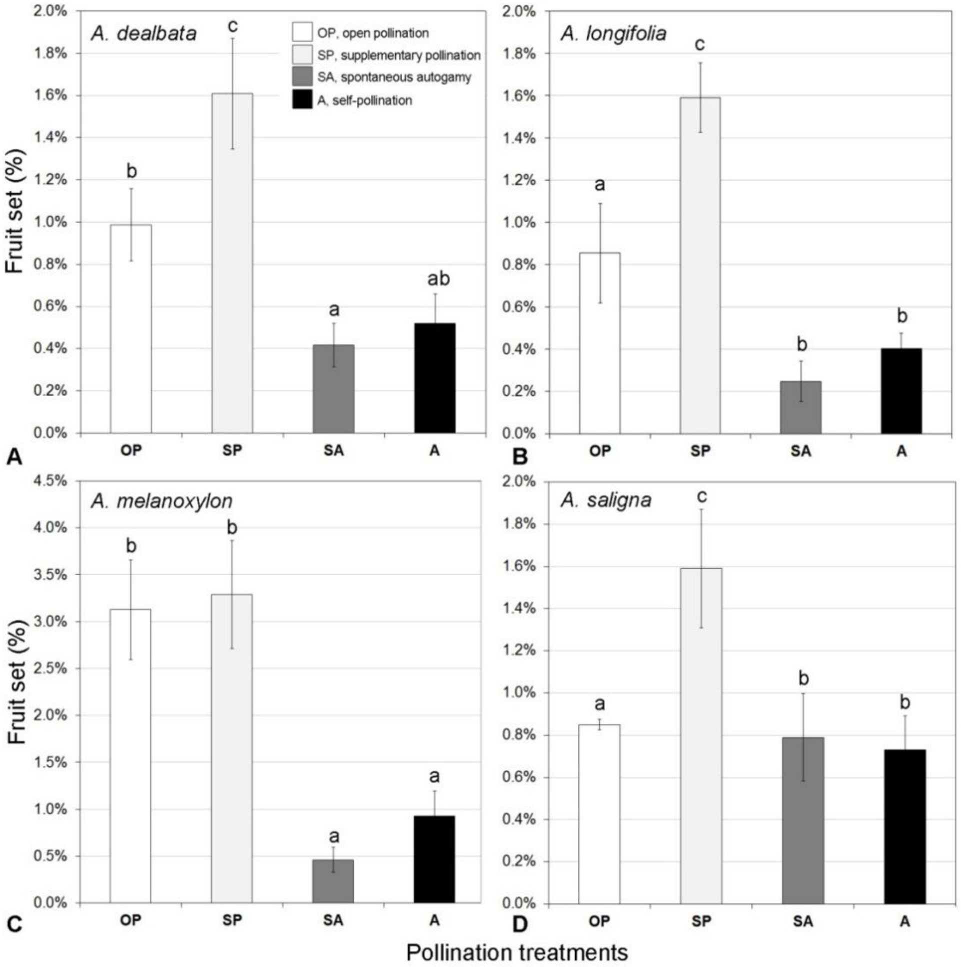

Figure 12. Fruit set from the hand pollination experiments for the four *Acacia* species studied. (A) *A. dealbata* (B) *A. longifolia* (C) *A. melanoxylon* (D) *A. saligna*. Fruit set is given as the percentage of flowers developing into fruit from the total number of flowers treated. Pollination treatments: OP, open pollination; SP, supplementary pollination; SA, spontaneous autogamy; A, Self-pollination. Values are given as mean and standard errors of the mean. Different letters reveal statistically significant differences at *P* < 0.05 among treatments within species. Different scale for *A. melanoxylon.*

Statistically significant differences were also observed in the seed to ovule ratio between treatments for all the species (*P* = 0.016 for *A. dealbata*; *P* < 0.001 for the remaining species). As observed in fruit set, the supplementary and open pollination treatments had significantly higher S:O value than spontaneous autogamy and self-pollination in all species except *A. dealbata* (Figure 13, Table VIII), despite no significant differences were observed between them (Figure 13, Table VIII)

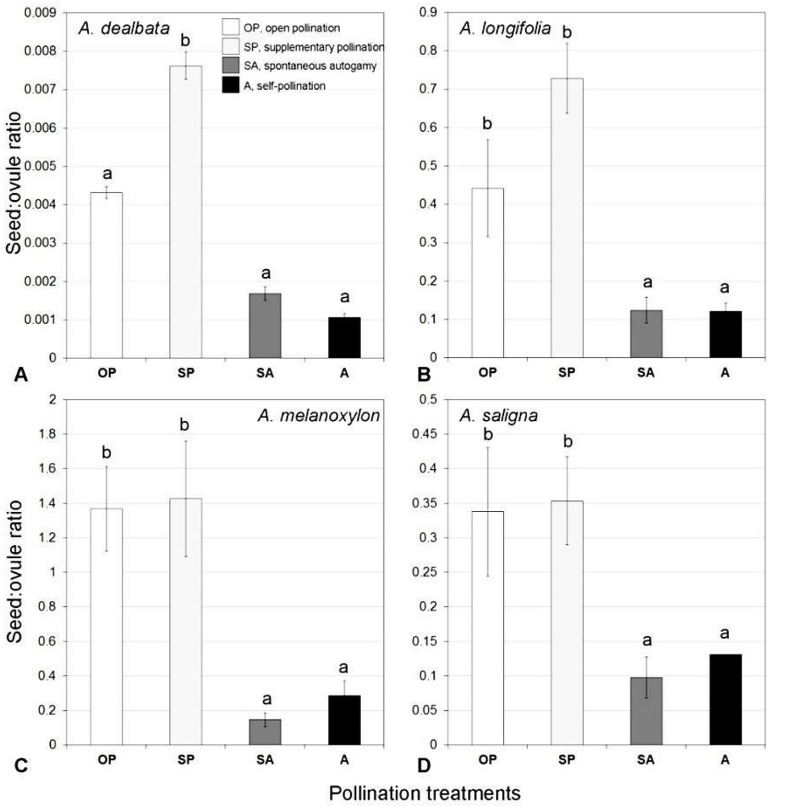

Figure 13. Seed to ovule ratio from the hand pollination experiments for the four *Acacia* species studied. (A) *A. dealbata* (B) *A. longifolia (*C) *A. melanoxylon* (D) *A. saligna*. Seed to ovule ration is given as the percentage of ovules that developed into morphologically viable seeds from the total number of ovules available in the hermaphrodite flowers treated. Pollination treatments: OP, open pollination; SP, supplementary pollination; SA, spontaneous autogamy; A, Self-pollination. Values are given as mean and standard errors of the mean. Different letters reveal statistically significant differences at *P* < 0.05 among treatments within species. Note different scales for different species.

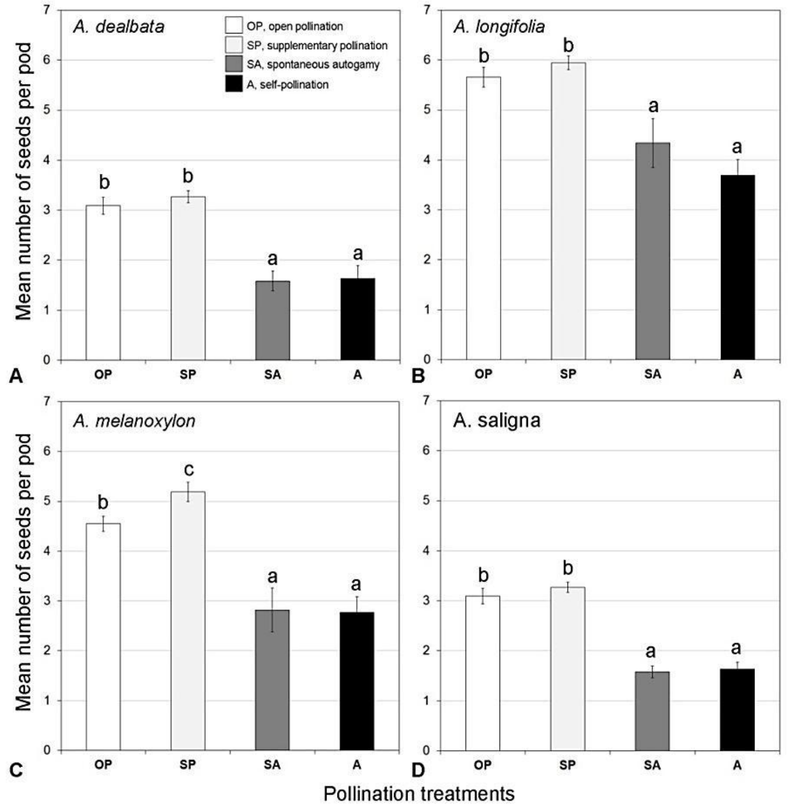

Figure 14. Seed production from the hand pollination experiments for the four *Acacia* species studied. (A) *A. dealbata* (B) *A. longifolia* (C) *A. melanoxylon* (D) *A. saligna*. Seed production is given as the mean number of morphologically viable seeds produced per pod. Pollination treatments: OP, open pollination; SP, supplementary pollination; SA, spontaneous autogamy; A, Self-pollination. Values are given as mean and standard errors of the mean. Different letters reveal statistically significant differences at *P* < 0.05 among treatments within specie

Finally, the production of viable seeds from the hand pollination experiments was significantly different between treatments for the four species (*P* < 0.001; Table IX), showing similar trends as the ones observed in fruit set and S:O (Figure 13). The mean number of seeds per pod was higher in *A. longifolia* and *A. melanoxylon* than in the other two species (Figure 14). The Overall, *A. dealbata* and *A. saligna* had a mean number of aborted seeds per pod higher than *A. longifolia* and *A. melanoxylon* (Appendix A, Figure 19).

The index of self-incompatibility (ISI) and percentage of pollen limitation (PPL) were calculated with the values of fruit set and S:O, respectively, obtained for each species (Table VI, Figure 14).

Table VI Indices of self-incompatibility (ISI) and percentage of pollen limitation (PPL).

Indices	*A. dealbata*	*A. longifolia*	*A. melanoxylon*	*A. saligna*
ISI	0.32	0.19	0.28	0.46
PPL	43.27	39.32	4.07	4.41

Notes: ISI followed Zapata and Arroyo (1978) and PPL followed Jules and Ranthcke (1999) (for details see Materials and Methods).

The mean values of ISI ranged between 0.19 in *A. longifolia* to 0.46 in *A. saligna*, with *A. melanoxylon* and *A. dealbata* having intermediate values of 0.28 and 0.32, respectively (Figure 14). This index takes into account the fruit set of supplementary and self-pollination treatments (see Materials and Methods section), and has a specific scale that allows a classification of the incompatibility levels of the species as follows: self-compatible species score > 1, partially self-incompatible species score < 1 and > 0.2, complete self-incompatible species score < 0.2 (sensu Kenrick and Knox, 1989). Thus, all the *Acacia* species studied are partially self-incompatible species, with *A. longifolia* having an ISI value in the limit between self-incompatible and partially self-incompatible species (Figure 14). Again, beyond the differences among species, a great variability in ISI values among individual trees of each species was observed (Figure 14), with individual trees of *A. dealbata*, *A. melanoxylon* and *A. saligna* ranging from self-incompatible to completely compatible (Figure 14).

The ISI values available for other populations and species of *Acacia* growing in native and invaded areas are similar to those found in this study (Figure 14, Appendix B: Table XI). No data is available for native populations of *A. longifolia* and *A. melanoxylon*. The value for *A. dealbata* in the native range was higher than the value found in this study (0.73 vs. 0.32) although both scores classify this species as partially self-compatible (Figure 14, Table VI). The values found in the literature for *A. saligna* in native and invaded areas in South Africa were very similar (0.77 v. 0.82) and classify this species also as partially self-compatible. These values were a bit higher than the value found in this study (0.49) (Figure 14, Table VI). The values for the index of percentage of pollen limitation (PPL) ranged between values close to 4% for *A. saligna* and *A. melanoxylon* to values around 40% for *A. dealbata* and *A. longifolia* (Table VI).

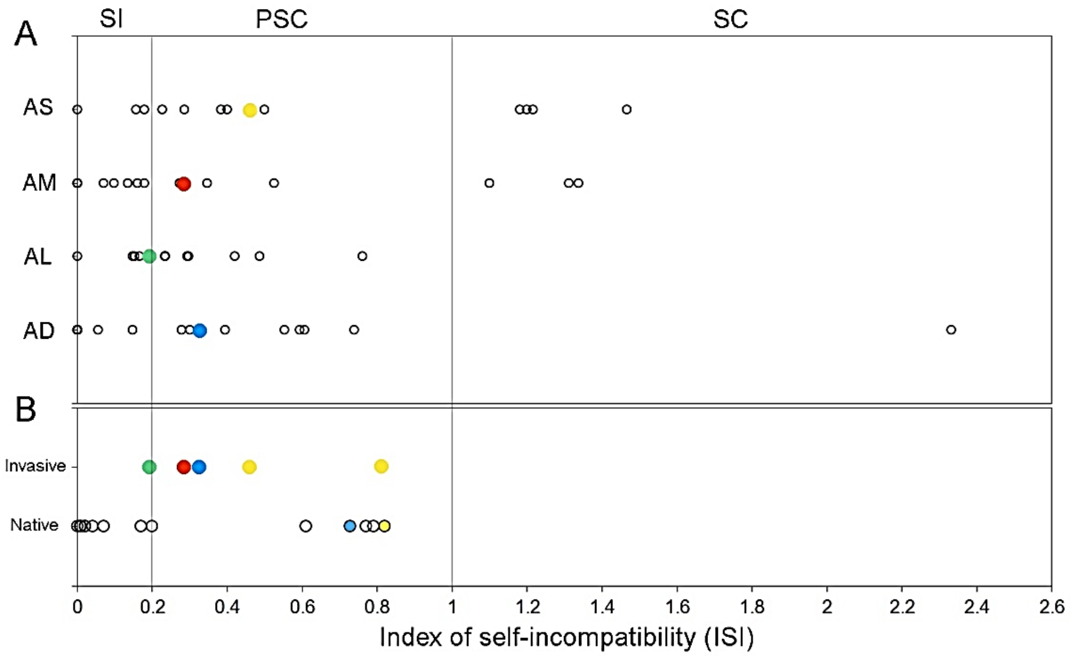

Figure 15. **Index of self-incompatibility (ISI**), followed Zapata and Arroyo (1978) and their score : **SI**– self-incompatibility (ISI<0.2); **PSC**–partially self-compatible (ISI between 0.2 and 1) and **SC**– self-compatible(ISI>1) A. ISI value obtained from the *Acacia* species studied in invaded areas in Portugal (mean in colour, values per individual trees as open circles) **AS** – *A. saligna*; **AM**- *A. melanoxylon*; **AL**- *A. longifolia* and **AD**- *A. dealbata*; B. ISI values found in literature for other *Acacia* species in native and invaded areas (for species identity see Table , in Appendix). The colour circles correspond to the data from this study (green AL; red AM; blue AD and yellow AS). *A. saligna* is the only species that has already been studied in another invaded area (South Africa, this ISI value is represented by another yellow circle). Data for *A. saligna* and *A. dealbata i*n Australia are presented as circles filled in yellow and blue respectively.

The overall reproductive success calculated with fruit set obtained in spontaneous autogamy treatment (Figure 20, in Appendix D) mimicking the complete absence of pollen vectors revealed that all species are able to produce seeds, with. *A. dealbata* being again the species with higher reproductive successful. Except for *A. saligna*, the success of open pollination was higher than spontaneous selfing, but statistical differences exist only for *A. dealbata* and *A. melanoxylon* (Table XVIII, Appendix XVII).

3.4 Offspring performance

The results from offspring performance resulted from the hand pollination experiments for the four *Acacia* species studied are presented in Figures 15 to 17, and in Appendix C (Tables XII to XVI, respectively).

The viable-looking seeds obtained in the different pollination treatments for the trees of each species were weighted and the results are presented in Figure 15 and Tables XII and XIII. Seeds produced in the open and supplementary pollination treatments were significantly bigger ($P < 0.001$) than those produced in the spontaneous autogamy and self-pollination treatments for *A. dealbata* and *A. melanoxylon* (Figure 15, Tables XII). No seeds were available for the self-pollination treatment for *A. longifolia* and seeds produced by spontaneous autogamy were significantly heavier than those produced by open and supplementary pollination (Figure 15, Tables XII). A more complex pattern was observed to *A. saligna,* with significant differences for seed weight after open and supplementary pollination treatments. However, these two treatments did not differ significantly from spontaneous autogamy. Self-pollination treatment yielded significantly lighter seeds that the other pollination treatments (Figure 15, Table XII).

The results of the germination assay for the four *Acacia* species studied are presented in Figure 16 and Table VII. Significant differences were found in seed germination between the two treatments of open and supplementary pollination and the treatment of spontaneous autogamy in *A. dealbata* ($P < 0.01$) and *A. melanoxylon* ($P < 0.05$). In *A. longifolia* seed germination of spontaneous autogamy treatment is significantly lower than supplementary and self-pollination treatments (Table

VII, *P* = 0.0196) but not from open pollination treatment (Figure 16). No differences were found in seed germination among treatments for *A. saligna* (Figure 16D, Table XIV).

Overall, seed germination was higher in the seeds produced by open and supplementary pollination than self-fertilization treatments (with rates around 50% against 10-20%, respectively).

Finally, the weight of one-month seedlings is presented in Figure 17 and Tables XV and XVI. For *A. dealbata* due to a small sample size only open and supplementary pollination treatments were used in the statistical analysis although there few seedlings in the other two treatments. Significant differences between treatments were only found for *A. longifolia* (Figure 17, Tables XV and XVI), with seedlings from the open and supplementary pollination treatments being bigger than those from self-fertilizing treatments.

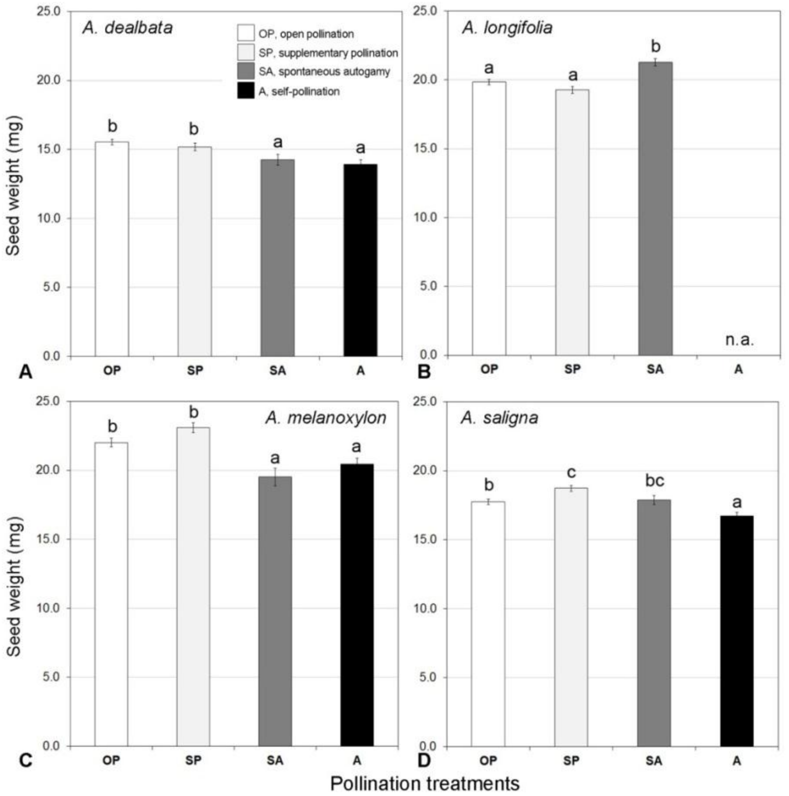

Figure 16. Seed weight from the hand pollination experiments for the four *Acacia* species studied. (A) *A. dealbata*; (B) *A. longifolia*; (C) *A. melanoxylon*; (D) *A. saligna*. Seed weight is given as the mean and standard error of the mean. Pollination treatments: OP, open pollination; SP, supplementary pollination; SA, spontaneous autogamy; A, Self-pollination. Different letters reveal statistically significant differences at *P* < 0.05 among treatments within species.

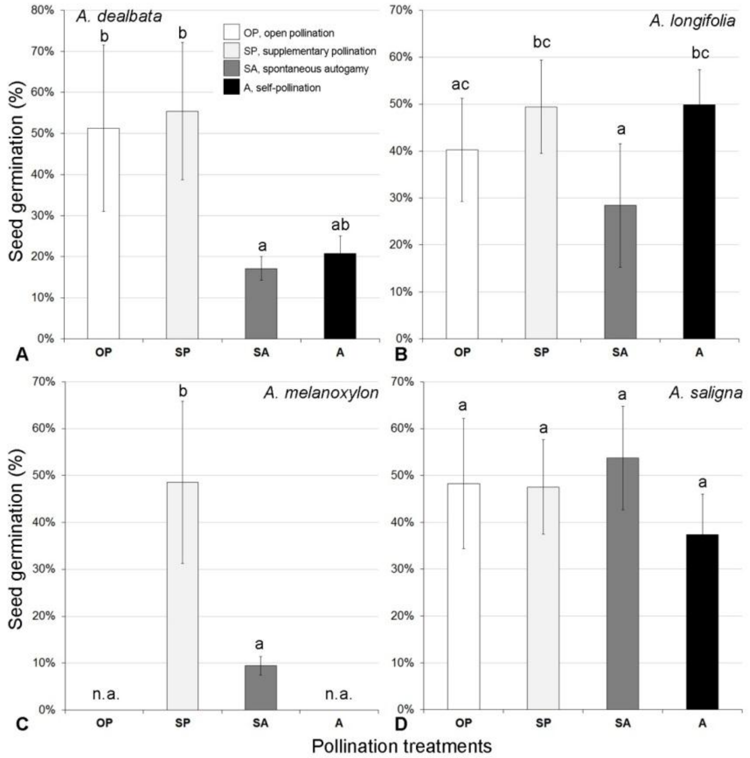

Figure 17. Seed germination (%) from the hand pollination experiments for the four *Acacia* species studied. Values are given as mean and standard errors of the mean. (A) *A. dealbata;* (B) *A. longifolia*; (C) *A. melanoxylon;* (D) *A. saligna.* Pollination treatments: OP, open pollination; SP, supplementary pollination; SA, spontaneous autogamy; A, Self-pollination. Different letters reveal statistically significant differences at *P* < 0.05 among treatments within species. n.a. means no available information.

For *A. dealbata*, only the interaction between treatment and tree was significant revealing the high variability found in seedling weight in the open-pollination treatment (Figure 10, TableVIII). No significant differences between treatments were found for *A. melanoxylon* and *A. saligna.* In *A. melanoxylon* the results were also unbalanced due to the reduced sample size of seedlings provided by the spontaneous autogamy (n=5). Overall, *Acacia saligna* seedlings were bigger than the seedlings of the other three species.

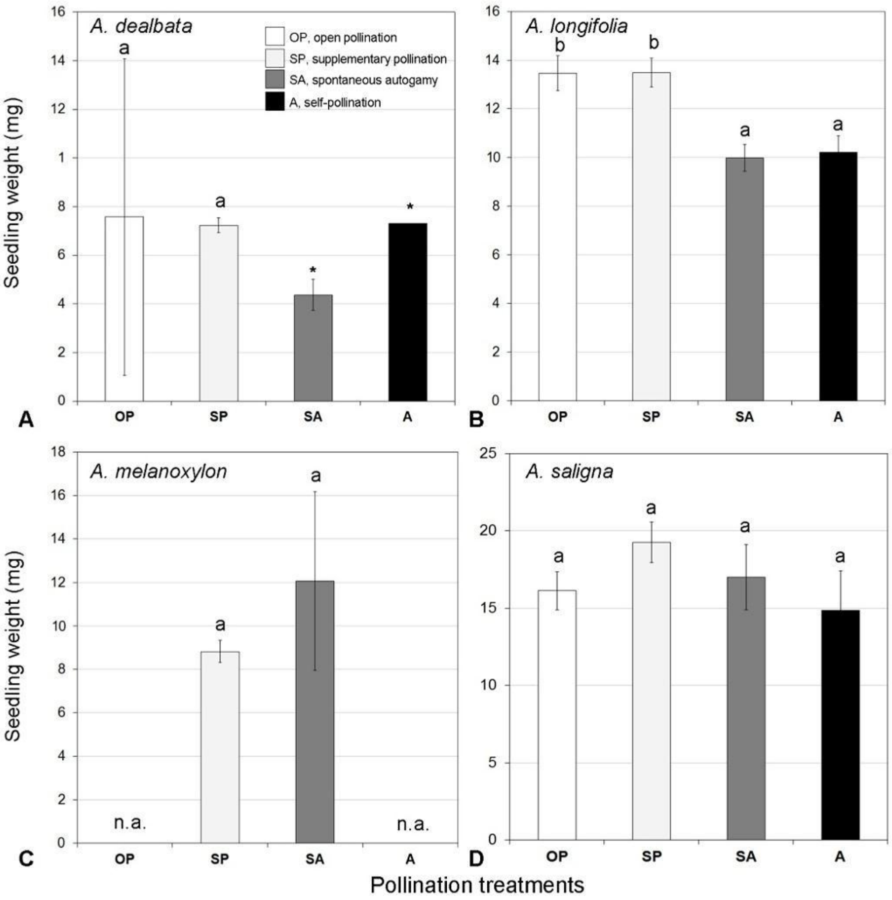

Figure 18. Seedling weight from the hand pollination experiments for the four *Acacia* species studied. (A) *A. dealbata*; (B) *A. longifolia*; (C) *A. melanoxylon*; (D) *A. saligna*. Seedling weight is given as mean and standard errors of the mean. Pollination treatments: OP, open pollination; SP, supplementary pollination; SA, spontaneous autogamy; A, Self-pollination. Different letters reveal statistically significant differences at $P < 0.05$ among treatments within species. n.a. means no available information. * Use to treatments that had been removed from the statistically analysis due to a small sample size (n <5).

4. Discussion

Discussion

Australian *Acacia* species have been widely introduced for forestry and gardening and, consequently, landscapes of many parts of the world are currently dominated by planted or invasive stands of *Acacia*s. Considering the area occupied, aggressiveness and impact on the native ecosystems, Australian *Acacia*s can be considered as the most problematic and widespread invasive plants in Portugal (Almeida *et al.,* 2006), among which are the four species studied in this work (*A. dealbata*, *A. longifolia, A. melanoxylon* and *A. saligna*).

Despite reproductive success is an essential factor in the colonization of new areas and in long-term establishment of viable populations, no information on any aspect of reproductive biology was available for invasive *Acacia* in Portugal.

The major achievements of this thesis are: a) the *Acacia* species studied have different investments in the production of reproductive units (flowers) and in natural reproductive success; b) *A. dealbata* has a massive production of flowers and a huge natural reproductive success, higher than any of the other three species; c) *A. melanoxylon* has a different reproductive strategy (andromonoecy); d) all species revealed to be partially self-compatible, although there is a high variability between individual trees; e) *A. dealbata* and *A. longifolia* suffered from pollen limitation; and f) pollen origin may affect offspring success with self-progeny having lower viability in *A. dealbata* and *A. melanoxylon*, while no patterns were observed for *A. longifolia* and *A. saligna*.

4.1 Floral morphology and display

Acacia species have small individual flowers remarkably similar and relatively simple in morphology and structural organization (Kenrick 2003). Such generalized morphology may facilitate invasion as it reduces the risk of pollinator limitation for introduced plants (Richardson *et al.,* 2000a). The tiny flowers are aggregated in dense flower heads attractive to pollinators that are considered the unit of pollination. The flower head (sometimes referred in literature as inflorescence) is a multi-stigmatic unit, relatively flexible in size and with its "surface" covered by many exposed anthers (Tybirk 1989).

In spite of having similar functional units of pollination (flower head), differences among the species were observed in several floral characteristics. Both the number of flowers per flower head and the number of stamens per flower vary substantially within and among species (Tybirk 1989, 1993; Sedgley *et al.,* 1992; Kenrick 2003), and this was also observed in the species studied. *Acacia longifolia* has more flowers per flower head and more stamens than the other three species, surprisingly with *A. dealbata* registering the lower numbers. The mean number of flowers per flower head in *Acacia* species can range from 2 or 3 (e.g., *A. myrtifolia*; Kenrick 2003) to 27 in *A. dealbata,* 50 in *A. longifolia* (this thesis) and up to 209 in *A. hindsii* (Stone *et al.,* 2003); while the mean number of stamens per flower varied from 33 in *A. dealbata* (this thesis) to 537 in *A. myrtifolia* (Kenrick 2003). The production of more robust flower heads or more floral rewards as pollen (through a bigger number of anthers) could be related with the pollinator assemblages that visit these species. Observations made in honeybees on a range of *Acacia* species suggest that flower heads with a small number of flowers are unable to support the weight of large insects and tend to collapse. In contrast, very small bees are able to gather a full pollen load from even sparse flower heads and are represented in the flower-visitor assemblages of all *Acacia*s so far studied (Stone *et al.,* 2003).

Data on pollinator's assemblage of *A. dealbata* and *A. longifolia* in Portuguese invaded areas has been collected this year in the scope of the project MUTUALNET and are currently being analyzed. In addition, the number of flower heads can fluctuate greatly depending on environmental conditions and resource availability (Sedgley, 1985; Gaol and Fox, 2002; Yates and Broadhurst, 2002).

Other distinctive features shared by Australian *Acacia* were observed in the studied species and confirm the pattern proposed for this group. They have asynchronized opening of flowers both, within flower heads and within single trees (Stone *et al.,* 2003), and individual flowers and flower heads are long-lived (Prescott, 2005). The opening of flowers on a single flower head over a series of days was observed in all the species and the flower head can last for up to 20 days (M. Correia, field observations; Stone *et al.,* 2003; George *et al.,* 2009).

4.1.1 Different strategies: hermaphroditism or andromonoecy?

Acacia species can have male and/or hermaphrodite flowers (Kenrick, 2003; George *et al.,* 2009). Hermaphroditism is the most common condition, while andromonoecy (i.e., individual plants bearing both male and hermaphrodite flowers) appears to be rarer (Zapata and Arroyo, 1978; Bullock

1985; Bernhardt *et al.,* 1984). Several *Acacia*s produce flowers (and often entire heads of flowers) containing only stamens (Tybirk 1989; Sedgley *et al.,* 1992; Baranelli *et al.,* 1995; Kenrick 2003) while others are complete hermaphroditic (e.g. *A. sinuata*; Raju *et al.,* 2006). In African species the positioning of the flowers seems to follow a pattern, with the hermaphrodite flowers being located distally in each flower head (Tybirk 1993). Further studies should be developed to assess the patterns of flower positioning within flower heads of Australian *Acacia*s.

Different strategies were also observed among the studied species. *Acacia dealbata*, *A. longifolia* and *A. saligna* were mostly hermaphrodite (>95% of the flowers of a flower head were hermaphrodite) while *A. melanoxylon* was clearly andromonoecious. In the studied year, only 30% of the flowers per flower head of *A. melanoxylon* were hermaphrodite. In addition, within flower head different types of flowers were found, from complete hermaphrodite flowers to male flowers without pistil, with transition flowers, some having smaller pistils and lower number of ovules per ovary and others with rudimental pistils without ovules. Transitions between perfect flowers and male flowers as the ones observed in *A. melanoxylon* were also observed in other species (Sedgley *et al.,*1992). Andromonoecy has been described in other *Acacia* species, such as *A. caesia* (Asia) and *A. macrantha* (Central America) (Raju *et al.,* 2006), *A. caven* (South America) in which about 50% of flowers are male (Peralta *et al.,* 1992). The Australian *A. mangium* was also reported as having andromonoecy, but with a tremendous variation in the percentage of staminate flowers per flower head (3 to 88%; Butcher *et al.,* 2004; and references there in).

The evolution of proximal male flowers seems to be a specialization in the subgenus *Acacia* (Tybirk 1989) and this tendency is further developed in other related genera having flowers specialized for nectar production and visual attraction (Arroyo 1981). Andromonoecy probably evolved due to resource limitation for the development of all fruits in a single flower head if all the pistils were successfully pollinated. Being cheaper to produce male flowers than hermaphrodite flowers, the resource investment in functionally male flowers improve male fitness by improving pollen donation and allows saving resources that can be reallocated to increase female fitness (Marín, M:V. and Rausher, M. D. 2006, references there in).

The mean number of aborted seeds per pod in *A. melanoxylon* was lower than in the other species, supporting this hypothesis and suggesting that this species may be reallocating resources to other traits, like cue-attracting pollinators. Other hypothesis, by contrast, suggests that staminate flowers are more effective at donating pollen than perfect flowers, they may produce more or larger pollen, have reduced pollen–pistil interference within flowers or among flowers on the same plant and may be more attractive to pollinators (Marín, M:V. and Rausher, M. D., 2006, references there in). Nevertheless, this breeding system is functional only when all plants flower simultaneously and when appropriate vectors transfer viable pollen to receptive stigmas on different individuals. In addition, the number of male flowers seems to be a highly variable and plastic character that allows individual plants to respond to environmental conditions and resource availability during floral development. For example, flower proportions may vary in response to changes in water availability (Aronson 1992). Different proportions of male flowers were also observed between seasons and positively correlated with intensity of flowering for *A. mearnsii* (Moncur *et al.,* 1991). Thus, future studies should address how the new conditions in the invasive area are driving this trait in comparison with the native range.

Many authors have described dichogamy (separation of male and female functions in time) as being widespread in *Acacia* species. Overall, Australian *Acacia* species have been described as having strictly protogynous flowers where the stigma is receptive before the anthers released the pollen (Stone *et al.,* 2003; George *et al.,* 2009). Future studies should confirm if the different development of anthers and pistil also coincide with different functional stages. Dichogamy has been proposed as a mechanism to prevent self-pollination in flowering plants (Lloyd and Weeb 1992) and also in *Acacia* (Stone *et al.,* 2003). However the opportunity for self-pollination in *Acacia* is high due to mass flowering (Bernhardt, 1989; Costermans, 2007), movement of pollinators within flower heads, and the tight organization of flower heads in the flowering branches (M. Correia, field observations).

4.1.2. Reproductive success

The massive flower display is a fundamental feature in most Australian *Acacia*s (Bernhardt, 1989; Costermans, 2007), and is a characteristic of the four studied species. In spite of this, only a small fraction of flowers develops successfully into fruit and seeds, so the natural overall reproductive success is low per tree in comparison with the number of flowers produced. Reproductive success is also highly variable within each of the studied species, with individual trees behaving in very different ways.

Acacia dealbata has the highest number of flower heads per flowering branch and per tree representing a huge investment in overall flower production, despite having small flower heads. This trait makes *A. dealbata* the species with higher natural reproductive success, which may contribute to its status as the most invasive *Acacia*. In support of this relationship between reproductive success and invasiveness, the two less widespread species in Portugal, *A. melanoxylon* and *A. saligna*, had a significantly lower investment in flower production, having a natural overall reproductive success less than 10% than that of *A. dealbata*.

Fruit set of *Acacia* species is low compared with other legumes. This low fruit set rate is in agreement with data from Baker (1983), who reported that usually only four or five flowers produce fruits from each inflorescence in the Mimosaceae. Such low values of fruit set may be considered as an adjustment of maternal resources to regulate flower and pod numbers (Baker *et al.,* 1983). In *Acacia*s, typically less than 1% of flowers result in fruit but fruits have a high seed to ovule ratios (Tybirk 1989). This has been proposed as a consequence of the polyad being capable of fertilizing all the ovules in the ovary just in one successful pollination event.

The correspondence between pollen grain number in the polyad and maximum seed number per pod in various species of *Acacia* led to investigation of ovule number in several *Acacia* species (Kenrick and Knox 1982). All studied species have 16 pollen grains per polyad and ovule number varies among species, generally being less than or even slightly greater than the pollen grain number (Kenrick 2003). The mean number of ovules per flower for the species studied in this thesis is 13 (10 for *A. saligna*), lower than the number of pollen grains in the polyad for all species.

Polyads are advantageous to maximize seed set if natural pod set rate is low. Pollen cohesion in polyads eliminates the chances of losing pollen and can be transported by any pollen vector (Knox and Kenrick 1983; Bernhardt 1989). Since polyads have enough pollen grains to fertilize all ovules in a flower, the seed to ovule ratio is expected to approach one. However, full seed set is rare in *Acacia*s, and in many species pods abort during the first weeks of development (Tybirk 1993), which is probably caused by reduced pollen viability and later seed abortion in the developing pod. All seeds in a pod may be full sibs as it was shown for *A. melanoxylon* using isozyme markers (Muona *et al.,* 1991). This will have an effect on competition during development within the pod and on competition and incompatibility relationships in a population, particularly in species where seeds tend to be retained in the pod. *Acacia saligna* and *A. dealbata* had the lower number of seeds per pod (one seed per pod is the most frequent value). This result show that total fecundation of ovules in a flower by the 16 pollen grains is very rare in all studied species. The mean number of seeds aborted per pod was higher in *A. dealbata* and *A. saligna* in all pollination treatments.

This could be related with high selfing rates (in agreement with the high levels of self-incompatibility observed in the study) and/or due to resource limitation (pollen or nutrients) that hinder the development of ovules to seeds. Even though, and as stated above, the massive production of flowers by the studied *Acacia* species counterbalance the low fruit set resulting in a huge seed crop in the invaded range that could be one of the factors involved in its invasions success.

4.2 Reproductive system

Reproductive success is essential to colonization of new areas and long-term establishment of viable populations. According to this, self-compatible plants have an advantage for the successful establishment in a new range because reproduction is less constrained by population size and pollinator availability; thus, self-compatible plants are expected to be more invasive than obligate outcrossing plants (Baker 1955; Gibson *et al.,* 2011). While outcrossing, when possible, might be beneficial for the evolution of invasive plants (Baker 1974), the capacity for autonomous seed production, which does not necessarily preclude outcrossing, is likely to be essential during several stages of the invasion process (Van kleunen and Johnson, 2007).

4.2.1. Self-incompatibility

Fruit set of studied *Acacia*s express significant differences between pollination treatments for all the species. The studied *Acacia* species are partially self-incompatible (ISI > 2; index of self-incompatibility), with *A. longifolia* having an ISI (index of self-incompatibility) value in the limit between self-incompatible and partially self-incompatible species (0.19). *Acacia saligna* has the higher value (0.46) followed by *A. dealbata* (0.32), and *A. melanoxylon* (0.28). For all studied species, fruit set, seed to ovule ratio and mean number of seeds per pot were significantly lower in the self-pollination treatments than in those involving cross-pollination. This result suggest that cross-fertilization is important for the reproductive outcome of invasive *Acacia*s, although in the absence of compatible partners or pollen vectors, self-fertilization can also contribute significantly to the spread in the new area.

Beyond the variability among species, a huge variation was found within species with complete incompatible trees, partially compatible trees and compatible trees growing within the population, which might result in complex patterns of the relative contribution of individual trees to the invasive populations. This intraspecific variation in self-compatibility rates appears quite common in Australian *Acacia* species (Philp and Sherry, 1946; Moffett and Nixon, 1974).

Australian *Acacia* species range from highly self-incompatible to completely self-compatible and autogamous (Moffett, 1956; Bernhardt *et al.,* 1984; Kenrick and Knox, 1989; Morgan *et al.,* 2002). Still, the selected Australian *Acacia* species have a clear preference for outcrossing (Broadhurst *et al.,* 2006; Gibson *et al.,* 2011). Generalist insects, mainly bees, are common pollinators of Australian *Acacia*s, so it is unlikely that they suffer from a lack of pollinators in the areas where they are introduced.

Hence, the main question is whether invasive populations maintain the same levels of self-incompatibility or have evolved mechanisms to increase autogamy rates as a mechanism of reproductive assurance.

The capacity to self-reproduction is known for six Australian *Acacia* species, five of which are invasive (*A. dealbata, A. decurrens, A. mearnsii, A. paradoxa* and *A. saligna*) (Gibson *et al.,* 2011, references in). Studies with species from other ranges of distribution, *A. retinodes* (Bernhardt *et al.,* 1984; kenrick and knox, 1985; Kenrick and Knox, 1989b), *A. myrtifolia* , *A. pycnantha*, *A. mearnsii* (Kenrick and Knox, 1989b), *A. decurrens* and *A. baileyana* (Morgan *et al.,* 2002) have shown seed set from self-pollination to be only 3 to 27% of that arising from cross-pollination. In contrast, seed set following self-pollination in A. *paradoxa* and *A. ulicifolia* was 82 to 95% of that arising from cross-pollination (Kenrick and Knox, 1989b) *A. sciophanes* also showed high levels of selfing with a comparatively low outcrossing rate of 0.61 (Coates *et al.,* 2006). Evidence suggests that self-incompatibility in *Acacia* could be the result of post-zygotic lethal genes (Kenrick, 2003).

When comparing the levels of incompatibility between native and invaded ranges for the studied species, there is no information for *A. longifolia*, for *A. dealbata* the results obtained in the invaded area confirm the information available to native area, where they are partial self-compatibility and self-incompatible when the populations are fragmented (Broadhurst *et al.,* 2006). However the preference for outcrossing is visible, with a higher production of pods and seeds in the open and supplementary pollination treatments. The same pattern was observed in *A. saligna* and *A. longifolia.*

A. saligna in native area has a mixed mating system, being partially self-compatible but predominantly out-crosser (George *et al.,* 2008) and in the invaded area of South Africa is partially self-compatible (ISI = 0.82; Gibson 2012). The results obtained in *A. saligna* population of Tocha are in agreement with these results. High outcrossing rates have been detected in *A. melanoxylon* R.Br. (Muona *et al.,* 1991) in native area and the same was found in the populations studied from Portugal. However *A. melanoxylon* also revealed a partially self-compatibility capacity, producing viable pods and seed in self-fertilizing treatments

Some of the levels of spontaneous selfing that were observed are most probably due to the proximity of sexual structures within flower head and between flower heads due to massive flowering. The prior or simultaneous deposition of self, incompatible, or related pollen by pollinators may interfere with the ability of plants to use available cross pollen, resulting in reduced seed set. Self-pollen grains may cause clogging or blocking of stigma surfaces preventing cross pollen from germinating or reducing cross-pollen tube development (Ramsey and Vaughton, 2000; and references there in). This was observed in *Burchardia umbelata* (Colchicacea) an Australian self-incompatible plant (Ramsey and Vaughton, 2000).

Plants could possibly reduce self-pollen nosiness by increasing the time between anther dehiscence and stigma receptivity to reduce autogamy, and by reducing the number of flowers open concurrently to reduce geitonogamy. Pollinators frequently move short distances and when genetic proximity exists, pollen transfer can occur between related individuals (Waser and Price, 1983, 1991a). The observations of *Acacia* floral visitor’s behavior reveal that some of this self-pollen interferences can happen in *Acacia* populations and might be responsible for the low fruit and seed set and the high seed abortion in some species. Some contingents imposed by the bagging procedure could also contribute for this output. *A. dealbata* which was the huge massive flowering had less seed viable per pod, more seeds aborted per pod and a low fruit set in natural conditions (near 1%), so geitonogamy is an unavoidable cost of requiring a large floral display to attract pollinators and could be a consequence of restricted pollinator foraging (Ramsey and Vaughton, 2000; references in). Indeed, in the complete absence of pollinators, all the studied species were still able to produce huge amounts of seeds per tree, despite in lower number than in open pollinated treatment.

This results support the hypothesis that invasive species like the studied Australian *Acacia* tend to have some level of self-compatibility, despite not higher than expected in comparison with native populations; suggesting that the ability to self-fertilize may predispose *Acacia* species to invasiveness and to spread at larger rates. However comparisons between the native and invaded area of the studied Australian *Acacia* species are hindered by insufficient data. Although this capacity may make species more likely to become invasive, it is not essential for invasiveness (Gibson *et al.,* 2011) *A. auriculiformis* and *A. pycnantha* are noticeable examples of invasive self-incompatible species.

Even being poor selfers, self-fertilization could ameliorate pollinator and mate limitation, two reproductive barriers that may occur in the initial steps of naturalization and invasion due to small size or low density of populations (Baker, 1955; Davis *et al.,* 2004).

Pollen limitation

Considering the generalised structure of the flower heads, generalist insects, mainly bees, are common pollinators of Australian *Acacia*s, so it was unlikely that *Acacia*s suffer from a lack of pollinators in the areas where they are introduced. Contrary to this expectation, *A. dealbata* and *A. longifolia* suffer pollen limitation in this invaded area. In natural conditions, fruit set and seed to ovule ratio were higher in the supplementary pollination treatment than in open pollination, indicating the occurrence of pollen limitation in these two species.

Pollen limitation occurs when pollen quantity is low, if pollinators are rare, or when plants compete for the services of pollinators and pollen quality is limited since pollinators deposit on stigmas self or incompatible pollen (Ramsey and Vaughton, 2000; and references in). The early flowering of *A. dealbata* and *A. longifolia* might limit the number and diversity of insects available for pollination, thus, explaining the results obtained. Since very few native species flower as early as these two *Acacia*, it might be hypothesized that they do not have a great impact on native pollination networks as it has been shown for other invasive species.

Acacia is an animal-pollinated invasive plant so has great potential to disrupt interactions between native plants and pollinators (Traveset and Richardson, 2006). Their integration into pollination webs is facilitated due to being pollinator generalist (Richardson *et al.,* 2000). Invasive plant (e.g. *Carpobrotus* spp., *Lantana camara, Mimosa pigra*) with rich floral resources, through huge or prolonged floral displays, could have a strong negative impact on the reproductive success of a native plant if it was chosen by pollinators (Traveset and Richardson, 2006).

The impact of flowering *A. saligna* on insect visitation to co-flowering native species has been recently assessed in in South Africa where *A. saligna* is an aggressive invasive plant (Gibson 2012). The results show that one of the native species most-visited by native honeybees (*Roepera fulva*) suffered significantly lower visitation when *A. saligna* was present (Gibson 2012). From a different point of view, pollen and nectar offered by *A. dealbata* and *A. longifolia* might be an important winter resource for insects but this remains unstudied.

4.3 Offspring performance

In spite of the differences observed in fruit set and mean number of seeds per pod with selfing pollination having lower success than cross-fertilization treatments, this pattern was not observed for all species in the studied seed and seedling traits. While, in *A. melanoxylon* and *A. dealbata* seed from open and supplementary pollination were significantly heavier than self-fertilizing seeds, *A. longifolia* had an opposite trend (seeds resulting from the self-pollination treatment were significantly heavier than seeds from the outcross pollen or open treatments). *A. saligna* has a different pattern, with differences after open and supplementary pollination treatments, which were not differ from spontaneous autogamy and could reflect high levels of self-pollination mediated by floral visitors. Self-pollination treatment yielded lighter seeds that the other pollination treatments. Regardless of the production of pods in sell fertilizing treatments a decrease in fertility and vigor of the self-produced seeds was expected (Moffet and Nixon, 1974). Thus, pollen origin may affect offspring success with self-progeny having lower viability in *A. dealbata* and *A. melanoxylon*, while no patterns were observed for *A. longifolia* and *A. saligna*.

Variation in germinability is a consequence of genetic, phenotypic, and environmental conditions under which the seeds mature and can be found among species, populations, and even among individuals within a population (Gutterman, 2000). The germination assay for the four *Acacia* species studied reveal significant differences in seed germination between cross-pollination and spontaneous autogamy treatments to all *Acacia* species with the exception of *A. saligna.* Overall, seed germination was higher in the seeds produced by open and supplementary pollination treatments than the seeds from self-fertilization treatments (with rates around 50% against 10-20%, respectively). The higher germinability of *Acacia* species is also a key factor in their invasion potential.

Finally, the weight of one-month seedlings did not differ between treatments in *A. melanoxylon* and *A. saligna*. In *A. melanoxylon* and *A. dealbata* the results were also unbalanced due to the reduced sample size. *A. longifolia* seedlings from the open and supplementary pollination treatments were larger than those from self-fertilizing treatments. *A. dealbata* seedling presented a high variability in the open-pollination treatment. *A. saligna* seedlings were bigger than the seedlings of the other three species. This result may suggest that *A. saligna* self progeny could have survival rates similar to outcross progeny. In seed germination and seedling growth experiments with *A. mearnsii, A. decurrens* (Moffett and Nixon, 1974) and *A. dealbata* (Gibson 2012, references in), their self-progeny had a reduced growth and survival than outcrossing progeny.

Despite that these differences could erode the reproductive assurance benefits of selfing (Herlihy and Eckert, 2002), self-progeny still had some viability, and thus can be an option for the establishment of *Acacia* species, although not so successful as outcrossing progeny. However, seedling growth was measured in very young seedlings to account for initial differences in the quality of seed resources, and further experiments should be performed to measure possible effects of inbreeding depression on reproductive maturity and seed set.

Several studies with *Acacia* species concluded that reproductive attributes, including flowering, pollination, seed set and dispersal, and seed viability, are improbable to constrain their natural recruitment. Germination and seedling establishment are fundamental aspects to the maintenance of long term and viable populations of *Acacia*s (Yates *et al.,* 2002; Coates *et. al.,* 2006).

5. Conclusion

General conclusions

1. The studied Australian *Acacia* species have different investments in the production of reproductive units (flowers and flower heads) and in natural reproductive success. But, a high intraspecific variability was also found in all studied reproductive features (flower characters, reproductive success, and incompatibility system and offspring performance).
2. Three of the four species are mostly hermaphrodite, while *A. melanoxylon* has a different reproductive strategy, andromonoecy.
3. Low pod production and, consequently, great floral resources loss, characterize the reproductive system of these species. Seed production is likely to be limited by resource availability. The environmental effects, such as rainfall, on reproduction and pollen viability of studied species need to be investigated.
4. Despite of the low reproductive success, there is a large production of seeds due to massive flowering: Australian *Acacia*s showed a low efficiency in the use of resources but a successful reproduction.
5. Regardless of the lower number of flowers per flower head in comparison with the other species studied, *Acacia dealbata* has a higher production of flower heads and higher natural reproductive success and can thus be considered the most aggressive invader of the studied species.
6. All species revealed to be partially self-compatible, although there is also a high variability between individual trees. Cross fertilization resulted in higher fruit set and seed to ovule ratios than self-fertilization.
7. *Acacia dealbata* and *A. longifolia* are early flowering species and suffered from pollen limitation despite the massive flowering.
8. Pollen origin may affect offspring success with self-progeny having lower viability in *A. dealbata* and *A. melanoxylon*. However, *A. saligna* has a self-progeny as viable as outcross-progeny.
9. The difficulty in comparing breeding systems of the studied Australian *Acacia*s from native and invaded areas is due to lack or scarce information available for both areas.

Future perspectives

Due to the high intraspecific variability obtained in all four species for all studied parameters, more populations should be studied in both native and invaded ranges. Future studies should address differences in pre-dispersal seed predation between native and invaded areas. Pre-dispersal seed predation by weevils occur in the native range of some Australian *Acacia*s and in Iberian woody legumes in Portugal but host switch between native and exotic legumes remains unstudied.

The control of invasive organisms is expensive, labor intensive, and often meets with little success. Therefore, it is important to prevent new introductions of potentially invasive species. Since there is a strong role of the breeding system in plant invasions, this factor should be studied before introduction and included in screening protocols for predicting invasiveness

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7. Appendices

Appendix A

Results from the hand pollination experiments for the four *Acacia* species studied.

Table VII Fruit set from the hand pollination experiments for the four *Acacia* species studied.

Treatment	*A. dealbata*	*A. longifolia*	*A. melanoxylon*	*A. saligna*
Open pollination	0.98 ± 0.172 (19)^{b}	0.90± 0.247 (12)^{a}	3.13 ± 0.531 (16)^{a}	0.85 ± 0.247 (12)^{a}
Supplementary pollination	1.61 ± 0.263 (19)^{c}	1.67 ± 0.171 (12)^{c}	3.29 ± 0.577 (16)^{a}	1.59 ± 0.282 (12)^{c}
Spontaneous autogamy	0.42 ± 0.102 (20)^{a}	0.26 ± 0.100 (12)^{b}	0.46 ± 0.132 (16)^{b}	0.79 ± 0.208 (12)^{b}
Self-pollination	0.52 ± 0.138 (20)^{ab}	0.38 ± 0.073 (11)^{b}	0.93 ± 0.266 (15)^{b}	0.73 ± 0.162 (12)^{b}
Statistical test	$F_{3,62}$=9.27, P<0.001	$F_{3,33}$=16.89, P<0.001	$F_{3,44}$ =17.05, P<0.001	$F_{3,33}$=11.03, P<0.001

Notes: Fruit set is given as the percentage of flowers developing into fruit from the total number of flowers treated. Values are given as mean and standard errors of the mean followed by sample size in parenthesis.

Table VIII Seed to ovule ratio from the hand pollination experiments for the four *Acacia* species studied

Treatment	*A. dealbata*	*A. longifolia*	*A. melanoxylon*	*A. saligna*
Open pollination	0.0043±0.00016 (16)^{b}	0.44±0.126 (10)^{b}	1.37±0.0246 (15)^{b}	0.34±0.093 (9)^{b}
Supplementary pollination	0.0076±0.00036 (18)^{b}	0.73±0.090 (12)^{b}	1.43±0.334 (14)^{b}	0.35±0.064 (12)^{b}
Spontaneous autogamy	0.0017±0.00017 (14)^{a}	0.12±0.034 (8)^{a}	0.15±0.040 (8)^{a}	0.10±0.030(10)^{a}
Self-pollination	0.0011±0.00010 (12)^{a}	0.12±0.021 (11)^{a}	0.29±0.088 (10)^{a}	0.13±0.043 (10)^{a}
Statistical test	$F_{3,26}$=6.12, P=0.0016	$F_{3,18}$ =32.30, P<0.001	$F_{3,28}$ =11.15, P<0.001	$F_{3,25}$=5.87, P<0.001

Notes: Seed to ovule ration is given as the percentage of ovules that developed into morphologically viable seeds from the total number of ovules available in the hermaphrodite flowers treated. Values are given as mean and standard errors of the mean followed by sample size in parenthesis.

Table IX Seed production from the hand pollination experiments for the four *Acacia* species studied.

Treatment	***A. dealbata***	***A. long folia***	***A. melanoxylon***	***A. saligna***
Open pollination	3.09 ± 0.171 (250)^{b}	5.66 ± 0.020 (198)^{b}	4.55 ± 0.155 (472)^{b}	2.82 ± 0.156 (209)^{b}
Supplementary pollination	3.27 ± 0.012 (437)^{b}	5.95 ± 0.137 (372)^{b}	5.19 ± 0.194 (252)^{c}	2.61 ± 0.102(315)^{b}
Spontaneous autogamy	1.58 ± 0.199(78)^{a}	4.34 ± 0.492 (29)^{a}	2.82 ± 0.436 (33)^{a}	1.14 ± 0.119 (83)^{a}
Self-pollination	1.63 ± 0.264 (78)	3.69 ± 0.321 (49)^{a}	2.77 ± 0.314 (93)^{a}	1.53 ± 0.140 (83)^{a}
Statistical test	$F_{3,687}$=5.13 P=0.0016	$F_{3, 631}$=11.96 P<0.001	$F_{3,751}$=19.62 P<0.001	$F_{3,592}$=17.46 P<0.001

Notes: Seed production is given as the mean number of morphologically viable seeds produced per pod. Values are given as mean and standard errors of the mean followed by sample size in parenthesis.

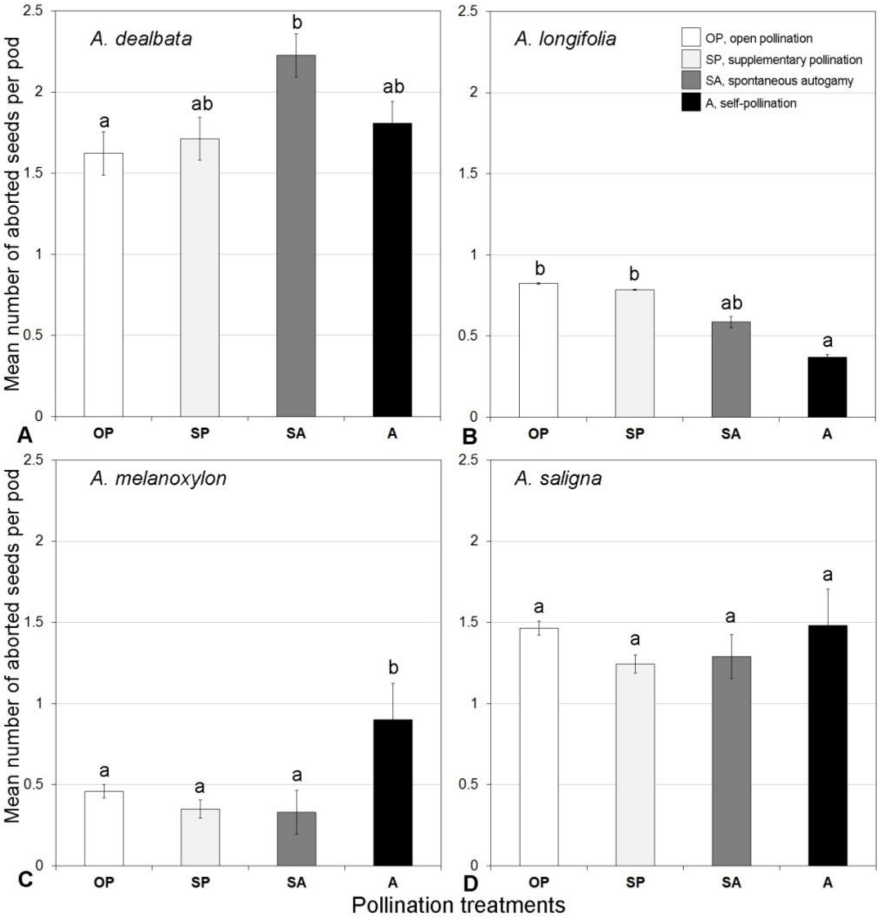

Figure 19. Mean number of aborted seeds from the hand pollination experiments for the four *Acacia* species studied. (A) *A. dealbata*; (B) *A. longifolia*; (C) *A. melanoxylon;* (D) *A. saligna*. Seed abortion is given as the mean number of aborted seeds produced per pod. Pollination treatments: OP, open pollination; SP, supplementary pollination; SA, spontaneous autogamy; A, Self-pollination. Different letters reveal statistically significant differences at $P < 0.05$ among treatments within species.

Table X Results of statically analysis of the number of aborted seeds per pod from the hand pollination experiments for the four *Acacia* species studied (GLZ with an error Gaussian and an identity function, plant was a random factor).

Treatment	*A. dealbata*	*A. longifolia*	*A. melanoxylon*	*A. saligna*
Statistical test	$F_{3,633}$=4.35 P=0.0048	$F_{3,633}$ =4.35 P=0.0048	$F_{3,831}$=5.85 P=0.0009	$F_{3,674}$ =0.75 P=0.524

Appendix B

Table XI Index of self-incompatibility (ISI) values of some *Acacia* species founded in literature (with respective references) mainly from native populations of Australia. This ISI values are presented in Figure as a dot (in Results).

Species	**ISI**	**Area**	**Status**	**References**
A. baileyana	0.02	Australia	Native	Gibson *et. al.,* 2012
A. dealbata	0.727			
A. mearnsii	0.0405			
A. mearnsii	0.00			Kenrick and Knox 1989
A. myrtifolia	0.17			
A. paradoxa	0.79			
A. pycnantha	0.008			
A. retinodes var. *uncifolia*	0.02			
A. terminalis	0.07			Millar *et al.,* 2008
A. saligna	0.77			
A. sciophanes	0.61			
A. caven	0.001	Argentina		Gibson *et. al.,* 2012
A. tortilis	0.2	Senegal		
A. saligna	0.82	South Africa	Invaded	Gibson 2012

Appendix C

Results from the offspring performance after different hand pollination experiments for the four *Acacia* species studied.

Table XII Seed weight from the hand pollination experiments for the four *Acacia* species studied.

Treatment	***A. dealbata***	***A. longifolia***	***A. melanoxylon***	***A. saligna***
Open pollination	15.5 ± 0.20 (193)^{b}	19.8 ± 0.21 (356)^{a}	22.0 ± 0.31 (191)^{b}	17.8 ± 0.20 (142)^{b}
Supplementary pollination	15.2 ± 0.28 (103)^{b}	19.3 ± 0.23 (190)^{a}	23.1 ± 0.35 (151)^{b}	18.7 ±0.21 (229)^{b}
Spontaneous autogamy	14.3 ± 0.39 (64)^{a}	21.3 ± 0.27 (140)^{b}	19.5 ± 0.66 (70)^{a}	17.9 ± 0.33 (79)^{b}
Self-pollination	13.9 ± 0.31 (55)^{a}	-	20.4 ± 0.44 (134)^{a}	16.7 ± 0.28 (79)^{a}

Notes: Seed weight (mg) is given as mean and standard errors of the mean followed by sample size in parenthesis. Different letters mean significant differences between treatments within each species.

Table XIII Results of the General Liner Model (GLM) analysis for the comparisons of the seed weight from the hand pollination treatments for the four *Acacia* species studied.

	A. dealbata			***A. longifolia***			***A. melanoxylon***			***A. saligna***		
	d.f.	*F*	*P*	d.f.	*F*	*P*	d.f.	*F*	*P*	d.f	*F*	*P*
Treatment	3	1.174	0.344	2	0.050	0.952	3	6.058	0.004	3	3.018	0.47
Tree	8	3.236	0.019	19	2.735	0.046	11	3.117	0.014	10	3.539	0.004
Treatment*tree	16	3.389	<0.001	11	10.408	<0.0001	19	11.077	<0.001	23	1.939	0.006

Table XIV Seed germination from the hand pollination experiments for the four *Acacia* species studied.

Treatment	***A. dealbata***	***A. longifolia***	***A. melanoxylon***	***A. saligna***
Open pollination	51.3 ± 20.27 (2)^{c}	40.2 ± 11.01 (10)^{ac}	-	47.6 ± 10.06 (9)^{a}
Supplementary pollination	55.4 ± 16.73 (2)^{c}	49.4 ± 9.90 (12)^{bc}	48.6 ± 17.26 (7)^{a}	48.3 ± 13.86 (10)^{a}
Spontaneous autogamy	17.1 ± 2.87 (6)^{ab}	28.4 ± 13.16 (7)^{a}	9.4 ± 2.00 (3)^{b}	53.7 ± 11.08 (10)^{a}
Self-pollination	20.8 ± 4.17 (5)^{bc}	49.8 ± 7.56 (11)^{bc}	-	37.4 ± 8.64 (11)^{a}
Statistical test	$F_{3,170}$=5.48 P= 0.0013	$F_{3,528}$=3.32 P=0.0196	$F_{3,129}$=5.85 P=0.0170	$F_{3,420}$=1.04 P=0.3758

Notes: Seed germination is given in percentage as mean and standard error of the mean followed by sample size in parenthesis.

Table XV Seedling dry weight from the hand pollination experiments for the four *Acacia* species studied.

Treatment	***A. dealbata***	***A. longifolia***	***A. melanoxylon***	***A. saligna***
Open pollination	7.6 ± 6.52 (24)^{a}	13.5 ± 0.71 (41)^{b}	-	16.1 ± 1.24 (51)^{a}
Supplementary pollination	7.2 ± 0.30 (40)^{a}	13.5 ± 0.59 (64)^{b}	8.8 ± 0.51 (39)^{a}	19.3 ± 1.31(49)^{a}
Spontaneous autogamy	4.4 ± 0.64 (3)*	10.2 ± 0.56 (25)^{a}	12.1 ± 4.11 (5)^{a}	17.0 ± 2.12 (15)^{a}
Self-pollination	7.3 (1)*	10.2 ± 0.69 (45)^{a}	-	14.9 ± 2.55 (12)^{a}

Notes: Seedling dry weight (g) is given as mean and standard error of the mean followed bay sample size in parenthesis.

Table XVI Results of the Generalized Liner Model analysis for the comparisons of the seedling weight from the hand pollination treatments for the four *Acacia* species studied.

	A. dealbata			***A. longifolia***			***A. melanoxylon***			***A. saligna***		
	d.f.	*F*	*P*	d.f.	*F*	*P*	d.f.	*F*	*P*	d.f	*F*	*P*
Treatment	1	0.893	0.436	3	3.913	0.021	1	0.003	0.967	3	1.081.	0.372
Tree	5	0.508	0.770	11	2.056	0.068	5	1.982	0.569	10	0.035	0.918
Treatment*tree	2	3.764	0.030	16	1.878	0.027	1	3.603	0.066	15	0.086	0.114

Appendix D

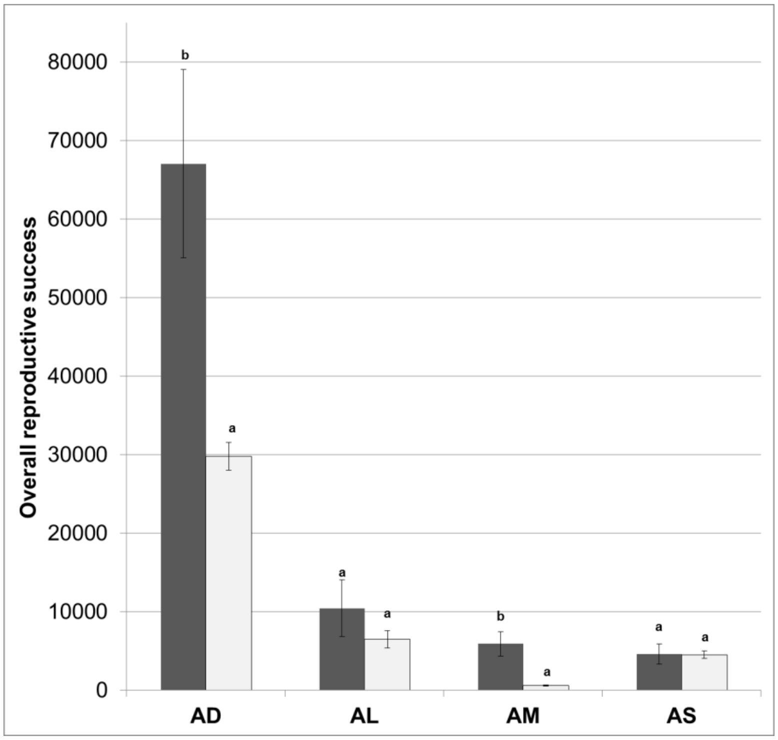

Figure 20. Estimated overall reproductive success for the four *Acacia* species studied. AD - *A. dealbata*; AL - *A. longifolia*; AM - *A. melanoxylon;* AS - *A. saligna*. Overall reproductive success of open pollination (dark grey bars) and spontaneous autogamy (light grey bars) treatments as a mean value of the tree success. Different letters reveal statistically significant differences at *P* < 0.05 among treatments within species.

Table XVII Results of the Generalized estimating equations (GEE) analysis for the estimated overall reproductive success after open and spontaneous autogamy pollination treatments for the four *Acacia* species studied AD - A. dealbata; AL - *A. longifolia*; AM - *A. melanoxylon*; AS - *A. saligna* (tree as a subject variable; success as a dependent variable). The reproductive success was modelled as a multinominal distribution, with cumulative logit used as the link function.

Species	Test of model effects		
	Wald x^2	df	*P*
AS	,056	1	,812
AL	1,494	1	,222
AD	6,609	1	,010
AM	19,835	1	,000

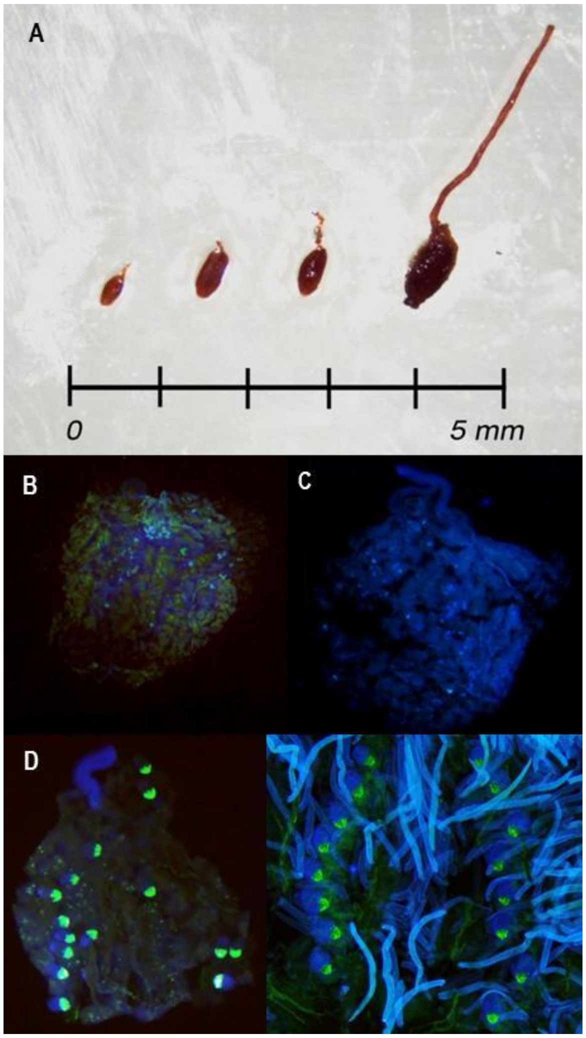

Figure 21. Different types of pistils found in *A. melanoxylon.* This species has transitional flowers between male to hermaphroditic. **A**: Dissecting microscope photos of pistils, with different sizes, from the smallest and rudimentary, up to the higher normal pistils (see the left to the right). **B** to **E**: fluorescent microscope photos (aniline blue) corresponding with types of pistils in the photo A. **B-C**: Rudimentary pistil without ovules, these flowers were classified as being males. **D-E**: hermaphrodite flowers; **D**: Small pistils with a high number of ovules per ovary; **E**: Well-developed ovules in a single pistil.

Appendix F

Table XVIII – Statistically analysis (GLZ) results for the differences in the characters used to flower head characterization among species.

Floral characters	Statistical test
No. anthers	$F_{3,386} = 740.20$ $P < 0.001$
Ovules	$F_{3,459} = 11.10$ $P < 0.001$
No. flowers per flower head	$F_{3,151} = 63.54$ $P < 0.001$
% hermaphrodite flowers per flower head	$F_{3,151} = 34.54$ $P < 0.001$