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Figueiredo Vasques**

**Avaliação da adequação de espécies de plantas
para restauro ecológico após graves perturbações**

**Assessment of higher plant species suitability for
ecological restoration following major disturbance**



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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Ciências e Engenharia do Ambiente, realizada sob a orientação científica do Doutor Jan Jacob Keizer, Investigador Auxiliar do Departamento de Ambiente e Ordenamento da Universidade de Aveiro e co-orientação da Doutora Maria da Conceição Lopes Vieira Santos, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro e Doutor Victoriano Ramón Valejo Calzada, Professor Titular da Universidade de Barcelona.



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Dedico este trabalho à memória do meu avô materno pelo seu exemplo de determinação e nobreza de espírito.

“The mind that opens to a new idea never returns to its original size.”

Albert Einstein

o júri

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palavras-chave

Restauração ecológica, germinação, desenvolvimento inicial de plântulas, stress hídrico, eco-fisiologia, arbustos mediterrânicos.

resumo

O presente trabalho é constituído por uma revisão bibliográfica e uma série de estudos científicos experimentais visando a melhoria do conhecimento da ecologia de germinação e resposta ao stress hídrico em arbustos autóctones. Estes estudos pretendem contribuir para o conhecimento da ecologia de espécies autóctones na fase de germinação e estabelecimento inicial das plântulas através da comparação da resposta de sementes de diferentes proveniências. Os primeiros três capítulos focam-se no estudo dos efeitos de pré-tratamentos na germinação de espécies arbustivas com diferentes estratégias de regeneração e dormência da semente, enquanto os dois últimos capítulos incidem sobre o efeito de diferentes condições de disponibilidade de água na germinação e estabelecimento inicial de plântulas com potencial para restauração ecológica.

Este estudo procura abordar aspetos importantes para o estudo e validação do uso de espécies alvo para a restauração ecológica em zonas Mediterrânicas, contribuindo deste modo para a melhoria do conhecimento da ecologia destas espécies.

A germinação foi estimulada por pré-tratamentos de calor em quatro das cinco espécies cuja época de dispersão da semente na primavera-verão. As sementes provenientes do sul tenderam a germinar melhor, sendo ao mesmo tempo mais pequenas. Por outro lado, a germinação em espécies cuja dispersão da semente ocorre no outono-inverno variou de acordo com o tipo de dormência da semente. A germinação foi em geral favorecida pela estratificação a frio na espécie apresentando dormência fisiológica mas foi negativamente afetada na espécie que não apresenta dormência. Esta resposta não foi, no entanto, inequívoca pois foi dependente da proveniência estudada. O efeito da estratificação a frio teve em geral um efeito mais positivo ou menos negativo nas sementes provenientes do norte em comparação com as outras proveniências.

O stress-osmótico teve um efeito negativo consistente na germinação de *Pistacia lentiscus* e outro fator que também afetou significativamente a germinação foi a variabilidade intra-populacional entre plantas mãe. As duas proveniências apresentaram diferentes graus de associação entre a germinação e/ou caracteres morfológicos da semente ou planta mãe.

Quanto à fase de estabelecimento inicial, o baixo conteúdo em água teve um claro efeito negativo no investimento relativo em biomassa acima do solo e um efeito positivo no investimento relativo em biomassa abaixo do solo em *Arbutus unedo*. Esta resposta não foi inequívoca, uma vez que as proveniências diferiram em adaptações morfológicas ao baixo conteúdo em água. As plântulas da proveniência mais húmida revelaram uma taxa de crescimento relativo superior mas, ao mesmo tempo, uma fraca adaptação às condições de baixa disponibilidade de água, quando comparadas com as outras proveniências. Ao contrário, as plântulas da proveniência seca com verão mais quente apresentaram semelhantes diâmetro do colo da raiz, peso seco de folhas e performance fisiológica sob os dois regimes de irrigação.

Os resultados obtidos revelam diferenças significativas na germinação e desenvolvimento inicial entre as distintas proveniências que poderão estar relacionadas com adaptações ao clima dos locais de origem. Os desenvolvimentos futuros do estudo dos mecanismos subjacentes às adaptações observadas poderiam contribuir para a melhoria das previsões de sucesso do estabelecimento inicial em diferentes populações de plantas, permitindo aumentar a confiança e efetividade de custos nas decisões relacionadas com ações de restauração ecológica em cenários de alterações climáticas.

keywords

Ecological restoration, germination, seedling early development, water stress, eco-physiology, Mediterranean shrubs.

abstract

The present work is composed by a conceptual scheme based on a literature review plus a series of experimental scientific studies. These studies aim at improving the knowledge base of germination ecology and response to water stress in autochthonous shrub species through the comparison of different provenances. The first three chapters focus on the study of the role of pre-treatments in the germination of shrub species with different regeneration and seed dormancy strategies while the two last chapters focus on the study of the effect of contrasting water availabilities in the germination and early establishment of species with restoration potential.

This study intends to address important aspects for the study and validation of the use of target species for ecological restoration actions in Mediterranean areas, thereby contributing to the improvement of the knowledge of the ecology of these species.

Germination in four out of the five species whose seeds are dispersed in spring-summer was enhanced by heat pre-treatments. The seeds from southern provenances tended to germinate better, being at the same time smaller. On the other hand, germination response in autumn-winter seed dispersed species varied according to the type of seed dormancy. Germination was, in general, enhanced by cold stratification in the species presenting physiological dormancy but negatively affected in the species with no dormancy. This response was, however, not unequivocal and depended on the role of provenance. The effect of cold stratification had, in general a more positive or less negative effect in the germination of the northern seeds when comparing to the other provenances.

Osmotic-stress had a consistent negative effect on germination of *Pistacia lentiscus* and another factor that significantly influenced germination was intra-population variability between mother-plant. The two provenances presented distinct degrees of association between germination and seed or mother plant morphological traits.

As to the early establishment phase, low water content had a clear negative effect on *Arbutus unedo* seedlings' aboveground biomass and a positive effect on belowground biomass relative investment. This response was not unequivocal, since provenances differed in morphological adaptations to low water content. Seedlings from the wettest provenance revealed a higher relative growth rate under high water content but a poor adaptation to limited water availability when compared to the other two provenances. By contrast, seedlings from the dry provenance with the hottest summer had similar root collar diameter, leaf dry weight and physiological performance under both watering regimes.

The present results revealed significant differences in germination and early development response among different provenances that could be related with adaptations to the climate of the seed source. Further studies on the mechanisms underlying the observed adaptations could contribute to the improvement of early establishment success predictions in different plant populations, allowing more reliable and cost-effective management ecological restoration-related decisions under climate change scenarios.

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List of Abbreviations

A - Net photosynthesis

Ci - Substomatal CO₂ concentration

E – Transpiration

Gs - Stomatal conductance

HWC – High Water Content

Hs – Shoot height

IWUE - Intrinsic Water Use Efficiency

LDW - Leaves dry weigh

LWC – Low Water Content

LWR - Leaf weight ratio (LWR)

Φ PS II - Yield Photosystem

MP- Mother-plant

RCD - Root collar diameter

RDW - Root dry weight

R:S- Root to shoot ratio (R:S)

RWR- Root weight ratio (RWR)

SDW - Stem dry weigh

SLA - Specific leaf area

SW:LA - Sapwood to leaf area ratio

TDW - Total dry weight

TLA - Total leaf area

T90 – Time when 90% of the total germination is attained

WC – Water content

Chapter 1

General Introduction

The development of modern societies has expanded and intensified the pressure over native habitats, leading to biodiversity loss in many places around the world. Besides that land management is an old practice in Europe, industrialization, construction and agriculture intensification have also affected biodiversity negatively in the past decades (Millenium 2005). In the Mediterranean, these processes have occurred along with inner-land abandonment and loss of land management traditional practices (Vallejo et al. 2001).

The understanding of the effects of human activities on species, communities and ecosystems allows the development of practical interdisciplinary approaches to protect and restore biological diversity (Primack 2000). Thus, ecological restoration aims at improving health, integrity and sustainability of a degraded ecosystem (SER 2004). Ultimately, ecosystem resilience should be restored through the enhancement of ecosystem biodiversity and functions (Walker 1995). In this context, species selection for ecological restoration should on one hand, promote biodiversity (Bischoff 2006; 2008) and on the other, contribute to ecosystem functions by promoting specific plant functional traits that were affected by disturbance and, according to the major ecosystem threats, will improve ecosystem resistance and resilience (Cortina et al. 2006).

The frequency and area of occurrence of wildfires in the Mediterranean has increased during the past decades (Pausas et al. 2008). Though fire is part of the natural processes occurring in Mediterranean ecosystems, plant regeneration can be hampered by repeated and/or high intensity wildfires (Naveh 1974). The increase and accumulation of fire-prone early-successional species in the ecosystem together with summer drought can aggravate this scenario (Vallejo et al. 2012a). The tendency to accumulate aerial death biomass (flammability) should then be considered as an important trait for species selection in fire-prone systems.

Various experiments and programs have been developed during the last decades throughout the Mediterranean in order to restore degraded lands (Chirino et al. 2009). These programs were focused initially in forestation actions for tree production – i.e. using single or few species and recently in a more ecological approach – i.e. using a

wide set of species that are previous selected according to their functions (Vallejo et al. 2006).

Ecological restoration in the Mediterranean should identify the actual threats to ecosystem resilience and resource sustainability, contributing to the reduction of future pressures' effects and desertification risk (Vallejo et al. 2012). In Mediterranean areas, however, the concepts of disturbance and reference ecosystem are often hard to define because human intervention by land management played a major role in shaping the actual ecosystems (Pausas 1999; Cortina et al. 2011). In this context, wildfires together with summer drought are major threats to the sustainability of ecosystem functions in the Mediterranean (Pausas et al. 2008). In addition, the foreseen increase of both threats due to climate change predictions had led to widespread doubts about the future resilience of Mediterranean ecosystems under the current management practices (Pausas et al. 2008).

The adjustment of management practices to the principles of ecological restoration in the Mediterranean aim at ecosystem recovering but also play a role in shaping sustainable ecosystems, namely by reducing the flammability of fire-prone communities through species composition adjustment (Vallejo 2012). The selection of strong resprouter, low flammable late-successional species has been advised as a management option to increase ecosystem resilience in the Mediterranean (Vallejo et al. 2012). This principle also meets the inclusion of species whose recruitment is not naturally favored by fire. In degraded forests of European ecosystems, mid to late successional species are less well represented in the soil seed bank and therefore its introduction is often justified (Bossuyt & Honnay 2008). In spite of this, the successful germination of early-successional species also represents an important feature in post-fire ecosystem resilience (Meira-Neto et al. 2011).

Seed mixtures could consider both short and long term restoration effects, such as soil loss prevention and habitat improvement. In this context, the positive effect of pioneer shrubs in the establishment and survival of late successional species through facilitation could justify a combined approach (Gómez-Aparicio et al. 2004).

Restoration actions with mid-to-late successional species often prefer planting over seeding, because that option naturally reduces germination and early survival uncertainties (Vallejo et al. 2012a). However, if germination and early establishment could succeed, hydroseding could be a suitable management option. This option could,

in particular, solve the main limitations of planting which include high nursery and irrigation costs, poor post-planting acclimatization and survival and difficulties in selecting proper planting sites (Chirino et al. 2009; Cortina et al. 2011). In spite of this, the use of hydroseeding in ecological restoration is still limited and mostly restricted to erosion mitigation through the use of commercial fast growing species (Oliveira et al. 2012).

The use of hydroseeding in post-fire or degraded lands' restoration is still uncommon in the Mediterranean. However, recent studies support the need to research their effectiveness using native species (Fernández-Abascal et al. 2003; Oliveira et al. 2012). The germination ecology of most of the native species remains, however, poorly understood (Clewett et al. 1997). This might be one of the causes for the actual dominance of the use of commercial seeds in ecological restoration actions in Europe (Oliveira et al. 2012). The use of commercial highly spread and fast growing species in ecological restoration should be avoided, as these species, by often competing and hampering regeneration of local species, can compromise biodiversity and ecosystem resilience goals (García-Palacios et al. 2010).

Once a certain set of target species is selected, their suitability needs to be assessed in order to predict if they will successfully germinate and establish given a set of conditions (Fig.1).

Since different species have different germination ecologies the assessment of species suitability requires considerable knowledge on species ecology. First of all, the knowledge on species distribution, habitats and seed dispersal phenology should be used to identify possible areas, at the regional scale, that might present sizable populations and the optimal time of harvest. After harvesting enough amounts of seeds, the appropriate seed cleaning and storage for each type of fruit/seed needs to be carried out. Then, germination potential and timing must be correctly addressed by selecting appropriate pre-treatments for dormancy breaking. Finally, the potential of seedling establishment under optimal and stressful conditions must also be assessed (Fig.2).

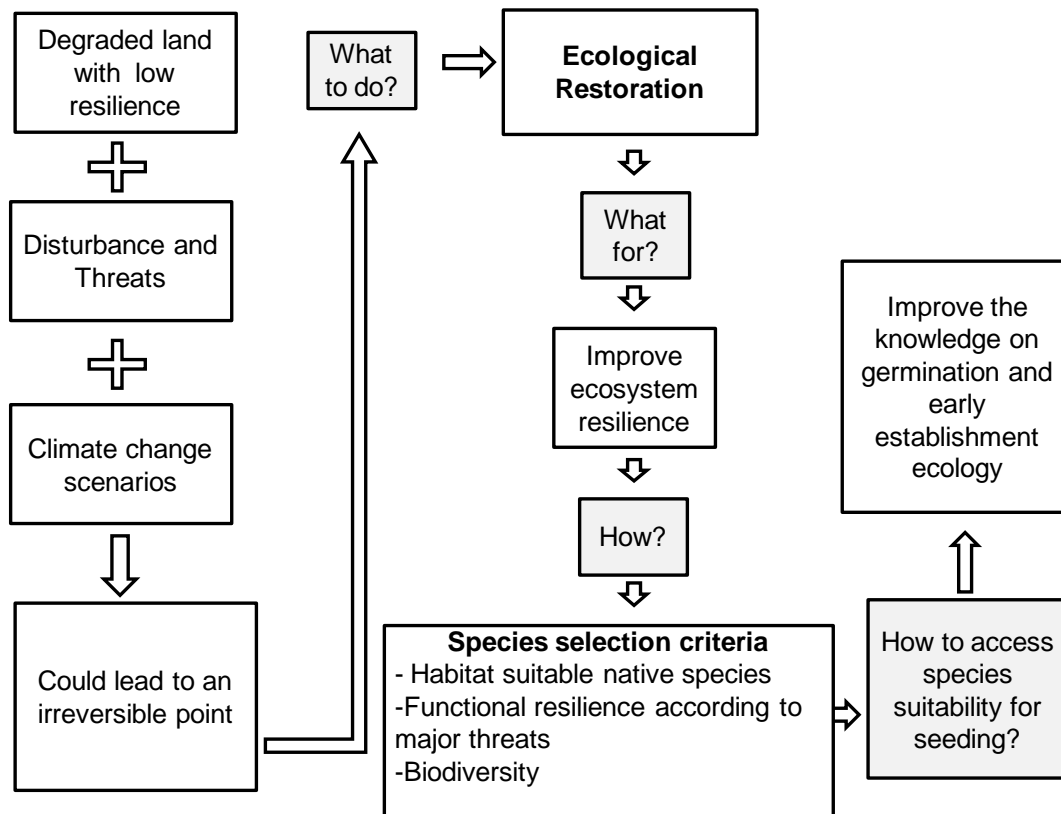


Figure 1 – Schematic representation of the criteria underlying species selection for ecological restoration and posterior assessment of their suitability for use in seeding actions.

To understand plant response and phenotypic plasticity is essential to assess the real effects of climate changes (Nicotra et al. 2010). Thus, in order to improve their success under climate change scenarios, ecological restoration actions need to adopt adaptive approaches, considering for e.g. intra-specific variations in plant response (Vallejo et al. 2012b). While addressing species suitability for seeding under ecological restoration, it is therefore important to consider that germination response may not be homogeneous throughout the distribution area of a certain species, as germination variability has been reported, either among populations, different individuals of the same population or between different branches from the same individual (Cruz et al 2003; Fig.2).

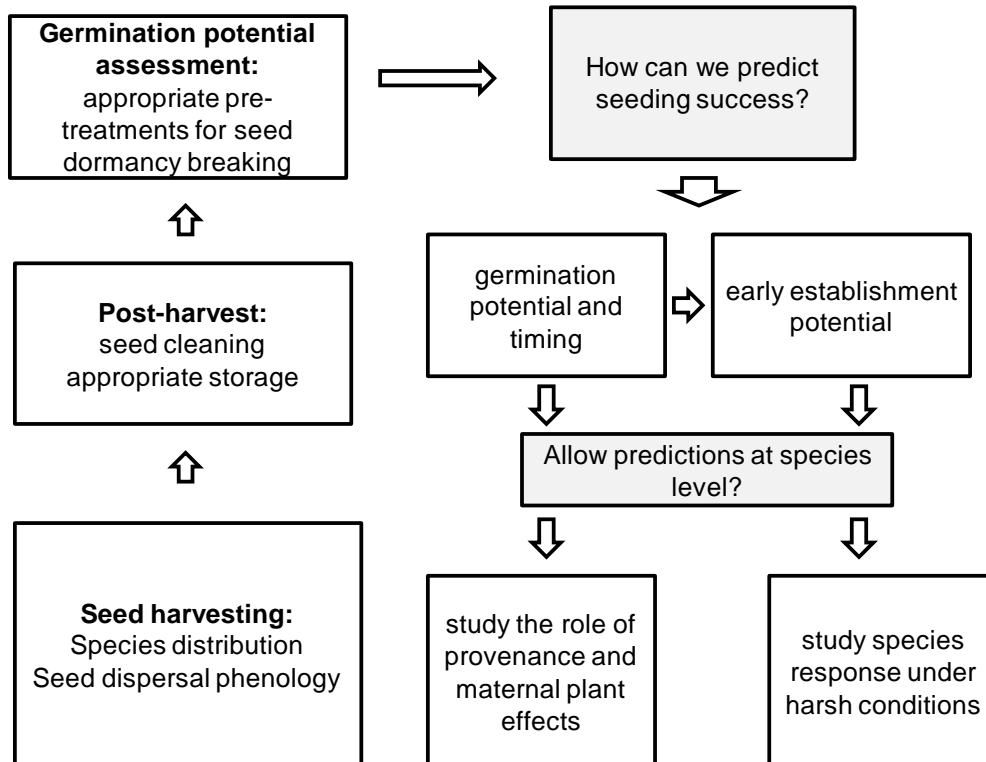


Figure 2- Important aspects to assess target species suitability.

The main objective of this thesis is to improve the knowledge base for selection and utilization of autochthonous habitat-specific shrub species for seeding-based ecological restoration following major disturbances, thereby contributing to more effective ecological restoration techniques.

This thesis is divided in two main parts: the first part (comprised by Ch. 2, 3 and 4) addressed overall germination ecology while the second part (comprised by Ch. 5 and 6) studied germination and early establishment under low water content conditions. The role of provenance is addressed throughout the thesis within each chapter.

In particular, Chapter 2 presents a conceptual framework of the germination ecology related-aspects that should be taken into account in germination predictions following ecological restoration actions. Due relevance is given to aspects such as, seed dispersion seasonality, dormancy type and dormancy breaking treatments. The next two chapters (Ch. 3 and 4) focus on the study the germination ecology in species with two contrasting post-dispersal seasons. The specific objectives of these chapters were to evaluate: i) the species-specific germination response to heat or cold pre-treatments according to their post-fire regeneration strategy and dormancy type (Ch.

3 and 4, respectively); ii) evaluate the effect of seed provenance in germination response and iii) explore possible relations between the observed response and the environmental/ climate conditions of the seed source.

The second part of the thesis researched germination and early establishment under low water content conditions (Ch.5 and 6, respectively). In chapter 5, the effect of seed provenance and maternal traits in germination under osmotic stress was researched using a case study species (*Pistacia lentiscus*). In particular, this chapter aimed to: i) assess the effect of low osmotic potentials in the germination phase; ii) test the role of provenance in germination under high and low water conditions and finally iii) investigate the role of mother-plant in germination potential through the assessment of a series of morphological indicators at the mother-plant, seed and seedling levels.

Finally, in chapter 6 the effect of seed provenance in the development of young seedlings under contrasting water content conditions was researched using *Arbutus unedo* as a case study. The specific objectives of this chapter were to: i) investigate the effect of low water content conditions in the morphology and eco-physiology of young seedlings; ii) test the provenance role in early development under high and low water conditions and iii) explore the relations of the observed responses with site-specific climate characteristics.

Two sets of species were selected for the present study according to their different seed dispersal seasons, i.e. during summer or winter. The criteria for species selection within the first set of species (Ch. 3) was based on their high representativeness in terms of species richness and cover (Maia et al. in prep.) in the understory of Portuguese forests (families: Cistaceae, Ericaceae and Leguminosae). Because of their high potential to accumulate aerial dead biomass, those species are not, however, indicated for ecological restoration in fire prone areas. To improve the knowledge-base on the germination of these species is still important to understand the potential for post-fire regeneration or their role in ecosystem resilience after major disturbance (Meira-Neto et al. 2011). Contrarily, the criteria for the selection of the second set of species were based on their high potential use for ecological restoration following wildfires. Thus, the second set of species (used in Chap. 4), from which two out of the three species' were further researched in Chapters 5 and 6, were low flammable resprouters with reported drought resistance, which are, at the same time, late sucessional species whose recruitment is not favoured by fire (Fig.1).

In summary, the management option of hydroseeding for ecological restoration opens the possibility of using a broad set of species that will germinate and get established according to their ecology. While opening a set of new opportunities, the success-related predictions of seeding native species under ecological restoration actions also rise up many challenges. To know when and to which extend a set of seeds will germinate and successfully establish requires an important and structured knowledge of native species germination and early development ecology, which will be further addressed in this thesis.

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Chapter 2

Seeding native plant species for ecological restoration in the Mediterranean – a conceptual framework for germination prediction

This chapter is in preparation for submission

Abstract

The use of native species over non-native commercial seeds is strongly advised for ecological restoration actions. Biodiversity conservation goals together with the higher adaptation potential of these species are among the reasons for this principle. At the management level, however, this option can represent an extra challenge. Native seeds are only commercially available for a few species and its origin is often uncertain. Furthermore, the germination ecology of native species is not well known and the knowledge available is not enough well structured to allow successful hydroseeding.

In the last decades, hydrothermal models for germination prediction have been developed and improved for several crop and model species. Up to date, however, the reliability of these models in predicting germination of native species has not been consistently explored. The main drawbacks for the use of native species in hydroseeding are related with difficulties in predicting the output, namely caused by seed dormancy breaking requirements and variations in germination timing (speed and seasonality).

We consider that the application of hydroseeding in ecological restoration should be based on a structured understanding of dormancy breaking mechanisms and seasonality plus a dynamic understanding of dormancy intensity. Seed dispersal phenology and seed source can provide useful information for the understanding of seasonal-related mechanisms of dormancy breaking and dormancy intensity, respectively. The development of germination prediction models considering multiple steps of seasonal effects in the change of dormancy intensity can improve

germination predictions following ecological restoration, facilitating the work of researchers and managers. Finally, we discuss in which extent the comprehensive use of germination ecology knowledge can assist seed harvesting and handling protocols, namely by: i) adjusting the best time for harvesting; ii) adjusting the use of pre-treatments and germination timing according to species-specific predictions and finally iii) acknowledging the importance of seed source in germination predictions.

2.1. Introduction

The principle of ecological restoration is to bring the ecosystem to a reference state which was likely to be present before disturbance took place (SER 2004).

It is commonly agreed that the use of seed mixtures of non-native species in ecological restoration actions should be avoided (Bischoff et al 2006; 2008; Mijnsbrugge 2010). Commercial seeds of native species are not common and, if available, its origin and reliability is often doubtful (Tishew et al. 2011). Furthermore, the use of commercial seeds can often contribute to lower genetic diversity and contaminate local gene pools (Hufford & Mazer 2003; Bischoff et al. 2008).

The introduction of native species is highly desirable to meet biodiversity conservation goals and ensure ecosystem functions following ecological restoration (Tishew 2011). Additionally, there are experimental evidences that local species can create good soil cover (Tormo et al. 2007). However, the uncertainties about the effective success of seeding of a high number of native species still hamper their application in practice (Bochet et al. 2010; Tishew 2011; Oliveira et al. 2012). In addition, the use of commercial species in seed mixtures can hamper the regeneration of native species by competition (Garcia-Palacios et al. 2010).

From a practical point of view, and at the management level, the use of commercial seed mixtures requires little efforts of seed acquisition and, given a set of recommendations provided by seed companies, also little knowledge on species germination ecology. The ease of sowing commercial seeds could therefore explain that, in spite of the current recommendations for ecological restoration, 70% of the seeding actions in Europe use commercial seeds instead of local native seeds (Tishew et al. 2011).

The main identified drawbacks causing uncertainties in the outcomes of the seeding of native species are i) slow germination; ii) seasonality; and iii) seed dormancy-breaking requirements (Oliveira et al. 2012). In order to predict germination after seeding, the aforementioned aspects should be duly considered and researched. After that, an effective transfer of the obtained knowledge to managers is also relevant to increase the use of native species (Bochet et al. 2010).

Genetic aspects and seed zones are now recognized to be important factors in ecological restoration actions (Bower & Aitken 2003; Valladares & Gianoli 2007; Bischoff et al. 2008). Though this debate has only recently aroused in ecological restoration research, provenance-studies for tree production improvement have a long history behind. In these studies, the best seed sources for a certain target site are often chosen by the inclusion of climate change scenarios (Matyas 1994; O'Neill et al. 2008). The effect of seed provenance in germination predictions following seeding should also be considered while improving the knowledge-base on germination ecology of native species. In this sense, germination success of local seeds can be predicted more reliably than it would be possible using species-wide information.

Underneath we propose a comprehensive conceptual framework for predicting the germination behavior of Mediterranean shrub species, addressing dormancy breaking requirements and speed and seasonality of germination. This framework combines the concepts of hydrothermal time models (Alvarado & Bradford 2002) and population-based threshold models (Batlla & Benech-Arnold 2010) for seasonal-related seed bank germination prediction. Our ultimate objective is to point out research directions to improve the prediction of native species germination, and, thereby, enhance their use in ecological restoration actions in the Mediterranean.

2.2. The use of hydrothermal models for germination prediction

In the last decades, hydrothermal models have been developed and improved aiming at predicting germination in several crop species. These models are based in the concept that, once dormancy is broken, temperature and water promote seed germination in an additive manner, i.e. a certain number of days above a certain temperature (minimum threshold) plus a certain number of days above a certain water potential will lead to germination (Alvarado & Bradford 2002; Allen 2003; Hardegree

et al. 2003). Based on this approach, the time and percentage of germination attained will depend on the exposure of the seed to temperatures and water potential between the species-specific thresholds.

In most plant species, germination is highly determined by temperature and water potential and thus these models are usually successful at predicting germination. Nonetheless, if the species show dormancy, the progress towards germination is blocked and these predictions can fail into a great extent. This is because of the fact that under dormancy, environmental conditions are received as stimuli that can lead or not to dormancy breaking before the progress towards germination can start. Hydrothermal models have lately evolved through great complexity, including dormancy and physiological aspects, which might allow their use for reliable predictions in a broader set of species (Bauer et al. 1998; Bradford 2002; Rowse & Finch-Savage 2003).

2.3. Seed dormancy type and dormancy breaking mechanisms

Dormancy is an adaptation mechanism that blocks germination under conditions that otherwise would favor it. In this manner, the set of stimuli needed to break dormancy may facilitate that germination will occur under conditions that would enhance seedling survival (Baskin & Baskin 2004). The most common type of dormancy found in seeds is physiological dormancy -imposed by embryo physiology but physical dormancy- imposed by the impermeability of the seed coat-is also a common strategy in the Mediterranean (Baskin & Baskin 2004; Moreira et al. 2010; Moreira & Pausas 2012). The phylogenetic classification of seed dormancy can be a valuable tool in the first approach to dormancy type identification (Baskin & Baskin 2004; Finch-Savage & Leubner-Metzger 2006). However different species of the same family can present contrasting dormancy breaking requirements (e.g. Vasques et al. 2012).

Under physiological dormancy, germination is prevented by the action of regulatory mechanisms (Bewley 1997; Baskin & Baskin 2004). The main regulatory mechanisms of seed dormancy at the physiological level are two important hormones: abscisic acid (ABA) and giberelins (GAs). The levels of ABA are largely responsible for primary dormancy induction and dormancy maintenance, whereas the levels of GAs increase with the embryo progress towards germination until radical protrusion (Bove et al. 2001; Finch-Savage & Leubner-Metzger 2006). Though the regulation of these

hormones depends on various environmental stimuli, temperature is known to play a major role in dormancy. Cold can promote the biosynthesis of gibberellins and dry conditions after dispersal can decrease ABA concentration (Finch-Savage & Leubner-Metzger 2006).

In seeds presenting physical dormancy, water imbibition is inhibited by impermeable layers of the seed coat and therefore, once this impermeability is broken, and if there is no other type of dormancy present, germination time can be determined by hydrothermal models (Baskin 2003). The use of seed coat damaging mechanisms, as mechanical or chemical scarification is usually successful in dormancy breaking (Patane & Gresta 2006). The occurrence of this dormancy-breaking mechanisms might not be, however, easily predictable in nature, since species survival and fitness is also dependent on the ability to remain dormant under the abrasion of microorganisms and soil particles (Baskin & Baskin 2000).

Temperature could also play a role in physical dormancy breaking as high and highly fluctuating temperatures are also described as mechanisms to break physical dormancy (Baskin 2003). Accordingly, in many Mediterranean species presenting physical dormancy, germination is known to be triggered by heat (Paula & Pausas 2008). Furthermore, cold stratification (the exposure to cold of imbibed seeds) was also reported to successfully break physical dormancy in some Leguminosae species (Baskin 2003; Van Assche et al. 2003). This might be explained, at least partly, by the previously reported role of gibberellins in the weakening process of the impermeable layers (Finch-Savage & Leubner-Metzger 2006). In this context, seasonality could also play a role in breaking physical dormancy.

The main threat to plant survival in the Mediterranean is drought during summer. As a consequence some Mediterranean species developed strategies to cope with summer drought by avoiding germination within the summer period or by ensuring seedling development before summer time. It is then expected that Mediterranean species present low maximum temperature thresholds, as an adaptation to avoid germination close to the summer period. This phenomenon was recently reported for *Arbutus unedo*, whose low maximum temperature threshold was maintained even after cold stratification (Bertsouklis & Papafotiou 2013). In Europe, the minimum and maximum temperatures thresholds for germination are consistently related with a latitudinal gradient, with northern seeds presenting higher minimum thresholds and southern species lower maximum thresholds (Fenner & Thompson 2005).

Consequently, special attention should be given to the definition of temperature and water potential thresholds of Mediterranean species.

2.4. Seasonality in germination predictions using soil seed bank models

Population-based thresholds models could already provide reliable predictions for the germination of summer and winter weed annuals of the soil seed banks of temperate regions (Benech-Arnold et al. 2000; Batlla & Benech-Arnold 2010). This was attained by using changes in environmental conditions, such as cold stratification and after-ripe heat and or dry conditions, in the prediction of seasonal changes in the dormancy intensity of the soil seed bank.

Similarly to what is observed for the temperate regions of Europe, spring and autumn are the two possible seasonal windows for germination in Mediterranean regions (Fig. 3). In this context the stimuli to overcome dormancy are often related with seasonal conditions observed after dispersion (Karsen et al. 1998). This reinforces the adaptation character of dormancy and its role in preventing germination under hostile environments.

In species that disperse seeds between autumn and spring (hereafter designated as winter species) dormancy is usually successfully broken after cold stratification, which allows germination in the following spring (Fig. 3). In the past, population models were also developed with the objective to access germination response after cold stratification (Benech & Sanchez 1995; Bradford 1996; Pritchard et al. 1996; 1999). Germination enhancement after cold stratification is also observed in species that are present in the Mediterranean, such as *Acer* spp., *Arbutus unedo*, *Celtis australis*, *Fraxinus* spp. and *Juniperus oxycedrus* (Garcia-Fayos 2011; Tilki 2004, 2007; Draghici & Abrudan 2011, Vasques et al. in prep). Cold stratification can however have a negative effect in the germination of winter-dispersed non-dormant species, as it was observed for *Pistacia lentiscus* (Anacardiaceae) and *Myrtus communis* (Myrtaceae; Garcia-Fayos & Verdú 1998; Garcia-Fayos 2001; Vasques et al. in prep).

Germination in species ripened after spring and before autumn (hereafter designated as summer species) is often enhanced by heat (Fig. 3). This is true for many Mediterranean species that present physical dormancy (e.g. Cistaceae and Leguminosae) but was also observed for Lamiaceae and Ericaceae species, which

do not present impermeable seed coats (Paula and Pausas 2008; Moreira et al. 2010). Moreira and Pausas (2012) attributed this response to fire-adaptation. However the high temperatures that can be attained under canopy gaps during summer (over 60°C) were also proven to significantly enhance germination in *Ulex parviflorus*, *Cistus albidus* and *Rosmarinus officinalis* (Santana et al. 2013), highlighting the importance of seasonal variations in the dormancy breaking process species with spring-summer seed dispersion.

Germination in summer species is therefore expected to occur during the following autumn, as observed by Céspedes et al. (2012a) in a seeder dominated seed bank. The success of germination seems to, however, be strongly dependent from the timing and duration of the wet season and, in this context, inter-annual variations should be properly considered in germination predictions (Moreno et al. 2011; Céspedes et al. 2012b). In this context, a delay of seedling emergence to the next germination window(s) caused by delays in rainfall was observed in several summer dispersed species (*Cistus ladanifer*; *Erica umbellata*, *Erica australis*, *Pterospartum tridentatum*, *Rosmarinus officinalis*; Moreno et al. 2011; Céspedes et al. 2013).

Interestingly, other species which are widely present within the European territory, such as *Calluna vulgaris* and *Crataegus monogyna* can experience germination enhancement by either cold or hot temperatures (Vera 1997; Garcia-Fayos 2001; Bujarska-Bokowska 2002; Paula and Pausas 2008). Germination prediction in these species should be carefully researched and the presence of both adaptation mechanisms in a broader set of species should be disentangled.

Given the species-specific literature or experimentally based information, a coherent conceptual framework for germination prediction can be created through the understanding of the germination seasonality in different species. Likewise, if appropriate, the seeding time can be adjusted to seasonal conditions and species adaptations in order to maximize the germination rates.

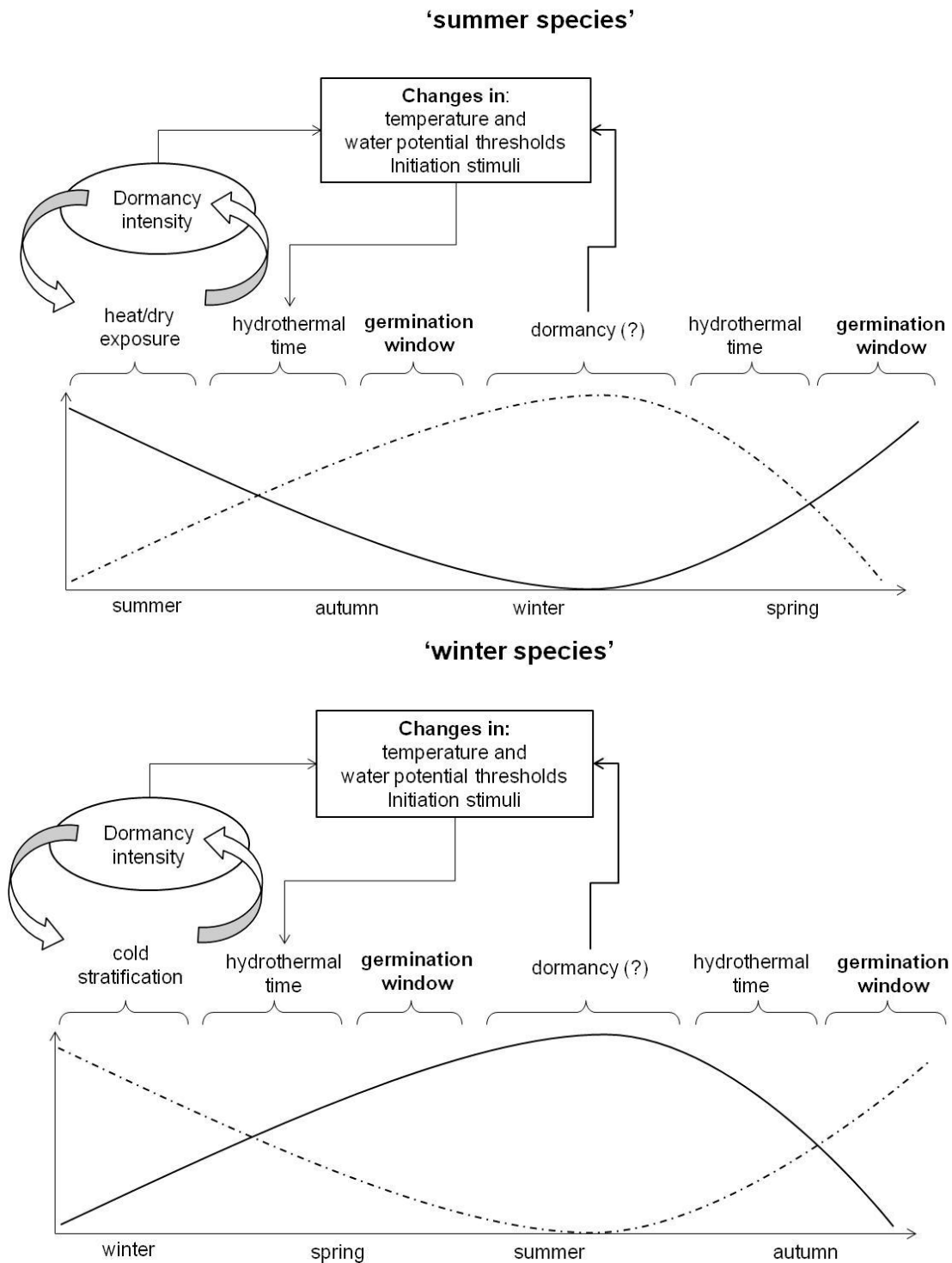


Figure 3 – Conceptual scheme for the study of germination ecology based in time of seed dispersal, dormancy breaking mechanisms and dormancy intensity. An illustration of seasonal variations in Temperature (full line) and Precipitation (broken line) in the Mediterranean and the correspondent changes in dormancy breaking and germination progress are presented for summer and winter species (up and down, respectively). The time of seed dispersal is indicated with an arrow.

2.5. Dormancy intensity

The biological clock of a seed can slow down or speed up depending on the intensity and duration of environmental stimuli and also on the information that is already present in the seed. Since the moment of fertilization the seed is constantly sensing and adjusting its biological clock. The intensity and effectiveness of these adjustments can vary according to the stage of seed development and continues to occur after dispersal (Probert 2000).

Several studies have reported the dynamic character of germination ecology and the complexity of environmental influences at different stages of seed maturity (Fenner & Thompson 2005). The origin of this complexity seems to be related with the dynamic character of seed dormancy whose intensity varies according to its exposure to different environmental conditions (Vleeshouwers et al., 1995; Bewley et al. 1997).

These adjustments can be translated in changes in temperature and water potential thresholds affecting the effect of environmental conditions in germination triggering (Alvarado & Bradford 2005; Fig. 3). Vergis (1964) proposed that the range of temperatures between thresholds narrows as dormancy intensifies and expands as dormancy declines. This idea was recently confirmed and, furthermore, changes in dormancy intensity were also proven to influence the sensitivity to initiation stimuli required for germination (e.g. light or nitrates; Alvarado & Bradford 2005).

Provenance differences can be originated in genetics, epigenetics or environmental differences. Seed provenance differences in germination that seem to be related with the climate of the seed origin were previously reported either following warm or cold stratification in many Mediterranean species, such as *Lavandula stoechas*, *Origanum vulgare*, *Thymus* spp., *Cistus ladanifer*, *Erica australis*, *Erica umbellata*, *Genista triacanthos*, *Pterospartum tridentatum*, *Arbutus unedo* and *Phillyrea angustifolia* (Pérez-García et al 2003; Vasques et al 2012; Vasques et al, in prep.). Moreover, a recent study also related the climate of seed origin and the minimum temperature requirements for germination in a latitudinal gradient of *Calluna vulgaris*' distribution in Norway (Spindelböck et al 2013).

Dormancy intensity could also be in the origin of these differences as seeds from warmer places could have less dormancy intensity and therefore germinate better (Guterman 2000; Murdoch and Ellis 2000). However the relation between seed source temperature and dormancy intensity is not straightforward and could depend

on the species present (Guterman 2000). In the development of a germination prediction model according to the presented conceptual scheme (Fig.3), the provenance-related variations in temperature thresholds would be assumed through different initial (and dynamic) dormancy intensities. This would represent a starting assumption that should be further adjusted and improved as the mechanisms involved in the relation between changes in dormancy intensity and sensitivity to terminator factors are not yet fully understood (Benech-Arnold et al. 2000). The understanding of provenance-related differences in seed dormancy can be hampered by the genetics role in mediating environmental responses (Fernández-Pascual et al. 2013). Even so, in order to include dormancy intensity in germination prediction models, the first approach using stratification or after-ripe dry/heat period time as dormancy intensity dependent steps could increase model reliability (Batlla and Benech-Arnold 2010). An estimation of the initial dormancy intensity state could, as aforementioned, assume that dormancy would be weaker for warmer provenances keeping in mind that these assumptions should be duly supported by species-specific literature and experimental data (Guterman 2000).

2.6. Implications for practice

2.6.1. Seed harvesting protocols

As mentioned before, seed provenance, through genetic, epigenetic or environmental influences, plays an important role in germination response, namely by influencing dormancy intensity which will, in its turn lead to differences in germination time and percentages. As a consequence, germination predictions of a certain species can hardly be based on just one provenance. Consequently, the study of germination ecology considering regional seed zones for seed harvesting seems to be the best management option for ecological restoration.

In the future, the causes and effects of provenances differences should be further researched, in order to enhance germination predictions following ecological restoration and also to obtain more reliable predictions of the speed and ability of species adaptation to climate changes.

The way forward for the inclusion of these findings in ecological restoration actions should consider the following steps: i) identification of potential seed sources at the

regional level; ii) literature review on seed dispersal timing iii) identify the best time for harvest by predicting the effects of the inter-annual environmental variations in seed dispersal phenology iv) when possible, use seed development conditions to estimate dormancy intensity at the time of harvesting.

2.6.2. Seeding-related decisions

The combination of knowledge on dormancy breaking mechanisms, species seasonality and dormancy intensity can give valuable insights for seed handling protocols. Special research attention should be given to the prediction of optimal germination temperature thresholds of a given species according to its seed dispersal seasonality and foreseen dormancy intensity.

The way forward in the adjustment of handling protocols, with the combined objective of increasing seeding success and giving valuable insight in germination prediction models, should include the following: i) literature review on species dispersal time and, according to that, most likely temperature thresholds and seed dormancy breaking mechanisms ii) model building using the information of temperature thresholds (and whenever possible water potential thresholds) obtained; iii) model building using dynamic steps for temperature threshold adjustment following changes in post-seeding environmental conditions (literature review and prediction of dormancy breaking mechanisms); iv) adjustment of the dormancy intensity initial conditions and inclusion of this variable in multiple steps with dynamic feedbacks; v) model validation using literature and, if possible experimental data; vi) identification of the most suitable time for seeding according to the adjustment of the time predicted for germination and the next favourable germination window.

Through a deeper knowledge of germination seasonality, further management actions, such as irrigation, can be taken, if that would represent a significant enhancement in germination success. In this context, irrigation practices can be advantageous in reducing competition pressures as they could favor the establishment of native species over invasive/exotic species (Wainwright et al. 2012). From a practical point of view, the species proportion in the seed mixtures should be carefully studied as the growth competition of commercially available generalist species can hamper the recruitment of more-local specific native-species (Oliveira et

al. 2013). Lastly, the inclusion of arbuscular mycorrhizal fungi could also be used to promote the establishment of late-successional species (Estaún et al. 2007).

Finally, germination in nature depends not only on temperature and rainfall but also on soil characteristics and other local influences. Consequently, the reliability of germination prediction models should be tested both under laboratory and field conditions, for different seed bed types, and a certain amount of viability loss should be considered both due to aging processes and predation (Allen & Meyer 1998).

2.7. Concluding remarks

Regarding the current drawbacks in the use of native species for ecological restoration, research steps must be taken in the direction of the development of comprehensive tools to turn the use of native species from regional sources more accessible and doable at the management level. In this context, the steps involved in model development of germination predictions should be based on an appropriate interpretation of germination seasonality based on the time of seed dispersion and seed dormancy type. Finally, the adjustment of the initial dormancy intensity based on seed development conditions and the inclusion of dormancy as a dynamic state should increase germination prediction reliability.

We consider that the development of reliable models for germination prediction in the Mediterranean can represent an important step to increase the confidence and structure the use of a broad set of native species in ecological restoration. Furthermore, the development of a comprehensive tool in this direction could increase the reliability of germination predictions under climate changes scenarios and therefore plan restoration actions according not only the current situation but also to possible future scenarios. Finally, this approach would contribute to improve the compliance of ecological restoration goals, towards a better ecosystem biodiversity conservation and resilience.

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Chapter 3

Germination in five shrub species of Maritime Pine understory – does seed provenance matter?

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Abstract

Context: Maritime Pine forests cover important mountain areas in Portugal and are known to be a particularly fire-prone forest type. Understory composition plays an important role in maintaining biodiversity and ecosystem services after recurrent wildfires.

Aims: This study aims to improve the knowledge on the germination ecology of understory species of Maritime Pine forests, focusing on the importance of seed provenance, including in relation to germination enhancement by heating.

Methods: The selected species were *Cistus ladanifer* L., *Erica australis* L., *Erica umbellata* L., *Pterospartum tridentatum* L. (Willk) and *Genista triacanthos* Brot. Seeds were collected from two or three distant populations. Besides a control treatment, two heating regimes were applied, i.e. 100 °C during 5 minutes and 80°C during 30 minutes.

Results: Heating treatments significantly enhanced germination in 4 out of the 5 species. Differences between provenances were most evident for *Cistus ladanifer* and *Erica australis*, especially following the heating treatments. Overall, the seeds from the southern provenances germinated better and, at the same time, were smaller.

Conclusion: The present results confirmed that seed provenance should not be ignored as a key-factor in germination ecology, so that further work is needed to untangle the roles of environmental and genetic factors in the observed differences between provenances.

Ana Rita Pessoa de Figueiredo Vasques

3.1. Introduction

Wildfires play an important role in the history of Mediterranean ecosystems and, as a consequence, Mediterranean plant communities have become fire resilient (Pausas and Vallejo 1999). The resilience of these communities is related to the ability of their principal species to resprout after fire and/or to attain elevated levels of recruitment by germination (Keeley 1986). Based on these traits, Mediterranean species can be divided into seeders and resprouters (Pausas 1999; Verdú 2000), presenting different levels of seedling density and survival following wildfires (Benwell 1998).

In Portugal, Maritime Pine (*Pinus pinaster* Ait.) plantations have been widely introduced and now are a predominant cover type of the northern and central mountain areas in particular (Godinho-Ferreira 2005; Nunes et al. 2005). However, they were strongly affected by wildfires in the past decades and, according to Silva et al. (2009), constitute the most fire-prone forest type in Portugal. Maritime Pine is an obligate seeder and, accordingly, has been found to germinate vigorously after fire, at least in León (Calvo et al. 2008) and Galicia (Vega et al. 2008). Post-fire seedling recruitment of Maritime Pine can be expected to vary amongst different regions/populations, due to differences in germination potential among provenances (Gil et al. 2009; Escudero et al. 2002).

Whilst Maritime Pine recruitment following wildfire has received little research attention in Portugal, post-fire regeneration of the understory species has hitherto been largely ignored (Maia et al. 2012). Knowledge of the germination ecology of understory species is nonetheless important, especially against the background of the likely climate changes and associated increase in fire hazard in Mediterranean regions (Pausas et al. 2008; Piñol et al. 1998). Namely, recurrent fires can markedly reduce the resilience of pine forests (Díaz-Delgado et al. 2002), so that the maintenance of the basic functions of the affected ecosystem may come to depend strongly on the understory species (Fisher et al. 2006; Srivastava and Vellend 2005). Furthermore, species with distinct regeneration traits increase the resilience of the community to subsequent fires (Keeley 1986; Pausas et al. 2004). Besides the importance of shrub species in maintaining biodiversity and ecosystem services (Hartley 2002), the selection of the understory species for introduction in fire-degraded communities is not being addressed by the present study because it would need to consider other aspects as well, in particular the species' expected

contribution to fuel load built-up and thus, future fire hazard as well as the foreseen management practices (Baeza et al. 2011; Fernandes et al. 2004; Vallejo et al. 2011).

The principal understory species of Maritime Pine plantations in Portugal are shrubs that pertain to the families of Ericaceae and Leguminosae and, to a lesser extent, Cistaceae (Godinho-Ferreira 2005). Five species were selected for the present study, i.e. two Ericaceae, two Leguminosae and one Cistaceae species. A key factor in selecting the two Ericaceae and the two Leguminosae species was the comparison of the above-mentioned, two main types of post-fire regeneration traits, i.e. of an obligate seeder or weak resprouter vs. a facultative resprouter or strong resprouter. In addition to the information underlying the classification of the five selected species in seeders and resprouters (Reyes et al. 2009, Paula and Pausas 2008) there is some evidence that provenance plays a role in the germination potential of one of the studied species, i.e. *Erica australis* (Cruz et al. 2003). This evidence, however, is limited to nearby populations in Spain. Such differences in germination potential between seed provenances can be related to seed morphological features, in particular seed size (González-Rabanal and Casal 1995; Moreira et al. 2010). Hanley et al. (2003) found smaller seeds to be more tolerant to high temperatures than larger seeds. Nonetheless, under field conditions the lesser tolerance of bigger seeds may be compensated by their capacity to emerge from greater depths (Bond et al. 1999).

The principal aim of this study is to get more insight in the germination of fire-adapted Mediterranean shrub species, in particular ones that are typical for the understory of Maritime Pine plantations in Portugal. For five selected species from three families, the study has as specific objectives: 1) to assess differences in germination between populations from 2 or 3 distant provenances within Portugal; 2) to determine if such differences vary with heating-based pre-treatments that can be expected to enhance the species' germination rates; 3) to evaluate if such differences are related to morphological seed characteristics, in particular seed size.

3.2. Material and Methods

3.2.1. Species and harvesting site selection

The five Mediterranean shrub species selected for this study were *Erica australis* L., *Erica umbellata* L., *Pterospartum tridentatum* L., *Genista triacanthos* Brott, and *Cistus ladanifer* L. Key selection criteria included wide and abundant occurrence in Portugal, elevated seed availability during the foreseen harvesting campaigns and, in the case of the Ericaceae and Leguminosae, contrasting post-fire regeneration traits (obligate seeders and weak resprouters: *E. umbellata* and *G. triacanthos*; facultative and strong resprouters: *E. australis* and *P. tridentatum*). Although perhaps less common in Maritime Pine plantation than the other four species, the obligate seeder *Cistus ladanifer* L. was selected for being particularly well-adapted to recruitment after fire (Delgado et al. 2008, 2001; Pérez-García 1997). The selected species present distinct types of dormancy. *Cistus ladanifer* has a hard seed coat that inhibits germination by impeding water entrance in the seed (Ferrandis et al. 1999) and this is also the principal cause of dormancy in the two Leguminosae species (Moreira et al. 2010). The two Ericaceae species, on the other hand, have a permeable seed coat but present physiological dormancy (Vera et al. 2010).

Selection of the harvesting sites was done in two phases. First, using available map information target areas were selected in mountainous areas that corresponded to the northern, central and southern ranges of the species' distribution areas in Portugal. These areas were then surveyed exhaustively, by car and foot, to locate a sizeable population for the different species, resulting in a total of seven harvesting sites (Fig.4). The species' northern provenances were sampled in Castro Laboreiro (41°55'N, 8°13'W: Ericaceae and Leguminosae) and Murça (41°25'N, 7°27'W: *Cistus ladanifer*), their central provenances in Castanheira de Pêra (40°03'N, 8°13'W: *Erica umbellata*, *Pterospartum tridentatum*), Góis (40°09'N, 8°02'W: *Cistus ladanifer*) and Alvares (40°04'N, 8°04'W: *Erica australis* and *Genista triacanthos*), and their southern provenances in Almodovar (37°45'N, 8°07'W: *Cistus ladanifer*) and Mu (37°37'N, 8°08'W: Ericaceae, Leguminosae). The harvesting sites of each species were thus at least 200 Km apart, minimizing the probability of gene flow occurrence and ensuring marked differences in climatic conditions (Slatkin 1987).

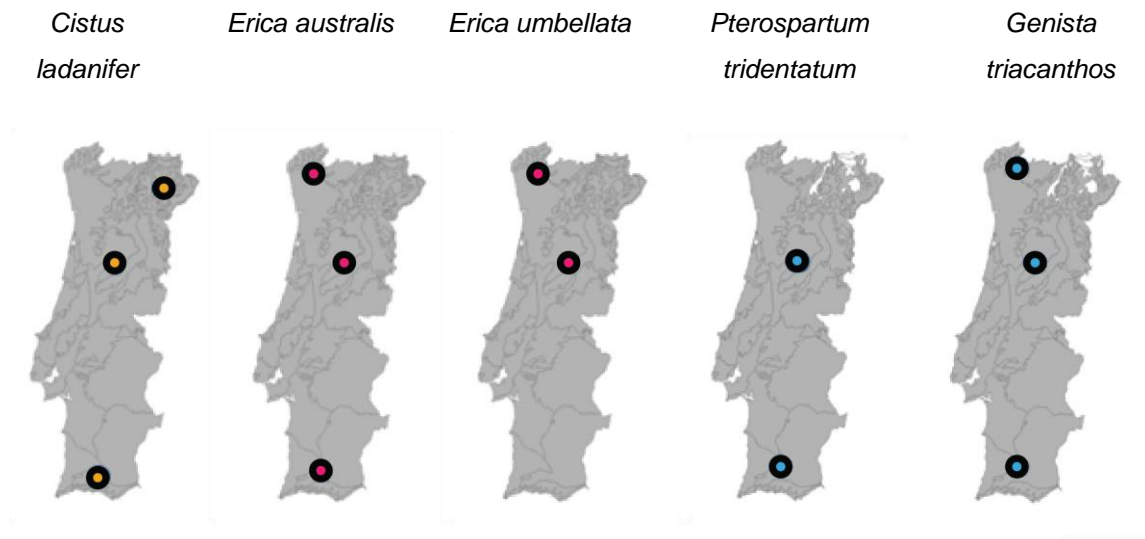


Figure 4 – Harvesting sites for the target shrub species of maritime pine understory according to the species distribution in mainland Portugal (species distribution in Fora Digital, available online at <http://jb.utad.pt/flora>).

3.2.2. Seed harvesting, handling and treatments

Seed harvesting was done in various field campaigns during June and July 2009. At each harvesting site, mature seeds were collected from 10 or, if not possible, at least 5 mother plants, which were selected randomly but avoiding those with clear signs of disease. In the laboratory, seeds were manually separated from fruits and other unwanted residues, using sieves, and carefully inspected for external signs of damage and immaturity. The selected seeds were then stored in paper bags under dark and low-moisture conditions for 4 to 6 months until the beginning of the experiments.

In three instances, the harvesting procedure ended up with insufficient mature seeds to warrant the inclusion of the respective provenance in this study. This was the case for the northern provenances of the two Leguminosae species and for the south provenance of *Erica umbellata*. The northern populations of the two Leguminosae species were comparatively small, on the one hand, and, on the other, many of the mother plants had already released the bulk of their seeds at the time of the harvesting campaigns. The fruits of the southern population of *E. umbellata* mainly comprised seeds with clear signs of immaturity.

Based on prior studies with the same species (Cruz et al. 2003; Delgado et al. 2001, 2008; González-Rabanal and Casal 1995; Pérez-García 1997; Rivas et al. 2006; Valbuena and Vera 2002; Vera et al. 2010), two heating treatments with different temperatures as well as different exposure times were defined that could be expected to enhance the germination of all five selected species. The same two heating treatments were applied to all species to facilitate comparisons between species, especially those of the same family. The treatments comprised heating at a comparatively high temperature for a short period (100°C for 5 minutes) and heating at a lower temperature for a longer period (80°C for 30 minutes). The treatments can be taken to represent a medium- and high-intensity fire, respectively (Reyes and Casal 2008). Heating was done in a standard laboratory oven. Both the heated and untreated seeds were then photographed (using a random selection of ten seeds per mother plant) and soaked during 24h in distilled water.

For each experiment, 15 seeds of each of the 5 to 10 individual mother plants were distributed in equal numbers over 5 petri dishes with agar-agar (10g/L). The Petri dishes were placed randomly in a germination chamber at 24 ± 2 °C with a 16 h day and 8 h night light period. Germination was recorded every week for at least 2 months until no more germination occurred for 2 subsequent weeks.

3.2.3. Data analysis

Germination response was characterized by germination percentage and time needed to achieve the 90% of the total germination (T90). In the case of the absence of germination, the maximum duration of the experiment was considered as the T90. Germination response as well as seed size were analyzed first by means of 2-way Analysis of Variance (ANOVA), using provenances and heating treatments as factors. When the assumptions of normality and/or homogeneity of variance underlying ANOVA were not met the role of provenance and of treatment were analysed separately. This was done using 1-way ANOVA or, for the species with only two provenances, the Student's t-test, if needed following transformation of the original data to $\ln(x+1)$ or square root (x). If the underlying assumptions could not be met, the tests' non-parametric equivalents - i.e. the Kruskal-Wallis test (KW) and Mann-Whitney U-test (MWU) - were performed instead. Post-hoc pair-wise comparisons between provenances and treatments were carried out using the Tukey or Dunn's

Test for ANOVA and KW, respectively. All statistical analyses were performed using Statistica, version 9 for Windows (StatSoft Inc.).

3.3. Results

3.3.1. Germination

3.3.1.1. Cistaceae: *Cistus ladanifer*

Cistus ladanifer achieved a very high maximum germination ratio of almost 100 % and the bulk of it occurred in a period as short as 2 weeks (Fig.5). This highest germination ratio was found for the southern population following heating at 80 °C during 30 minutes. This treatment was also the most effective in breaking seed dormancy in the other two populations.

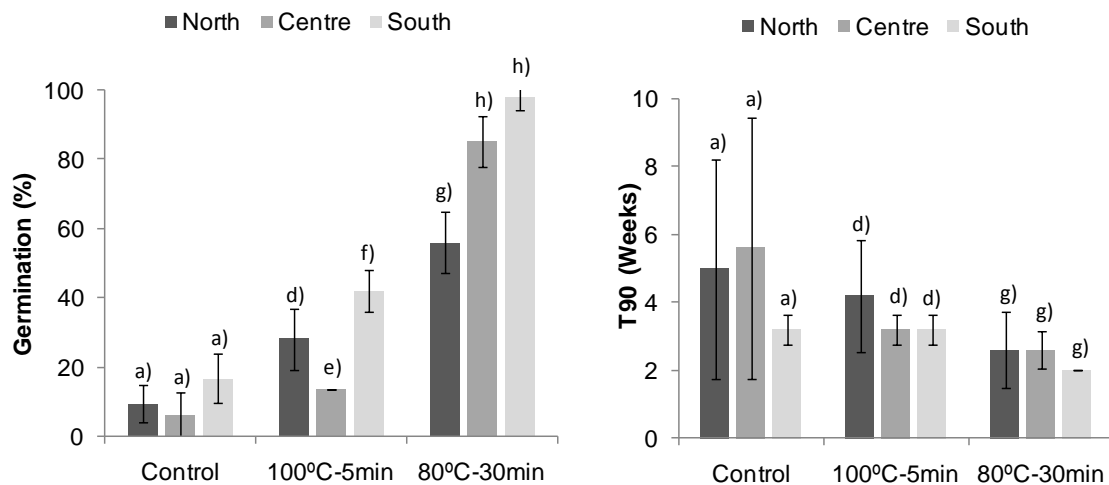


Figure 5 – Germination percentage (left) and time-to-germination of 90 % of the final germination (T90; right) for *Cistus ladanifer* (average and standard deviation) for all provenances and treatments (number of mother plants: north = centre = south = 10). Different letters indicate significant differences between provenances within treatments, at $\alpha = 0.05$, using the Tukey Test.

The germination ratios revealed a significant interaction between provenance and heating treatment (2-w ANOVA: $p = <0,001$), so that the role of provenance depended on the treatment. In the case of control experiments no significant differences existed between the three provenances, whereas the opposite was true for the 100°C-5min treatment (Tukey: p 's = $<0,001$). The 80°C-30min treatment produced intermediary results, with significantly higher germination ratios for the central and southern provenances than for the northern provenance (Tukey: p 's = $<0,001$). The 80°C-30min treatment was more effective in enhancing germination than the 100°C-5min treatment, producing significant effects on all three provenances (Tukey: p 's = $<0,001$) as opposed to on the north and south provenances only (p 's = $<0,001-0,70$).

The time needed for 90 % of the final germination ratio to occur (T90) did not vary significantly between the three provenances within the treatments (p 's = 0,27-0,42). The treatments, on the other hand, presented significant differences in the time needed for germination T90 (KW: $p = <0,01$). The post-hoc comparisons, however, only identified significant differences for the south provenance, with the 80°C-30min treatment significantly decreasing the T90 compared to the control and 100°C-5min treatment (Tukey: p 's = $<0,05$).

3.3.1.2. Ericaceae: *Erica australis* and *Erica umbellata*

Erica australis reached a markedly lower maximum germination ratio (45 %) than *Cistus ladanifer* and also germinated somewhat slower, with a T90 of at least 4 weeks (Fig.6). A noticeable contrast in final germination ratios existed between the southern and the other two provenances, at least following heating.

The role of provenance in germination ratios was tested for the individual heat treatments separately, since the assumptions underlying 2-way ANOVA were not met. This role was significant for the two heating treatments (ANOVA of $\ln(x+1)$ transformed data: 100°C-5min, $p <0,001$; KW: 80°C-30min: $p = 0,001$). Both treatments produced significantly more germination of the southern than the other two provenances (Dunn's and Tukey: p 's $<0,05$). By contrast, the control experiments revealed an ambivalent role of provenance, since the overall test result was significant (KW: $p = <0,01$) but all pair-wise comparisons were non-significant (Dunn's: $p = >0,05$).

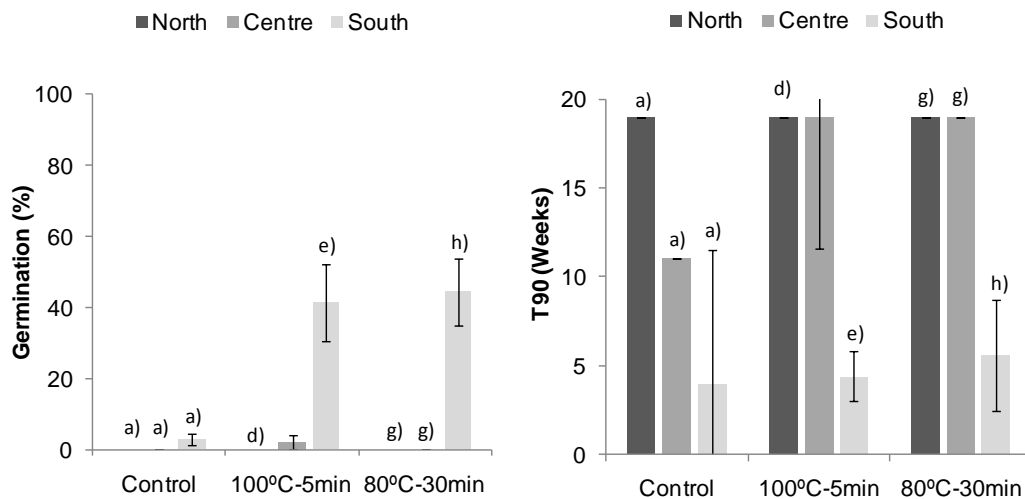


Figure 6 – Germination percentage (left) and time-to-germination of 90% of the final germination (T90; right) for *Erica australis* (average and standard deviation) for all provenances and treatments (number of mother plants: north = 10; centre = 9; south = 10). Different letters indicate significant differences between provenances within treatments, at $\alpha = 0.05$, according to the Dunn's test.

The T90 were also found to differ significantly amongst the three provenances but, again, just for the heating treatments (100°C-5min: ANOVA of $\ln(x+1)$ transformed data: 100°C, $p < 0.001$; 80°C-30min: KW, $p = 0.001$). In both instances, these significant differences reflected significantly lower T90's for the south than the other two provenances.

Erica umbellata consistently revealed very limited germination, not exceeding 5% even after 18 weeks (Fig.7), but these figures did not differ widely from those of *E. australis* for the same, northern and central provenances. Neither provenance (t-test and MWU: p 's = 0.20-0.70) nor heating regime (ANOVA and KW: p 's = 0.34-0.37) had significant effects on the germination ratios. The differences in T90, while shown in Figure 7, were considered of little interest due to the low germination ratios.

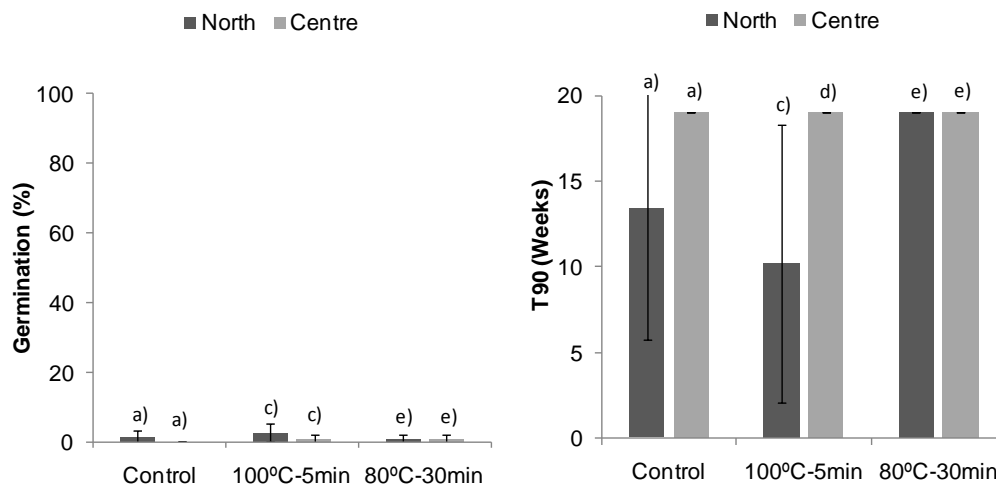


Figure 7 - Germination percentage (left) and time-to-germination of 90% of the final germination (T90; right) for *Erica umbellata* (average and standard deviation), for all provenances and treatments (number of mother plants: north = centre = 10). Different letters indicate significant differences between provenances within treatments, at $\alpha = 0.05$, according to the Mann-Whitney U-test and Student's - t test for germination percentage and T90, respectively.

3.3.1.3. Leguminosae: *Pterospartum tridentatum* and *Genista triacanthos*

The maximum germination ratio of *Pterospartum tridentatum* (80 %) was intermediate between that of *Cistus ladanifer* and that of *Erica australis* (Fig.8). Germination was comparatively slow, however, as T90 amounted to 13 weeks against 4 weeks or less in the cases of *C. ladanifer* and *E. australis*. Whilst the stimulating effect of heat exposure on germination was apparent, the role of provenance was not.

Like in the case of *Cistus ladanifer*, germination ratios revealed a significant interaction between provenance and treatment (ANOVA: $p = 0.04$). Accordingly, the differences between the two provenances were only significant following the 100°C-5min treatment (Tukey: $p = <0.01$ vs. 0.31-0.61). The two heating treatments both significantly enhanced germination ratios for the central as well as south provenance (Tukey: p 's = <0.001). However, differences between the two treatments were not significant for either of the two populations (Tukey: p 's = 0.41-0.60).

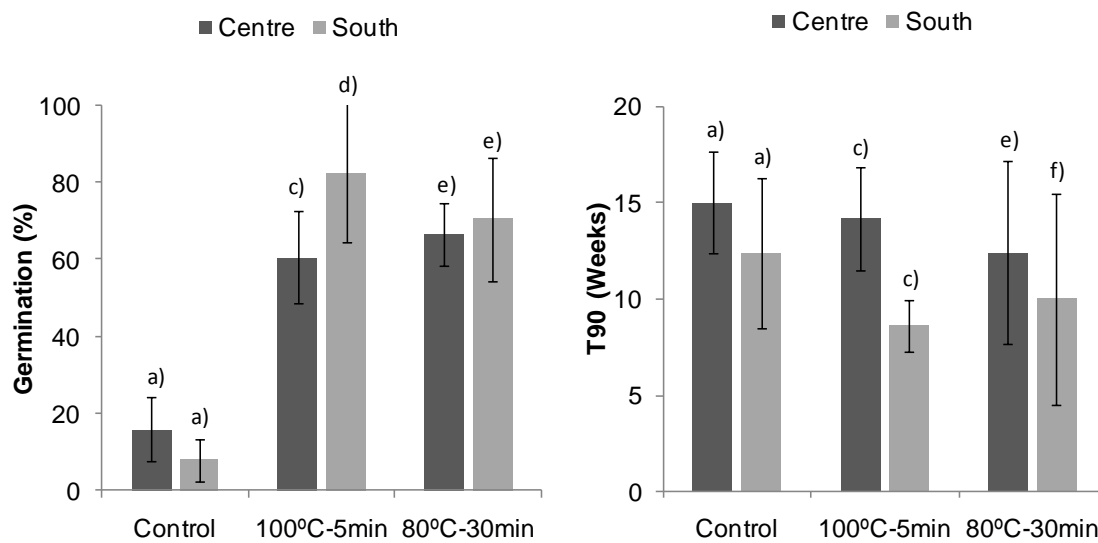


Figure 8 – Germination percentage (left) and time-to-germination of 90% of the final germination (T90; right) for *Pterospartum tridentatum* (average and standard deviation) for all provenances and treatments (number of mother plants: centre = 10; south = 5). Different letters indicate significant differences between provenances within treatments, at $\alpha = 0.05$, according to the Tukey test for germination percentage and according to Student's t-test for T90.

The T90 values were consistently smaller for the south than centre provenance. This differences were significant for the 80°C-30 min treatment (t-test: $p < 0.01$) but not for the control or 100°C-5 min treatment (t-test: p 's = 0.25-0.48). When analyzing the two provenances, treatments did not influence T90 in a significant manner (ANOVA: $p = 0.33$).

The results for *Genista triacanthos* coincided well with those of *Pterospartum tridentatum* in three aspects: (i) a similar maximum germination rate (80%), and for the same, 100°C-5min treatment; (ii) a apparent enhancement of germination by heating; (iii) a comparatively slow germination, with T90 frequently exceeding 10 weeks (Fig.9).

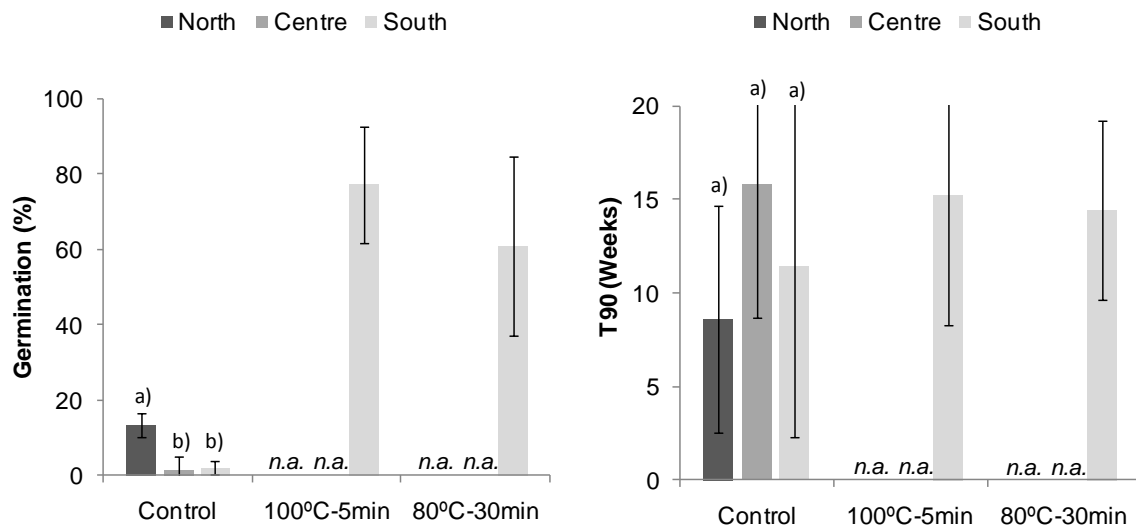


Figure 9 - Germination percentage (left) and time-to-germination of 90% of the final germination (T90; right) for *Genista triacanthos* (average and standard deviation) for all provenances and treatments (number of mother plants: control – north = 10; centre = 9; south = 10; 100°C – 5 min – south = 7; 80°C – 30 min – south = 7). Different letters indicate significant differences between provenances within control, at $\alpha = 0.05$, according to the Dunn's test for germination percentage and according to the KW test for T90 to compare provenances within the control treatment. "n.a." indicates that no data existed for the respective provenance.

Although this could only be assessed for the control experiments, germination ratios differed significantly amongst the three provenances (KW: $p < 0.01$). The northern population exhibited significantly more germination than either of the other two populations (Dunn's: p 's < 0.05). Likewise, germination ratios differed significantly amongst the three treatments, at least in the case (south) for which this could be evaluated (ANOVA: $p < 0.001$). The two heating treatments both resulted in a significant enhancement of germination compared to the control experiments (Tukey: p 's < 0.05) but the enhancements themselves were not significantly different (Tukey: $p = 0.30$).

Unlike germination ratios, T90 did not vary significantly with either provenance (KW: control experiments, $p = 0.17$) or treatment (KW: south provenance, $p = 0.52$).

3.3.2. Seed size

For the four species that revealed a significant role of provenance in germination ratios – i.e. *Cistus ladanifer*, *Erica australis*, *Pterospartum tridentatum* and *Genista triacanthos* - seed size was also determined (Fig.10). Without exception, the average seed size of the southern population was smaller than those of the northern and central populations. Statistically significant differences, however, were limited to three of the species and, for each of these species, restricted to the southern population and only one of the other two populations (ANOVA and t-test: p 's = <0,001-0,03). In the case of *Cistus ladanifer*, the differences in seed size between provenances were not significant (ANOVA: $p = 0,36$).

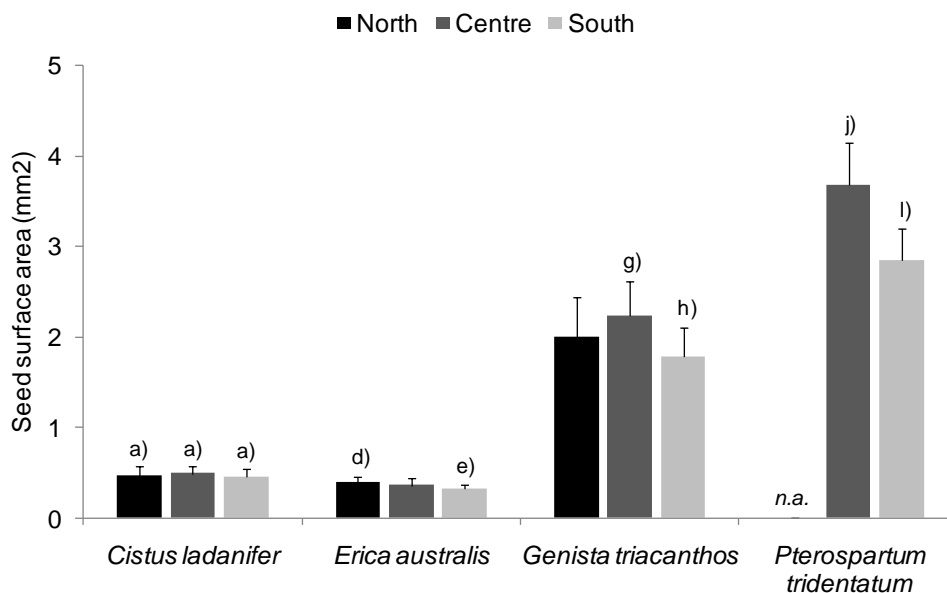


Figure 10 – Seed surface area (average and standard deviation; mm²) for the four species revealing significant differences in germination ratios between the provenances (number of mother plants: *Cistus ladanifer*, north = 10, centre = 10, south = 10; *Erica australis* and *Genista triacanthos*, north = 10, centre = 9, south = 10; *Pterospartum tridentatum*, north = 5, south = 10). Different letters indicate significant differences between provenances, at $\alpha = 0.05$, using the Student's t-test for *Pterospartum tridentatum* and the Tukey test for *Erica australis* and *Genista triacanthos* (after $\ln(x+1)$ transformation); "n.a." indicates that no data existed for the respective provenance.

3.4. Discussion

Four of the five shrub species studied here revealed that heating can enhance their germination in a significant manner. For all four species, this evidence for heating-enhanced germination agreed well with the findings of prior studies (Cruz et al. 2003; Delgado et al. 2008; Pérez-García 1997; Rivas et al. 2006; Valbuena and Vera 2002; Vera et al. 2010). A more quantitative comparison of this stimulus, however, was seriously hampered by differences in heating regimes and in particular their temperatures, including amongst the various prior studies as well illustrated for *Cistus ladanifer* by Pérez-García (1997) and Delgado et al. (2008). Such methodological differences could contribute to the discrepancy between the present results for *Erica umbellata* - no apparent response to heating - and those by Gonzalez-Rabanal and Casal (1995) – a positive effect. This is rather unlikely, though, since Gonzalez-Rabanal and Casal (1995), besides the positive effect of heating, found similarly low germination ratios following 5 - minutes heating at 80 °C and 110 °C. A strong heating response was certainly not to be expected for *E. umbellata*. Reyes and Casal (2008) characterized *E. umbellata*'s response to a fire stimulus as weak, notwithstanding its plant functional regenerative type (PTRF) of germination dependence. Reyes et al. (2009) even suggested a negative heating effect for *E. umbellata*, presenting a higher germination percentage without treatment (17%) than after fire (thermic shock or smoke: 6 %). Moreira et al. (2010) found that smoke rather than heat stimulated the germination of *E. umbellata*, producing a germination ratio of 57 % as opposed to 0 - 2 % without treatment as well as for a wide array of heating treatments. Thus, the germination response of *E. umbellata* to heating could vary from weakly positive in the NW region of the Iberian Peninsula (Gonzalez-Rabanal and Casal 1995; this study) to absent in the southern and eastern regions (Moreira et al. 2010). This would imply that the failure to include *E. umbellata* seeds from the south provenance in this study had no notable implications.

Unlike the two Ericaceae species, the two Leguminosae species revealed marked similarities in their germination response, in terms of their - high - maximum germination ratios (80 %) and the significant role therein of heating. Rivas et al. (2006) also reported a significant increase following heating for both species, i.e. for their heating treatment that was most similar to that applied here (110 °C for 5 min). The corresponding germination increase by heating, however, were considerably lower than in this study, especially for *Genista triacanthos* (21 %) but also for

Pterospartum tridentatum (50 %). Comparison of the present results and those of Rivas et al. (2006) suggested that the exposure time was a key factor in the effectiveness of heating at 80 °C. Whilst the two heating treatments of this study produced similar results, the 80°C-5min and 80°C-10 min treatments of Rivas et al. (2006) lacked a significant effect in *G. triacanthos* and resulted in roughly twice as little increase in germination for *P. tridentatum* than the 110°C-5min treatment. A marked stimulation of germination by fire/heating was equally found by Reyes et al. (2009) for both Leguminosae species and by Moreira et al. (2010) for *G. triacanthos*. Also the fire/heating-enhanced germination ratios of these two studies (*P. tridentatum*: 65 %; *G. triacanthos*: 80-90 %) did not differ widely from those reported here. The same applied *mutatis mutandis* to the germination of the untreated seeds (<20 %), with the exception of the figure for *G. triacanthos* in Reyes et al. (2009: 65 %). This exceptionally high value could involve differences in provenance, being in line with the significantly higher germination ratio of untreated *G. triacanthos* seeds from the northern than the other two populations studied here. It could further imply that the fire dependence of *G. triacanthos*, as defined by Reyes and Casal (2008) would vary markedly from NW Spain to SE Spain and Portugal.

Ample evidence was provided here that seed provenance should be an important consideration when comparing germination response studies. Four of the five species and all three families studied revealed significant differences between provenances. This proof-of-principle was surprisingly robust against “missing provenances” in the case of two of the three species (*Pterospartum tridentatum* and *Genista triacanthos*). In the case of *Erica umbellata*, on the other hand and as argued above, there was reason to believe that the south provenance would not have revealed a significantly different response from the other two provenances. Further work to verify this hypothesis would seem of interest especially for the implied contrast in germination response with *Erica australis*.

The heating treatments proved an essential element in this study, plainly justifying the additional efforts. Whilst the control experiments revealed a significant role of provenance for one species (*Genista triacanthos*), the heating experiments did for three species (*Cistus ladanifer*, *Erica australis*, *Pterospartum tridentatum*). This heating-dependent role of provenance could be due to the low germination rates in the control experiments, indicating the need for larger sample sizes. Even so, the

germination ratios in the control experiments of *Genista triacanthos* were also low but, at the same time, differed significantly.

The current findings sustaining the importance of provenance would seem to fit in well with species' adaptation to a regime of recurrent fires (Paula and Pausas 2008). Namely, the southern populations of three of the species germinated significantly better following heating than the other population(s), on the one hand, and, on the other, fire hazard can be expected to increase along the strong climate gradient from north to south Portugal. The divergent results obtained here for *Genista triacanthos* (more germination for the northern than other two populations) would plainly justify a follow-up study that tested the heating response of the northern and central populations as well. Such a study would face important logistic challenges. Repeated field campaigns would be needed to harvest sufficient mature seeds, for three reasons in particular: (i) the species' short harvesting period, due to the seed dispersion mechanism of "explosive" pods (Lopez et al. 2000); (ii) the limited size of its northern populations in particular; (iii) the lack of knowledge on the timing of its seed ripening process, including with respect to the geographical and inter-annual variations therein. Similar considerations would apply in the case of *Pterospartum tridentatum* and, except for the first constraint, *Erica umbellata*.

The tendency for seed size to be smallest in the southern populations of *Cistus ladanifer*, *Erica australis* and *Pterospartum tridentatum*, even if not always statistically significant, could suggest a genetic basis for the role of provenance in the species' response to heating. This would need further research, however, since such tendencies could also be the result of environmentally-determined phenotypic plasticity, especially because of the large distances between the sampled populations and the associated, marked differences in climatic and meteorological conditions. According to Silvertown (1989) and, more recently, Galloway et al. (2009), differences in seed size between populations are a non-heritable trait. Further work should definitely address the genetic/phenotypic basis for inter-population differences in germination behaviour and seed characteristics as observed here, especially to sustain forest management for future climate change scenarios (Chambel et al. 2005).

Independent of its genetic or phenotypic origin, seed size could play a role in germination through its relation with seed dormancy. Silvertown (1984) found that smaller seeds often presented stronger dormancy than larger seeds. According to

Delgado et al. (2001) and González-Rabanal and Casal (1995), on the other hand, seed size affected the germination response to heating by being related to the resistance of seeds to heating damage. A relation between seed size and resistance to heating damage was also found by Hanley et al. (2003) but in the opposite sense of smaller seeds germinating better than larger seeds at higher temperatures. The latter finding agreed well with the present results, with the southern provenances having smaller seeds as well as higher germination ratios following heating.

3.5. Conclusions

The principal conclusions of this study into differences in germination response between two or three distant populations of five widespread shrub species in Portugal from three different families (*Cistus ladanifer*; *Erica australis* and *E. umbellata*; *Genista triacanthos* and *Pterospartum tridentatum*) were the following:

- 1) germination ratios of untreated seeds were typically less than 20 % for all five species but were markedly enhanced by heating in the case of four of the five species, typically exceeding 50 %;
- 2) these same four species also revealed a significant effect of provenance on germination ratios and in three instances this role of provenance would not have been detected without the heating treatments;
- 3) all three families provided pointers towards a possible link between provenance - heating-enhanced germination and seed size but this could equally well involve genetic differences as phenotypic plasticity;
- 4) notwithstanding the gaps in the present data set due to missing provenances, the obtained results made a clear case for the need to contemplate seed provenance in comparing as well as predicting germination response.

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Chapter 4

The role of cold storage and provenance in the germination of three autumn-winter dispersed Mediterranean shrub species with contrasting dormancy types

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Abstract

Context: The increase of fire frequency in the Mediterranean is a major threat to plant regeneration and biodiversity conservation, and thus the artificial seeding of native species may be considered to restore degraded lands.

Aims: This study aimed at testing the effects of cold storage at high and low moisture in the germination of different provenances of three autumn-dispersed Mediterranean species with contrasting dormancy types.

Methods: Ripped fruits from three autumn-winter dispersed species were harvested in two or three different provenances from Portugal. After cleaning and drying, seeds were subjected to three differential types of storage: cold at low moisture or high moisture and a control. Five petri-dishes per provenance and treatment were placed under constant temperature (20°C) and germination was recorded weekly until no more germination occurred.

Results: The effect of cold storage at high moisture varied according to the species studied. Germination was negatively affected by this treatment in non-dormant species (*Pistacia lentiscus*), its speed was enhanced in seeds with physiological dormancy (*Arbutus unedo*) and there was no effect in the germination of seeds with physical dormancy (*Phillyrea angustifolia*). Significant differences between provenances were found in all the three species studied here. In *A. unedo* and *P. angustifolia* those differences could be caused by differences in dormancy intensity.

However, a possible adaptation to high moisture conditions was also visible in the northern seeds of the non-dormant species (*P. lentiscus*).

Conclusion: The germination response to storage mimicking field post-harvesting conditions was highly dependent on the species and dormancy type, varying also among different seed provenances. The low germination rates attained in *A. unedo* and *P. angustifolia* seem to be related with the existence of a Mediterranean syndrome in germination, so that the maximum temperature threshold should be further adjusted for germination tests using these species.

4.1. Introduction

During the past few decades, the frequency of wildfires and the area affected by them has increased noticeably in Mediterranean Europe and, in particular, in the Iberian Peninsula (Pausas et al. 2008). This tendency, together with its possible future intensification under likely climate-change scenarios, has raised widespread concerns about the resilience of Mediterranean ecosystems to repeated high-intensity wildfires (Díaz-Delgado et al. 2002; Pausas et al. 2008).

Ideally, restoration actions should contemplate both short-term and long-term objectives, ranging from rapid provision of a protective soil cover to reduce soil erosion risk to the enhancement and conservation of biodiversity as well as of resilience to future disturbances (Vallejo et al. 2012). Post-fire emergency measures typically involve seeding with grass species for soil protection, whilst mid- to long-term measures generally involve the planting of shrub species to re-establish or reinforce biodiversity-related functions (Vallejo et al. 2005; 2012).

It is widely recognized that seeding of shrub species for ecological restoration purposes should be done with native species but, at the same time, that this often implies major challenges (Tischew et al. 2011; Oliveira et al. 2012). The difficulties in seeding native shrub species are largely related to the presence of dormancy in many species and also to the lack of knowledge on the appropriate dormancy breaking mechanisms (Oliveira et al. 2012).

Dormancy is defined as the inability to germinate under conditions that otherwise would favour germination (Baskin and Baskin 2004). It constitutes an adaptive trait that prevents germination, both before dispersal - the so-called primary dormancy -

and after dispersal during permanence in the soil seed bank – the so-called secondary dormancy (Murdoch and Ellis 2000). Secondary dormancy has been classified into five major groups, of which this study addresses the two main types, physiological and physical dormancy (Baskin and Baskin 2004; Taiz and Zeiger 2010). In the case of physiological dormancy, germination is impeded by a physiological block that requires specific external stimuli to be terminated while in the case of physical dormancy, germination is inhibited by the lack of water imposed by seed coat impermeability (Murdoch and Ellis 2000; Finch-Savage and Leubner-Metzger 2006).

Different dormancy types can occur within a certain family as well as within a certain genus, but the type of dormancy present does appear to be species-specific (Finch-Savage and Leubner-Metzger 2006). The three Mediterranean shrub species studied here were selected to represent the two above-mentioned dormancy types - physical (*Phillyrea angustifolia*, according to García-Fayos (2001)) and physiological (*Arbutus unedo*, according to Tilki (2004)), as well as the absence of dormancy (*Pistacia lentiscus* after removal of the fruit pulp, according to García-Fayos and Verdú (1998) and García-Fayos (2001)). All three species, however, disperse their seeds during the same time of the year – i.e. autumn-winter – so that they face similar environmental conditions after seed dispersal. During their permanence in the soil seed bank, the seeds of the three studied species will typically be exposed to cold and humid conditions, characteristic of the wet Mediterranean winters. Exposure of imbibed seeds to cold conditions has been found to induce the production of gibberellins that are known to promote germination in physiologically dormant seeds (Penfield et al. 2005). Therefore, we hypothesized that cold storage under high moisture conditions would: (i) enhance germination in *Arbutus unedo*; (ii) either increase germination or have no effect in *Phillyrea angustifolia* and (iii) either decrease germination in *Pistacia lentiscus* or else have no effect, depending on the seeds' susceptibility to lose viability under these storage conditions (Roberts 1973; Murdoch and Ellis 2000).

Germination behaviour not only depends on the type of dormancy but also on its intensity. This is due to the fact that dormancy is a dynamic state that can vary markedly in intensity due to differences in environmental conditions, both during seed development as well as following dispersal, during permanence in the soil seed bank (Finch-Savage and Leubner-Metzger 2006). Various studies have also shown differences in germination response between populations from different geographical

areas with distinct climate conditions (Bischoff et al. 2008; Mijnsbrugge et al. 2010; Vasques et al. 2012). At the same time, however, the effects of geographical variations remain hard to predict, including because species differ in their responses to similar developmental or post-dispersal conditions. Provenance-related differences in germination are also not always easily interpreted. For example, cold stratification has been found to both eliminate and enhance germination differences between seed provenances in different species (Milberg and Andersson 1998). Finally, the particular relevance of seed provenance for ecological restoration actions is perhaps worth stressing, as seeds from regional sources are highly recommended to avoid inbreeding or outbreeding depression (Hufford et al. 2012).

The overall aim of this study was to further the understanding of the germination behaviour of three Mediterranean shrub species with an elevated interest for ecological restoration of fire-prone ecosystems. The specific objectives were to: (i) assess if the species' germination response to cold storage - mimicking typical field conditions following seed dispersal - would fit in with their contrasting types of dormancy; (ii) determine if the species' germination response varied significantly between populations of distant provenances in Portugal with distinctive temperature and rainfall regimes; (iii) explore if these provenance-related differences in germination could be linked to differences in site-related environmental conditions during permanence in the soil seed bank in winter and, as such, could represent adaptations to regional conditions.

4.2. Material and Methods

4.2.1. Species and site selection

Three evergreen Mediterranean shrub species - *Pistacia lentiscus* (Anacardiaceae), *Arbutus unedo* (Ericaceae) and *Phillyrea angustifolia* (Oleaceae) – were selected to represent different types of dormancy (as detailed in the introduction) but comparable timings of seed dispersal (during autumn-winter). The three species were furthermore chosen for their elevated potential for ecological restoration in fire-prone ecosystems. First, they are mid- to late-successional species, whose spontaneous recruitment is typically compromised by recurrent and/or large wildfires (Mesléard and Lepart 1991). Second, they have a low to moderate flammability (Liodakis and Kakardakis 2008), so that they reduce the risk of future disturbances by recurrent fires. Third, they are

strong resprouters, so that their presence can increase ecosystem resilience to future disturbances (Vallejo et al. 2005; 2012). Finally, they have wide geographical distributions in Portugal (Table 1), so that they could be used for ecological restoration actions across most of the country.

The species' large distribution areas also allowed studying the role of provenance in germination behaviour by comparing three or, due to limited seed availability in the case of the north provenance of *Phillyrea angustifolia*, two populations separated by large distances of at least 200 km (Table 1). Due to Portugal's strong climate gradient from north to south (Peel et al. 2007) the selected populations involved clear differences in temperature and especially rainfall regimes. Mean annual rainfall was roughly 30 to 40 % lower at the southern than central study sites and about 45 to 65 % lower at the southern than northern sites (Table 1). The exact locations of the populations were selected by field surveying in previously-defined target areas for populations with sufficiently large numbers of plant specimen with ripe seeds.




4.2.2. Seed harvesting and handling

Seeds were harvested during the autumn of 2009 by randomly selecting, whenever possible, ten mother plants. This was done using the nearest neighbour method but excluding plant specimens located at less than 5 meters distance, showing clear signs of disease or lacking ripe fruits. As referred earlier, no suitable population of *Phillyrea angustifolia* for harvesting could be found in the target area in northern Portugal. Also the populations of *Phillyrea angustifolia* in central and southern Portugal comprised less than the targeted number of mother plants with ripe fruits (5 and 9, respectively). In the case of *Pistacia lentiscus*, it was not possible to avoid the harvesting of red fruits from various specimens, even though red fruits can be expected to contain higher percentages of empty seeds than black fruits (Verdú and García-Fayos 1998; 2000). To somehow compensate for this effect, the fruits were subjected to a floating test and, if floating, were excluded from further processing.

The selected fruits were handled for each mother plant separately. The first step involved using a blender to separate the seeds from the fruit pulp. The seeds were then washed under running water and set to dry over filter paper for 2 weeks (García-Fayos 2001). Afterwards the seeds were stored in hermetic glass bottles, using a layer of cotton and silica to guarantee low-moisture (6-8%) conditions. The bottles

were kept in the dark and at room temperature for 2-3 months until the start of the treatments. The experiments started with a careful visual inspection of all seeds, excluding those with apparent signs of damage.

Table 1 – Species distribution (Digital Flora Online), average annual temperature and total precipitation (IPAM, Meteorological data from 1981-2010) for the seed harvesting sites of *Pistacia lentiscus*, *Arbutus unedo* and *Phillyrea angustifolia* at the studied provenances.

| Species | Region | Site | Distribution in Portugal | Average annual temperature (°C) | Average total precipitation (mm) |
|-------------------------------|---------|--------|-------------------------------------------------------------------------------------|---------------------------------|----------------------------------|
| <i>Pistacia lentiscus</i> | Aveiro | north |  | 15.6 | 938 |
| | Setúbal | centre | | 16.6 | 735 |
| | Faro | south | | 17.9 | 509 |
| <i>Arbutus unedo</i> | Braga | north |  | 15.0 | 1449 |
| | Coimbra | centre | | 16.0 | 886 |
| | Faro | south | | 17.9 | 509 |
| <i>Phillyrea angustifolia</i> | Setúbal | centre |  | 16.6 | 735 |
| | Faro | south | | 17.9 | 509 |

4.2.3. Treatments and experimental setup

The germination potential of the three species was determined for one control treatment and two cold storage treatments to simulate conditions following seed dispersal in winter. The cold storage treatments consisted of storing the seeds in a cold chamber at 4-5 °C and in the dark for 10 weeks prior to the actual germination tests, either under low moisture conditions (LM) or under high moisture conditions (HM). The LM conditions involved maintaining the seeds in the hermetic glass bottles as explained in the previous section while the HM conditions involved adding vermiculite that was previously wetted till “field capacity” (free drainage) to the hermetic glass bottles and mixing it homogeneously with the seeds. The control treatment consisted of maintaining the seeds as they were kept before the cold storage treatments.

The experimental setup of the germination tests consisted of 5 replicates, each comprising a Petri dish in which up to 30 seeds were distributed in a regular pattern. Three seeds per mother plant were placed in each petri-dish. The Petri dishes were then placed randomly in a room with controlled temperature (20 °C) as well as photoperiod (16:8). During a period of four months germination was assessed at weekly intervals. Germination was considered to have occurred when radicle had emerged from the testa.

4.2.4. Data Analysis

Final germination percentages at the end of the 4-month period were first analysed by means of 2-Way ANOVA, using treatment and provenance as factors. In case these factors had a significant overall effect, the individual contrasts were tested using Tukey's HSD post-hoc test. In addition, the role of the treatments was assessed for each provenance individually, using 1-way ANOVA and Tukey's HSD post-hoc test. The assumptions underlying ANOVA were assessed by visually analysing the distribution of the residuals and by testing homoscedasticity by means of the Levene's test. To meet the latter assumption, the germination percentages of both *Pistacia lentiscus* and *Arbutus unedo* had to be transformed to $\ln(x+1)$ and \sqrt{x} , respectively. The germination percentages of *Phillyrea angustifolia*, however, could not be transformed successfully to pass the Levene's test. Therefore, the non-parametric Kruskal-Wallis test was applied to separately assess the overall

significance of treatment and provenance. The Kruskal-Wallis test was also employed to determine if treatment had a significant effect on the individual provenances, using the Dunn's test to evaluate the different contrasts. All statistical analyses were carried out using SPSS v.18.

4.3. Results

4.3.1. Overall effects of cold storage

Under control conditions, the three species differed clearly in the time required for germination (Fig. 11). *Pistacia lentiscus*, lacking dormancy, reached its maximum germination percentages after 2 to 3 weeks, while the other two species (with physical or physiological dormancy) took 12 to 14 weeks.

The three species revealed clear differences in their germination response to the three treatments. The cold high moisture treatment had a significant overall effect on the final germination percentages of *Pistacia lentiscus* but not on those of the two species with dormancy (Table 2). In the case of *Arbutus unedo*, the effect of treatment was not unequivocal due to a significant interaction with provenance (Fig. 11; Table 2). In the case of *Phillyrea angustifolia*, there was no significant overall treatment effect which could also be due to the very low germination percentages obtained in all tests. The possible interaction of treatment with provenance, however, could not be tested for *P. angustifolia*, as the homoscedasticity assumption underlying ANOVA could not be met.

The significant overall treatment effect on *Pistacia lentiscus* was due to a negative effect of cold storage under humid conditions. Tukey's post-hoc test results showed that the final germination percentages following cold storage under high moisture were significantly lower (7 %) than those under control conditions (13 %) as well as those following cold storage under low moisture conditions (18 %; Table 2).

Table 2 – Statistical test results for the germination percentages of *Pistacia lentiscus*, *Arbutus unedo* and *Phillyrea angustifolia* from two or three provenances in Portugal and for three storage treatments, i.e. control conditions (CT) and cold storage under low and high moisture conditions (LM and HM, respectively). Significant results at $\alpha = 0.05$ are presented in bold.

| <i>Pistacia lentiscus</i> | | | | | |
|-------------------------------|--------------------------------|--------------|-------------------------|-------------------------|------------------------|
| Treatment | 2 Way-ANOVA | | Tukey-Test | | |
| | F value | p value | CT vs. HM | CT vs. LM | HM vs. LM |
| | 11,26 | 0 | 0,02 | 0,19 | 0 |
| Provenance | 7,1 | 0,003 | centre vs. north | centre vs. south | north vs. south |
| Treat*Prov | 1,83 | 0,15 | 0,002 | 0,27 | 0,08 |
| | provenance-wise ANOVA | | Tukey-Test | | |
| | F value | p value | CT vs. HM | CT vs. LM | HM vs. LM |
| north | 1,76 | 0,214 | | | |
| centre | 4,59 | 0,033 | 0,107 | 0,786 | 0,03 |
| south | 5,64 | 0,019 | 0,282 | 0,227 | 0,015 |
| <i>Arbutus unedo</i> | | | | | |
| Treatment | 2 Way-ANOVA | | | | |
| | F value | p value | | | |
| | 6,13 | 0,01 | | | |
| Provenance | 81,79 | 0 | | | |
| Treat*Prov | 3,02 | 0,03 | | | |
| | provenance-wise -ANOVA | | Tukey-Test | | |
| | F value | p value | CT vs. HM | CT vs. LM | HM vs. LM |
| north | 3 | 0,09 | | | |
| centre | 6,28 | 0,01 | 0,01 | 0,19 | 0,26 |
| south | 0,75 | 0,49 | | | |
| <i>Phillyrea angustifolia</i> | | | | | |
| Treatment | Kruskal-Wallis | | | | |
| | QS | p | | | |
| | 4,49 | 0,106 | | | |
| Provenance | 8,64 | 0,003 | | | |
| | provenance-wise Kruskal-Wallis | | Dunn's test | | |
| | QS | p | CT vs. HM | CT vs LM | HM vs LM |
| centre | 0 | 1 | | | |
| south | 6,71 | 0,03 | 1 | 0,418 | 0,02 |

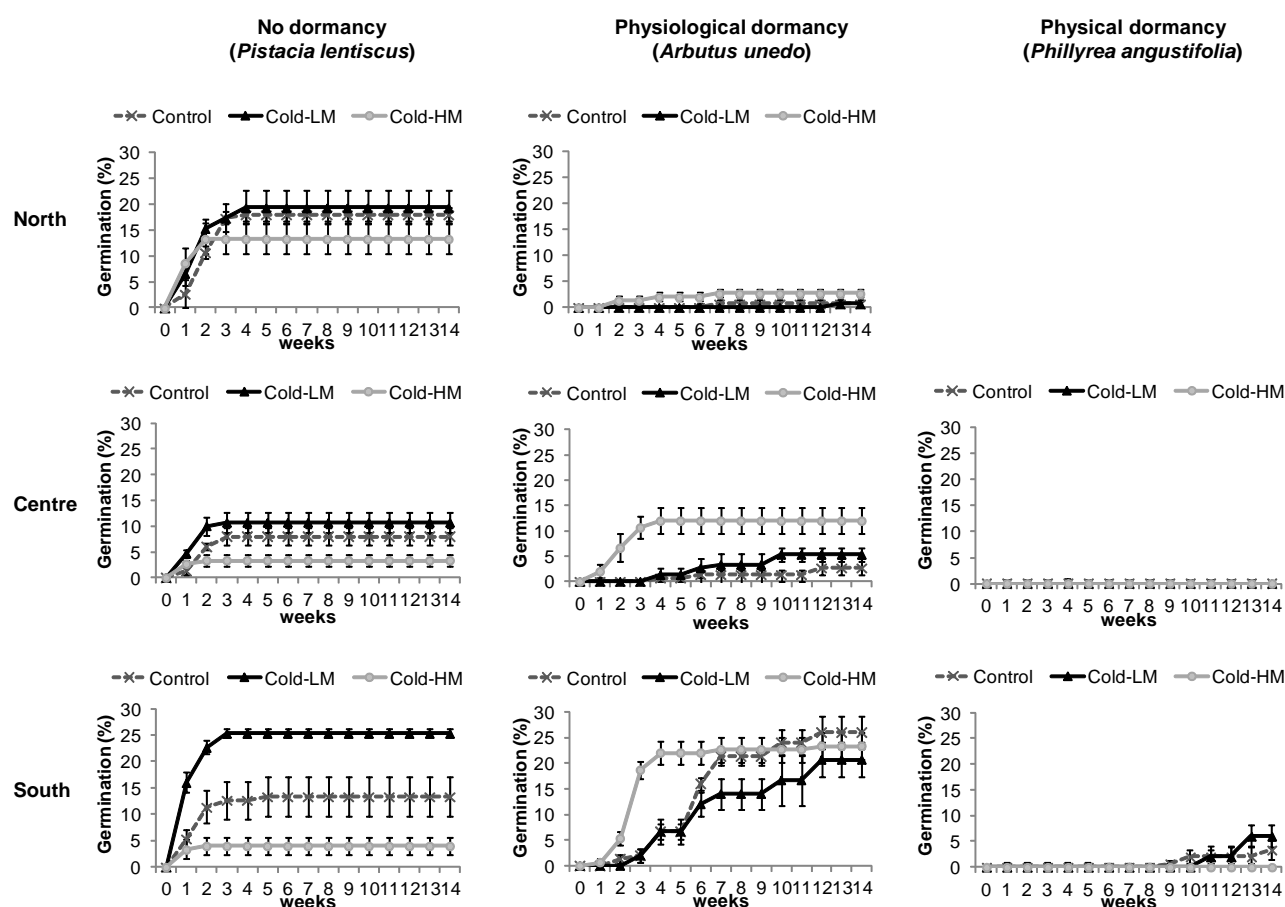


Figure 11 – Cumulative germination percentage (average and standard error) over time (weeks) for all the studied provenances of the three species representing three dormancy types (*Pistacia lentiscus*, *Arbutus unedo*, *Phillyrea angustifolia*) under control and cold storage at low (LM) and high moisture (HM).

4.3.2. Overall role of provenance

Provenance had a significant overall effect on the germination percentages of *Pistacia lentiscus* but not of *Arbutus unedo*, due to the above-mentioned significant interaction with treatment (Table 2). It also played a significant role in the case of *Phillyrea angustifolia*, albeit the possible interaction with treatment could not be tested, as also explained earlier.

Independently of the treatments, the germination percentages of the north provenance of *Pistacia lentiscus* were significantly higher than those of the centre provenance. They also tended to be higher than the germination percentages of the south provenance, as was suggested by the marginal p-value of 0.08 and as was

apparent from figure 11 for the HM treatment. *Phillyrea angustifolia* revealed significantly higher germination percentages for the south than centre provenance; however, even the southern germination percentages were low, remaining below 10 % in all individual germination tests.

4.3.3. Provenance-specific effects of cold storage

Not only *Pistacia lentiscus* but also the other two species showed or suggested different provenance-related responses to the treatments. In the case of *Pistacia lentiscus*, the final germination percentages of the three treatments differed in a consistent manner for all three provenances (cold-LM > control > cold-HM) but in an increasingly pronounced fashion along the north-south gradient (Fig. 11). Accordingly, significant post-hoc differences were inexistent in the case of the north provenance and were limited to one contrast in the case of both the centre and the south provenance. In both cases, germination percentages were significantly lower under high than low moisture conditions.

In the case of *Arbutus unedo*, a significant treatment effect was restricted to a single provenance (centre; Table 2). It corresponded to a positive impact of cold storage under high moisture conditions (12%) as opposed to under control conditions, with mean germination percentages amounting to 12 and 3 %, respectively. This positive effect of cold storage under high moisture conditions on *Arbutus unedo* was also suggested in various other instances, i.e.: (i) in the centre provenance when compared to cold storage under dry conditions; (ii) in the north provenance when compared to both other treatments and (iii) in the south provenance when compared to both other treatments but only during the initial germination response between weeks 2 and 6.

In the case of *Phillyrea angustifolia*, the south provenance revealed a significant role of treatment which corresponded to a negative impact of cold storage under high as opposed to under low moisture conditions. On the other hand, the centre provenance failed to produce any germination for all treatments (Table 2).

4.4. Discussion

Seed storage conditions were found to have distinct effects on three Mediterranean shrub species selected for representing contrasting types of dormancy, including the lack of dormancy. In general or for specific provenances, storage under cold conditions enhanced germination in *Arbutus unedo* (in terms of final percentages or rates), had no effect on germination in *Phillyrea angustifolia* but reduced germination in *Pistacia lentiscus*. These contrasting results fitted in well with the species' dormancy types, as also hypothesized in the introduction. A cold stimulus following imbibition could very well trigger the breaking of physiological dormancy in *Arbutus unedo* seeds, and, at the same time, be irrelevant to the breaking of physical dormancy in *Phillyrea angustifolia* seeds, while causing cold-induced damage to germination-ready seeds in *Pistacia lentiscus*. Also the germination results under control conditions could be explained well by dormancy presence/absence and type, with the seeds of *Pistacia lentiscus* germinating faster than those of the other two species and with the seeds of *Phillyrea angustifolia* germinating clearly less than those of the other two species.

In general, the observed germination percentages under control conditions agreed well with those reported by previous studies. In the case of *Arbutus unedo*, most studies found germination under standard conditions to vary between 0 and 5 % in (Smiris et al. 2006: 0 %; Demirsoy et al. 2010:1-3 %; Tilky 2004: 4 %; Ertekin and Kirdar 2010: 5 %); even so, Hammami et al. (2005) reported a considerably higher result (19 %). In the case of *P. lentiscus*, the present results were within the range of the values reported by Quaoud (2007: 10 %), on the one hand, and, on the other lower than those reported by Piotto and Di Noi (2001: 40-80 %). No prior germination studies seem to have been carried out with *Phillyrea angustifolia* but Takos and Efthimiou (2003) also found (near to) no germination for *Phillyrea latifolia* (physical dormancy according to Garcia-Fayos 2001). The authors carried out three additional treatments to get further insight in how to break physical dormancy of *Phillyrea angustifolia*. Mechanical scarification and application of a heat shock (120°C for 5 min) did not substantially enhance germination, as final germination percentages remained below 5 %. By contrast, mechanical scarification followed by germination under a 12h thermo-period (20:10 °C) resulted in germination percentages of 81 and 88 % for the seeds of the centre and south provenance, respectively. These latter

results clearly suggested the importance of the adequate selection of temperature in germination tests.

The positive effects of cold stratification under humid conditions on germination in *Arbutus unedo* observed in this study were in line with the findings of prior studies by Demirsoy et al. (2010), Ertekin and Kirdar (2010) and Tilky (2004). The present degree of germination enhancement was also of the same order of magnitude as that reported by Demirsoy et al. (2010: + 3-20 %) but it markedly less than that found by Ertekin and Kirdar (2010: 50 %) and especially Tilky (2004: 80 %). These discrepancies in germination enhancement could involve, among other factors, provenance-related differences in dormancy intensity.

Dormancy intensity can influence the temperature thresholds by narrowing the range of favourable temperatures for germination as dormancy intensity increases (Fenner and Thompson 2005). In the present case, the results of each of the three treatments pointed towards a decrease in dormancy intensity in *Arbutus unedo* along the geographical gradient from north to south Portugal, as suggested by an increase in final germination percentages. In turn, this geographical pattern in dormancy intensity could be explained as an adaptation to the prevalent climate conditions, allowing *Arbutus unedo* to germinate earlier in regions where temperatures are favourable earlier in the winter season. On the other hand, the low germination percentages observed in this and other studies, could be associated with the presence of a Mediterranean syndrome in this species, i.e. a relatively low maximum temperature threshold for germination progress (Fenner and Thompson 2005; Bertsouklis and Papafotiou 2013). This would agree well with the previously hypothesized decrease in dormancy intensity from north to south provenances, and could explain the results found for both *A. unedo* and *P. angustifolia*.

The negative effects of cold storage under high moisture conditions on germination in *Pistacia lentiscus* were contradictory to the marked enhancement by cold storage at high moisture found by Quaoud (2007: + 20 %). Likewise, Piotto (1995) found that cold storage at high moisture enhanced germination in *Pistacia lentiscus*, albeit just by speeding it up. A possible explanation would be that the duration of the cold treatment played a key role in its effect on the viability of *Pistacia lentiscus* seeds, as both Quaoud (2007) and Piotto (1995) involved much shorter periods than this study (21 and 30 vs. approx.. 70 days). Contrarily, García-Fayos and Verdú (1998) reported

poor persistence of *Pistacia lentiscus* seeds in the soil, suggesting an elevated susceptibility to viability loss during storage.

Finally, provenance could be an important source of variability in germination results among different studies. This was also clearly suggested by the present results, revealing a significant overall role of provenance as well as the lack of a significant treatment effect in one out of three provenances. The provenance-related germination differences observed here could well represent adaptive features. The lack of a negative effect of cold storage under humid conditions on the north provenance would agree well with the wetter climate conditions in north Portugal. Also the suggested positive effect of cold storage under dry conditions on the south provenance, in strong contrast with the negative effect of cold storage under wet conditions, would fit in with the drier climate conditions in south Portugal. These results agree well with previously mentioned population-related differences in storability in *Pistacia lentiscus* seeds (García-Fayos (2001)).

4.5. Conclusions

The main conclusions of the present study into the germination behaviour of three Mediterranean shrub species that have elevated potential for ecological restoration and disperse their seeds during the same time of the year (autumn-winter) but exhibit contrasting dormancy types were the following: (i) *Arbutus unedo*, *Phillyrea angustifolia* and *Pistacia lentiscus* differed markedly in their germination responses to cold storage treatments simulating conditions in the soil following dispersal; (ii) the distinct responses, especially in terms of germination percentages, agreed well with the presence/absence and type of dormancy in these species, with cold storage under humid conditions enhancing germination in *Arbutus unedo*, (physiological dormancy), reducing it in *Pistacia lentiscus* (no dormancy) and not affecting it in *Phillyrea angustifolia* (physical dormancy); (iii) provenance was a key factor in the germination response of all three species, either by having a significant overall effect or interacting with the effects of the treatments; (iv) provenance-related differences in germination behaviour could be tentatively explained as adaptive features, taking into account the dormancy types of the respective species. Further work is needed to disentangle the role of seed developmental conditions in dormancy intensity and to further refine germination test protocols for Mediterranean species, by selecting

proper dormancy breaking conditions and, especially, adequate optimal germination temperatures.

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Chapter 5

The role of osmotic stress in germination of *Pistacia lentiscus* seeds from different mother-plants and provenances

This chapter was submitted in:

Vasques, A.R., Pinto, G., Dias, C., Santos, C., Keizer, J.J. The role of osmotic stress in germination of *Pistacia lentiscus* seeds from different mother-plants and provenances. Plant Species Biology.

Abstract

The increase in land degradation and biodiversity loss in the Mediterranean has been subject of many scientific reports. In extreme cases of land degradation, ecological restoration actions can improve biodiversity and/or enhance resilience against major threats. An important constraint for seeding-based restoration actions in the Mediterranean is low water availability, both during germination and early establishment

This study aimed at improving the knowledge of the germination behavior of *Pistacia lentiscus* under low water content conditions. The specific objectives were to: i) evaluate the effect of osmotic stress on the germination of seeds from different provenances; ii) assess maternal-related associations of germination and seedling growth potential with seed or mother-plant morphological traits.

Osmotic-stress had a consistent negative effect on germination, resulting for both seed provenances, in a decrease in germination of approximately 65% under low water potential conditions (-2.13 MPa) and 100% under very low water potential conditions (-8.38 MPa). Another factor that significantly influenced germination was intra-population variability between mother-plant. Poor (<5%) to zero germination was observed in approximately 35% and 60% of the mother-plants of the north and south provenance, respectively.

The two provenances presented distinct degrees of association between germination and seed or mother plant morphological traits. Even so, seed density was a good overall indicator for seed germination, as plant height was for establishment potential. Although, a causal relation between these traits cannot be established based on the present results, the present study did highlight the importance of taking into consideration seed and maternal plant traits when harvesting seeds for ecological restoration purposes. Without clear criteria for mother-plant selection and given the poor germination observed for many mother-plants, seeds of *P. lentiscus* should be harvested from more mother-plants than advised in standard restoration protocols. Further research is needed to disentangle the origins of the observed variability in germination behavior of *P. lentiscus* and assess the implications for the recruitment in its natural populations.

5.1. Introduction

Wildfires have an important role in the natural regeneration of Mediterranean ecosystems. However, the increase in fire frequency and burnt area during the last decades have converted fire into one of the principal threats to forest ecosystems in the Iberian Peninsula (Pausas et al., 2008). Frequent fires tend to favour early stages of succession that, in turn, comprise highly flammable and, thus, fire-prone communities (Baeza et al., 2011). If the disruption of ecosystem resilience and biodiversity losses attain a critical level beyond recovery through natural regeneration, ecological restoration actions must be duly considered (Vallejo et al., 2012).

The practice of seeding actions is widely established to restore ecosystem functions and enhance soil cover after major disturbances, such as road constructions (Bochet et al., 2009). In the case of post-fire restoration, however, shrub species are typically (re-) introduced by planting. A key reason for planting instead of seeding is associated to the difficulties of predicting germination success under the harsh conditions that characterize disturbed sites (Pausas et al., 2004). These harsh conditions can be (partially) overcome by hydroseeding but it is a costly technique (Oliveira et al., 2012; Prats et al., 2013). More recently, the coating of seeds with surfactant has been shown to increase germination and seedling establishment under repellent soil conditions (Madsen et al., 2012), as it typical in recently burnt as well as long unburnt forests in the study region (e.g. Keizer et al., 2005a, 2005b, 2008).

Once dormancy is broken, seeds require optimal temperatures and moisture conditions to progress towards germination (Allen, 2003). This means that if a seed is under optimal temperature conditions, the rate of water uptake will determine the germination rate. Water uptake occurs in three different phases: imbibition, metabolic preparation for germination, and embryo and seedling growth. In the first phase, water uptake is rapid, slowing down in the second phase and again increasing during the third phase (Manz et al., 2005).

Dry seeds can exhibit extremely low water potentials of under -300 MPa at less than 10% R.H. (Bewley et al., 2013). Even that, because of these water potentials, seeds tend to absorb water from the external environment, many species fail to complete germination under relatively high water potentials, presenting a critical seed water potential for germination completion that rounds -1.5 MPa, depending amongst others on temperature and oil content (Bewley et al., 2013). It has been hypothesized that species from drier places have adaptations to avoid germination under low water potentials (Evans and Etherington 1990). In addition, differences in water stress tolerance have been observed for distinct varieties and even provenances (Tilki and Dirik, 2007).

Seed development can be influenced by local environmental conditions through the maternal plant. Those conditions are known to play an important role and can be determinant in germination rate (Donohue, 2009). In this context, seed size is described as being a highly plastic trait which is largely determined by the maternal-plant environment (Bischoff et al., 2006; Castro, 1999). Positive correlations between seed size and germination and/or seedling growth were previously reported at the mother-plant level (Castro, 1999). However, the mechanisms that underlie such variations are far from being entirely understood. This is especially true for natural populations and consequently for most of the target species used in ecological restoration. In this context, maternal effects on seed size may be a source of adaptation contributing to offspring fitness (Biere, 1991; Galloway, 2002; Galloway and Etterson, 2007).

Harvesting from approximately 50 maternal plants from nearby populations is advised by ecological restoration protocols regarding local gene pool conservation (Bischoff et al., 2008; Ensconet, 2009). The number of target mother-plants can therefore be properly questioned if most of the mother-plants fail to germinate.

The species studied here, known as mastic tree (*Pistacia lentiscus*) is a dioic evergreen shrub or small tree that is often dominant in Mediterranean sclerophyllous formations. It is considered a suitable target species for ecological restoration in fire-prone ecosystems, mainly due to its high sprouting ability and low flammability and it is also described as highly resistant to drought (Liodakis and Kakardakis, 2008; Fillela et al., 1998). Furthermore, this species has a high cultural value due to mastic ancient use in culinary, cosmetics and medicine.

The combination of these particular characteristics plenty justifies the selection of this species as a case study. In addition, the authors had previously addressed germination for seeds harvested at the same sites in another year, reporting differences in germination response of the provenances to cold stratification (Vasques et al., in prep). The observed differences could be originated in different moisture exchange balances in seeds from different provenances. In addition, and in spite of the high variability observed, the role of maternal-plant was not properly addressed in the previous study and was, therefore, considered in the present study.

This study aims at improving the knowledge on seedling emergence of *Pistacia lentiscus* by simulating drought conditions immediately after seeding. The specific objectives are: i) to evaluate the effect of osmotic stress in the germination of different seed provenances; ii) to investigate maternal-related associations between germination and seedling growth potential and seed or mother-plant morphological traits.

5.2. Material and Methods

5.2.1. Seed harvesting and mother-plant morphological traits

Two harvest sites were selected, according to a latitudinal gradient of *Pistacia lentiscus*' distribution in mainland Portugal, one in the north-western limit of the distribution (40°17' 8°90'), and the other in the south-western limit (37°28' 8°79'). Seeds were harvested from 30 mother-plants at each location, ensuring a minimum distance of 5 meters between plants and avoiding those with external signs of disease. At each mother-plant, approximately 300 fruits were harvested at all canopy aspects, aiming at harvesting the maximum number of black fruits available per

mother-plant. For each mother-plant, height and basal diameter were measured and the total number of stems was estimated.

5.2.2. Seed handling and morphology

Seeds were separated from fruit pulp with the help of a blender, at low power to avoid damage, and were then cleaned under running water with the help of a sieve. The floating fruits and/or seeds were excluded at this stage. Seeds were then air dried over various layers of filter paper. After two weeks, seeds were placed in hermetic glass bottles and covered with cotton and silica to ensure dry storage and viability maintenance (Vasques et al., in prep.). Seeds were stored at 4-5° C for 9 months, until the start of the experiment. After storage every seed was visually inspected for external signs of damage. From each mother-plant 140 seeds were selected randomly for the germination tests, 30 were selected for seed weight (3 replicates of 10 seeds) and other 5 seeds were selected randomly to measure maximum and minimum surface length and external seed thickness. Seed area was calculated through maximum and minimum seed length and volume comprised also seed thickness. Seed density was calculated through the formulae: seed density = seed weight/seed volume.

5.2.3. Germination and seedling growth

Before sowing, seeds were disinfected with 2% of bleach for 5 minutes and then soaked in distilled water for 24 hours. Thirty seeds from each mother-plant were spread in petri dishes with 4 layers of filter paper imbibed with water or with Polyethylene glycol 6000 solution, according to the treatment. The control consisted in two petri-dishes for each mother-plant (replicates) soaked with water. For the medium water stress treatment (MWS), PEG was prepared at 10% (corresponding to -2.13 MPa at 22°C; Michel and Kauffmann, 1975) and in the high water stress treatment (HWS) a concentration of ~30% of PEG was used (-8.38 MPa at 22°C). The aforementioned treatments aimed at testing a medium and a severe water stress respectively. Seeds were placed in a germination chamber at a constant temperature of approximately $22 \pm 2^{\circ}\text{C}$ and photoperiod 16:8. Germination monitoring (i.e. when radical emerged from the testa) was recorded every week until no germination occurred for 2 consecutive weeks.

Seedlings from the control treatment were transferred to petri-dishes with clean moistened filter paper and, after 3 weeks of sowing, the total length of the seedlings

that presented cotyledons were measured. Seedling growth was considered zero in mother-plants that didn't germinate and this measurement was called establishment potential.

5.2.4. Statistical analysis

The effect of the water content treatments (control and water stress) and provenance in germination response and seedling emergence was evaluated through 1-Way Nested ANOVA when the homocedascity assumption was accomplished. If, after standard transformations the assumptions were not meet, the non-parametric *Kruskal-Wallis* (KW) test was carried. When differences were significant and more than three groups were present, *Tukey* or *Games-Howell* (GH) post-hoc tests were carried for ANOVA and KW, respectively. The *Spearman* rank correlation coefficient was computed to test the association of the germination observed in both control replicates and MWS and also to evaluate possible correlations between germination or seedling growth and seed or maternal-plant morphological traits. The unit used in the correlation test was the average value for each mother-plant. All statistical analyzes were performed using SPSS v. 10.

5.3. Results

5.3.1. Germination and seedling growth

Germination started to occur after 7 days and presented a gradual increase, reaching the maximum germination 30 days after sowing (Fig. 12). Moderate water stress (-2.13 MPa) lead to a decrease in germination of approximately 63 and 67%, for the north and south provenances and high water stress (-8.38 MPa) affected germination in the maximum extend (100%). Hence, both osmotic-stress treatments affected germination in a significant manner in respect to the control and also differed between each other (KW: $p < 0.001$; GH: $p < 0.01$). On the other hand, provenance didn't affect germination significantly ($F = 1.15$; $p = 0.28$). Accordingly, the final average germination percentages attained in the control were poor for both north ($11.78 \pm 2.93\%$) and south ($8.83 \pm 2.37\%$) provenances.

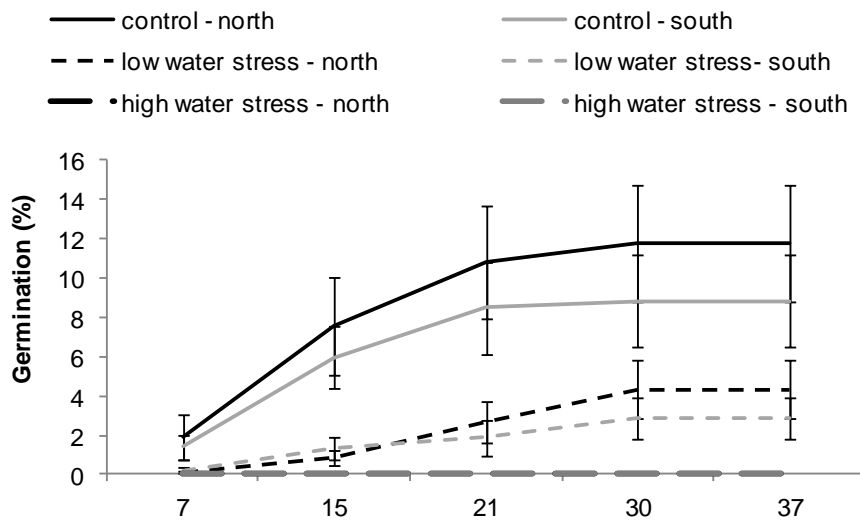


Figure 12 – Average and standard error of the cumulative germination over time for north and south provenances under control and water stress.

The germination observed for each mother-plant was correlated between replicates of the control ($\rho=0.77$; $p<0.01$) and also between moderate water stress and control ($\rho=0.48$; $p<0.01$). The role of mother-plant in the overall variability was traduced in large differences in the germination percentages attained both for the control (north: 0-78%; south: 0-52%) and water stress (north: 0-33%; south: 0-23%) treatment (Fig.13). Furthermore, poor to null germination ($<5\%$) was observed in approximately 35% and 60% of the mother-plants in the case of north and south provenance, respectively. These results justified the choice of the mother-plant as the unit in the subsequent tests for correlation between seed and maternal plant morphological traits.

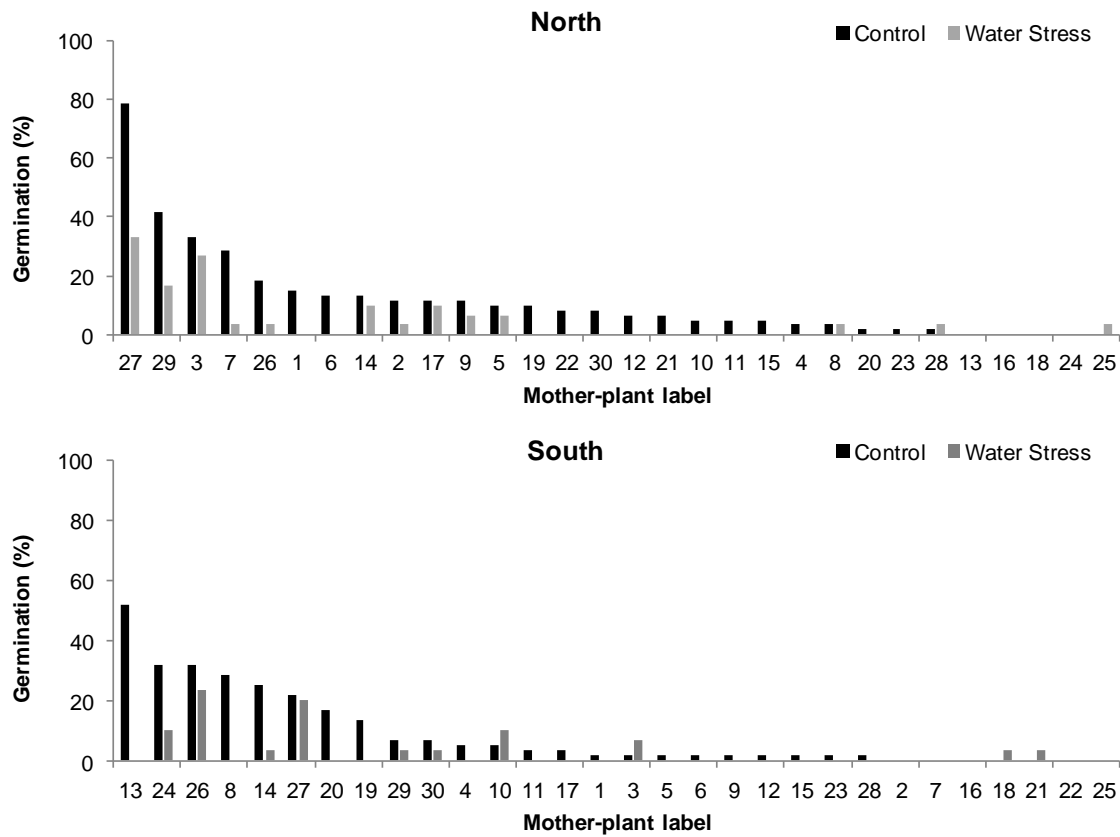


Figure 13 – Average of the final germination observed for the control treatment and total germination observed for the water stress treatment per mother-plant. Results are displayed for north and south provenances (up and down, respectively).

Three weeks after sowing the seedling growth potential (estimated as total length) ranged between 3 and 7.5 cm for both provenances (1 Way ANOVA: $F= 2.57$; $p=0.11$; Fig. 14).

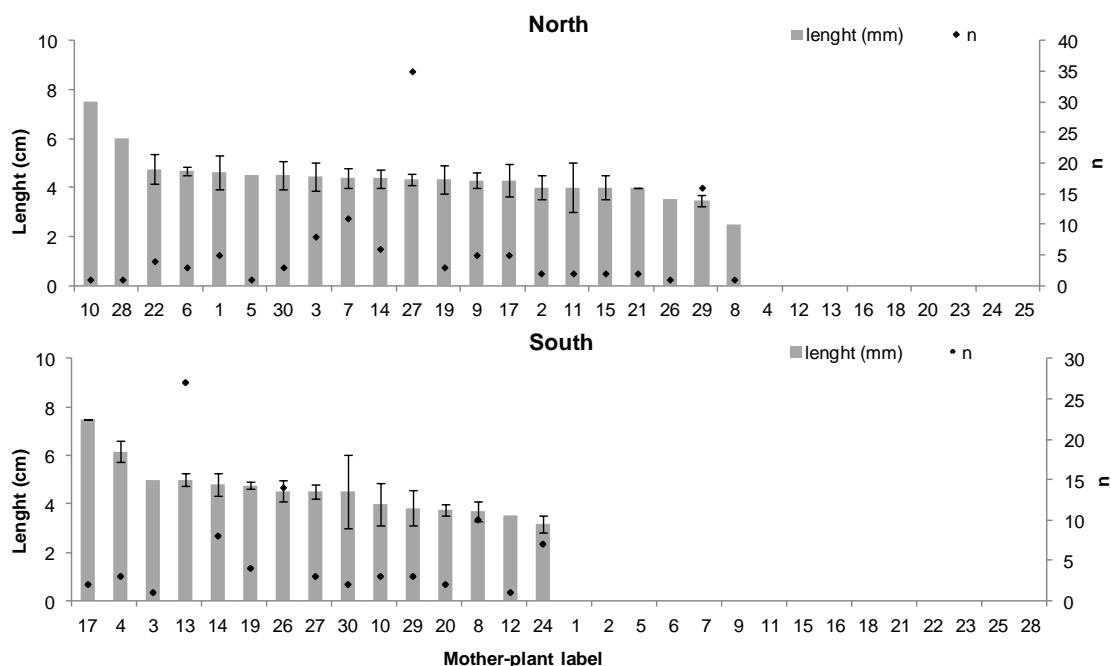


Figure 14 – Average, standard error and length of the seedlings with cotyledons 3 weeks after sowing under the control treatment. Results are displayed for all mother-plants and provenances.

5.3.2. Fruit colour and seed morphology

The percentage of black fruits was, on average, higher for the south (50%) than for the north provenance (35%), but this difference was not statistically significant ($F=2.91$; $p=0.09$; Fig.16).

Seeds from the south provenance tended to be bigger than those of the north provenance for all four seed traits measured (Fig.15). Differences were however only statistical significant for seed area ($F=10.07$; $p<0.01$), seed weight ($F=9.54$; $p<0.01$) and seed volume ($F=5.63$; $p<0.05$).

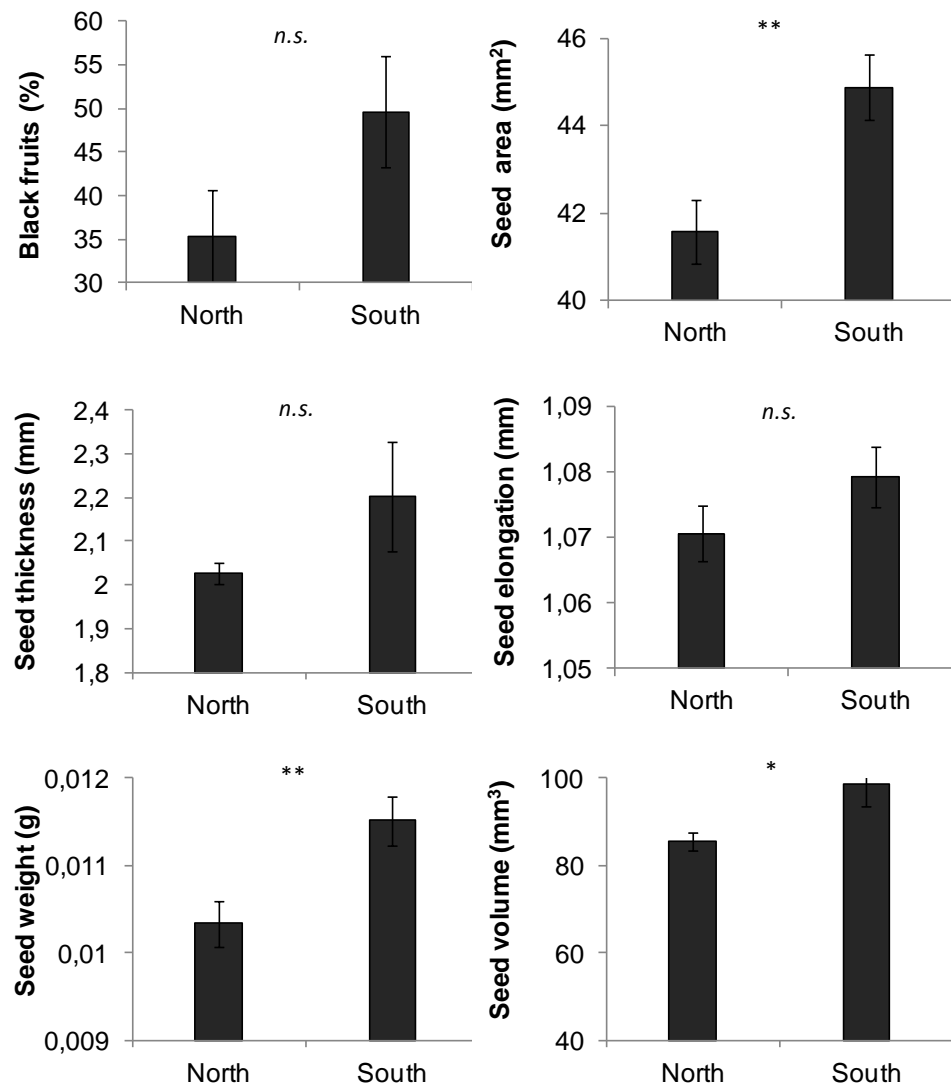


Figure 15 – Average and standard error for the fruit and seed characteristics: black fruits (percentage), seed area, seed thickness, seed elongation, seed weight and seed volume. Significant differences between provenances are indicated for $\alpha < 0.05$ by *, $\alpha < 0.01$ by ** and non significant differences are indicated by *n.s.*

Germination was positively correlated with seed density ($\rho = 0.41$; $p < 0.01$) but with no other seed trait (Table 2). The two provenances presented, however, distinct degrees of association between germination and seed morphological traits. Whether germination in the northern provenance was positively correlated with seed density ($\rho = 0.39$; $p < 0.05$) and significantly negatively correlated with seed thickness ($\rho = -0.44$; $p < 0.05$), no seed morphological trait was correlated with germination for the south provenance. The establishment potential was also correlated with seed density

(rho= 0.39; p<0.01) but yet again, this was only true for the north provenance (rho=0.39; p<0.05; Table 3).

Table 3– Spearman correlations between germination potential or seedling growth and seed or mother-plant morphological traits. Significant correlations ($\alpha \leq 0.05$) are presented in bold.

| | | Germination potential | | | | | | Establishment potential | | | | | |
|------------------|--------------------|-----------------------|-----------------|--------------|-----------------|--------------|-----------------|-------------------------|-----------------|--------------|-----------------|-------|------|
| | | overall | | north | | south | | overall | | north | | south | |
| | Traits | rho | p | rho | p | rho | p | rho | p | rho | p | rho | p |
| Fruit | Black (%) | 0.14 | 0.29 | 0.37 | 0.06 | 0.14 | 0.46 | 0.06 | 0.68 | 0.30 | 0.12 | -0.04 | 0.85 |
| | Area | -0.15 | 0.27 | -0.13 | 0.50 | -0.02 | 0.91 | -0.19 | 0.16 | -0.04 | 0.84 | -0.19 | 0.33 |
| | Thickness | -0.18 | 0.17 | -0.44 | <0.05 | 0.04 | 0.82 | -0.17 | 0.21 | -0.30 | 0.11 | 0.13 | 0.51 |
| Seed | Elongation | -0.03 | 0.81 | 0.09 | 0.62 | 0.03 | 0.87 | -0.08 | 0.55 | -0.11 | 0.57 | -0.05 | 0.80 |
| | Weight | 0.14 | 0.30 | -0.04 | 0.84 | 0.34 | 0.07 | 0.12 | 0.39 | 0.06 | 0.74 | 0.17 | 0.37 |
| | Volume | -0.20 | 0.13 | -0.36 | 0.05 | 0.02 | 0.94 | -0.22 | 0.09 | -0.25 | 0.18 | 0.01 | 0.96 |
| | Density | 0.41 | <0.01 | 0.39 | <0.05 | 0.25 | 0.18 | 0.39 | <0.01 | 0.39 | <0.05 | 0.16 | 0.40 |
| Mother -plant | Height | -0.11 | 0.42 | -0.31 | 0.09 | 0.06 | 0.76 | -0.26 | <0.05 | -0.48 | <0.01 | -0.26 | 0.16 |
| | Basal perimeter | -0.24 | 0.07 | -0.28 | 0.13 | 0.14 | 0.45 | -0.22 | 0.09 | -0.20 | 0.30 | 0.10 | 0.61 |
| | Number of stems | -0.06 | 0.65 | -0.23 | 0.23 | -0.37 | <0.05 | 0.04 | 0.77 | -0.22 | 0.24 | -0.19 | 0.30 |

5.3.3. Maternal-plant traits

The mother plants were significantly higher in the north provenance (1.29 m) than in the south (1.07 m; $F = 4.74$; $p < 0.05$; Fig. 16). At the same time, the northern plants tended to have a smaller basal diameter than the southern plants (8.78; vs. 9.92 cm) and fewer stems (north= 1.29; south= 1.07), but these differences were not statistically significant ($F = 0.77$ -1.21; p 's = 0.28-0.39).

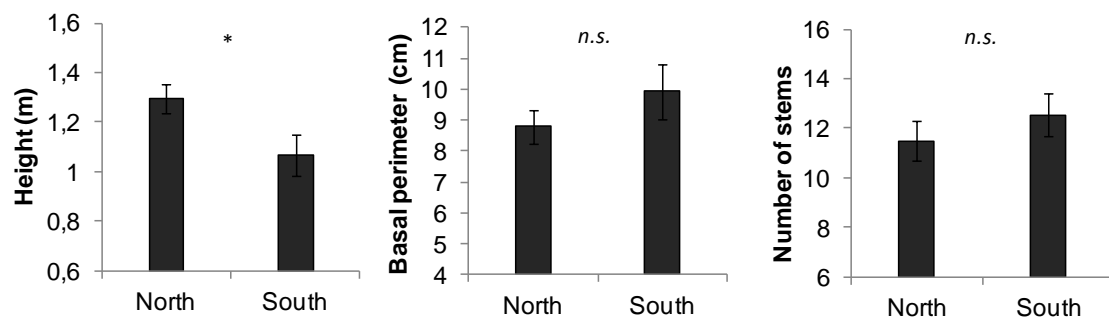


Figure 16 - Average and standard error for the maternal-plant morphological traits: height, basal perimeter and number of stems. Significant differences between provenances are indicated for $\alpha < 0.05$ by *, $\alpha < 0.01$ by ** and non significant differences are indicated by *n.s.*

The number of stems was negatively correlated with germination for the south provenance ($\rho = -0.37$; $p < 0.05$) but this was not true for the north provenance. No other maternal plant trait revealed correlations with germination. The establishment potential, however, was generally negatively correlated with mother-plant height ($\rho = -0.26$; $p < 0.05$) but, at the provenance level this association was only significant for the north provenance ($\rho = -0.48$; $p < 0.01$; Table 3).

5.4. Discussion

There was a clear negative effect of the water stress treatments in the germination of *Pistacia lentiscus*, with the treatment at -2.13 MPa exhibiting significantly lower germination and complete absence of germination in the -8.38 MPa treatment. This result reveals that water availability after imbibition (representing heavy rainfall) is important for germination completion in *P. lentiscus*. Germination can be, therefore considerably hampered in post-fire or highly disturbed scenarios. Garcia-Fayos and Verdu (1998) highlighted the importance of consecutive days with heavy rain for the germination of this species. On the other hand, as previous studies indicate that germination in several species can be inhibited under -1.5 MPa, the present results reveal an important drought resistance strategy in *Pistacia lentiscus*.

This species is distributed throughout the Mediterranean, being riped in autumn-winter and germinating readily if the conditions are favourable. Many Anacardiaceae species present physical dormancy, however information present in literature

suggests that *P. lentiscus* does not present dormancy (Garcia-Fayos 2001; Garcia-Fayos and Verdu (1998). On the other hand, Garcia-Fayos and Verdú (1998) reported the low storability of *P. lentiscus* seeds. This result was agrees with a previous study of the authors where vernalization negatively affected germination (Vasques et al. in prep). Under the natural population's recruitment point of view, this could mean that, if the species fail to germinate after dispersion and if the population is not adapted to overwinter, the germination of that generation might be compromised. In this context, the observed germination at water potentials lower than -2 MPa could represent an important adaptation of the species since it could allow new recruitment under a hot and dry autumn expected under climate changes.

Most of the results of the present study did not support the main overall hypothesis that provenance could play an important role in germination under low water availability. However, a part of results supported the importance of the provenance role, since different associations between germination and/or establishment potential and seed or mother-plant traits were found for different provenances. In spite of this, seed density was an overall good indicator of germination potential. This correlation could be explored aiming at finding floating techniques in denser solutions than water that could be applied in seed sorting.

There was also an overall negative correlation between seedling growth and mother-plant height, i.e. the taller plants originated smaller seedlings. This could be related with the spatial germination patter of higher germination under the canopy than in open sites described for this species (Garcia-Fayos and Verdu, 1998). In this context, bigger plants could provide a better nursery effect and therefore the offspring seedling could survive with less initial growth investment. These adaptation mechanisms have been described previously as changing inter-generations in response to different light environments in plants (Galloway and Etterson, 2007).

Contrarily to the observed in previous studies, the percentage of black fruits was not in the origin of the poor germination percentages (Vasques et al. in prep).

The germination failure in a big part of the harvested seeds together with reproductive problems reported for the species, namely embryo abortion in stressful habitats (Verdú and García-Fayos 1998), seed predation by wasps (Verdú and García-Fayos 1998), plus the low storability of its seeds in the soil rise concerns about the success

of new seedling recruitment of this species in natural populations. Similar recruitment threats were described for *P. therebinthus* (Traveset 1994).

Genetic fitness and diversity are key factors in ecological restoration and therefore a high number of mother-plants should be selected for harvest (Ensconet 2009). Nevertheless, when a high number of seeds from these maternal plants fail to complete germination, the selection of mother-plants through fitness indicators could be considered in order to ensure recruitment after ecological restoration in extremely harsh conditions.

Some morphological traits at the seed and mother-plant level were proven to have a relation with germination/seedling emergence and, although, the causal relation cannot be proved, the present study highlight the importance to take into consideration not only seed provenance but also mother-plant when harvesting seeds for ecological restoration. Similar associations were found recently for a perennial grass used in ecological restoration (Kulpa and Leger 2012).

The present study illustrates well the importance of intra-population variability in the germination of *P. lentiscus*. The ecological, economic and cultural relevance of this species plenty justify further research to disentangle the origins of the observed variability and its implications in the recruitment of natural populations. The observed challenge in finding suitable fruits in sizeable populations jointly with the recruitment difficulties reported in literature, associated with seed abortion and wasp predation reinforce the need of such studies. Without clear insights for mother-plant selection, seeds should be harvested in a higher number of mother-plants than those established in restoration protocols to ensure genetic diversity in the new offspring and improve resilience. However, intensive harvesting to ensure germination needs might threat regeneration in natural populations. This knowledge could contribute either to a better adjustment of harvesting protocols for ecological restoration and to a better planning of the natural population conservation.

5.5. Conclusions

The main conclusions of the present study to address the role of osmotic stress in germination and seedling growth of different mother-plants and provenances are the following:

1. Low water potentials (approximately -2MPa and -8MPa) hampered germination significantly in *P. lentiscus*, affecting both provenances in a similar manner.
2. There was a high variability in germination and seedling growth associated with mother-plant.
3. The degree of association between germination or seedling growth and seed or maternal morphological traits was limited. However, seed density was a good indicator for seed germination and plant height for establishment potential.

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Chapter 6

The role of seed provenance in the early development of *Arbutus unedo* seedlings under contrasting watering conditions

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Abstract

In the last decades, several studies have reported the increase of land degradation and desertification in the Mediterranean Basin. Depending on degradation severity, ecological restoration might be needed in order to promote ecosystem recovery. The ecology of the selected species and intra specific variability should be considered in order to improve restoration options, especially facing climate change.

The present study tested the hypothesis that seedlings from drier provenances would be better adapted to low water content conditions. Seeds were germinated under controlled temperature after which seedlings were grown in a phytotron under two contrasting watering regimes. Seedling performance was analysed using morphological and physiological parameters.

Low water content had a clear negative effect on the seedlings' aboveground biomass (total dry weight, root collar diameter, leaf dry weight and leaf weight ratio) and a positive effect on belowground biomass (root weight and root:shoot ratio). This response was not unequivocal, since provenances differed in morphological adaptations to low water content. Seedlings from the wettest provenance revealed a higher relative growth rate under high water content but a poor adaptation to limited water availability when compared to the other two provenances. This was observed by the absence of a significant belowground investment in this provenance. Seedlings

from the wettest provenance also presented a significant reduction of total leaf area that was not observed in the other two provenances. This can however be hardly considered as a successful adaptation to cope with drought since this provenance produced less sclerophyllous leaves, less belowground biomass and also lower sapwood to leaf area ratio independently from the water content conditions. By contrast, seedlings from the dry provenance with the hottest summer had similar root collar diameter, leaf dry weight and physiological performance under both watering regimes.

The observed adaptations to water regimes seem to be related with the climate of the seed source and highlighted the importance of seed provenance in ecological restoration actions using Mediterranean species. This knowledge could improve early establishment success predictions for different plant populations, allowing more reliable and cost-effective management decisions under climate change scenarios.

6.1. Introduction

In the past decades, the Mediterranean Basin has suffered a considerable increase in the area affected by land degradation and desertification (Díaz-Delgado et al., 2002; Pausas et al., 2008). The principal underlying causes are land abandonment and poor management of forest areas, which then result in recurrent and high-intensity wildfires (Vallejo et al., 2012). Although Mediterranean plant communities are well adapted to fire, recurrent wildfires tend to favour fire-prone species, i.e. species that accumulate large amounts of dead biomass and, thereby, not only increase fire hazard but also fire intensity (Baeza et al., 2011).

The Mediterranean climate is typically seasonal, with a summer period of warm temperatures and the bulk of rainfall occurring in spring and autumn. Water is considered as the main factor regulating plant activity and plant survival. In forest seedling plantations, plant survival is strongly influenced by the intensity and duration of drought periods right after plantation (Vallejo and Alloza, 1998). According to climate change projections, the frequency and intensity of drought is expected to increase in the Mediterranean region (Solomon et al., 2007). Although Mediterranean plants are well adapted to post-fire regeneration and summer drought, these adaptations may not be sufficient to ensure plant regeneration under extreme land degradation scenarios (Pausas et al., 2008).

In general, plants respond to stressful environmental conditions by shifting carbon allocation to the organs collecting the most limiting resource (Chambel et al., 2005). It is therefore expected that under drought conditions, plants increase belowground biomass allocation to improve water uptake capacity. However, drought-induced adjustments usually involve complex adaptation mechanisms, such as leaf area reduction, osmotic regulation and heat dissipation (Chaves et al., 2002). In addition, different plant species have different strategies to cope with drought, i.e. drought avoidance in the case of winter and spring annuals or drought resistance in the case of sclerophyllous Mediterranean species (Chaves et al., 2002).

In the past decades, our understanding of plant water relations and plant adaptation mechanisms was considerably improved through research focused on genetic determinants and environmental triggering factors that regulate plant activity of economically relevant species (Price and Courtois, 1999; Hamanishi and Campbell, 2011).

It is now increasingly recognised that wild species play a key role in ecosystem resilience under climate change scenarios. In this context, the intra-specific variability in plant functional traits can be determinant to improve species' fitness (Bischoff et al., 2006; 2008). This variability can result from ecotypic differentiation caused, in its turn, by specific morphological and physiological adaptations to different habitats (Hufford et al., 2003).

Depending on the degree of plant regeneration impairment, the implementation of ecological restoration actions by seeding or planting can be crucial to prevent the disruption of ecosystem resilience. Among other management practices, the selection of local-specific low flammable and high resilient species, can contribute to interrupt the established degradation processes by promoting resilience against major perturbations (Vallejo et al., 2012).

The first studies on the genetic variation of forest tree provenances date back to the beginning of the 19th century (Langlet, 1971). Under the concept of ecological distance, the benefits of matching seed source and target site conditions have been widely reported for tree production purposes (Matyas, 1993; O'Neill et al., 2008).

Though ecological protocols address the importance of local gene pool conservation (SER 2004), the role of intra-specific variability and seed source adequacy has only been addressed recently for a limited number of herbaceous species (Bischoff et al.,

2008; Mijnsbrugge et al., 2010). In this context, the improvement on the knowledge base of provenance-related adaptations might not be set aside in plant performance predictions following restoration actions.

The strawberry tree (*Arbutus unedo*) is an evergreen shrub, native to South Europe, North Africa, Palestine, Ireland and Macaronesia. It is widely spread in Portugal and commonly present in understory of oak and pine forests. This species presents several characteristics that justify its selection as a case study for the improvement of knowledge based for ecological restoration protocols, namely: low standing dead biomass, being catalogued as low to intermediate flammable (Liodakis and Kakardakis, 2008); strong resprouting ability after fire, contributing to ecosystem resilience (Vallejo et al., 2012); late successional, whose recruitment is often compromised by recurrent wildfires (Mesléard and Lepart, 1991) and, finally an intermediate resistance to drought, in comparison to other Mediterranean species (Gratani and Varoni, 2004).

Few decades ago, *A. unedo* was used as a model species to study environmental factors limiting CO₂ assimilation in Mediterranean species (Harley et al., 1986). Previous studies with *A. unedo* reported a conservative water use strategy, through Gs reduction or increase in root investment under drought conditions (Castell and Terradas, 1994; Navarro-García et al., 2011). Water stress resistance mechanisms, such as anti-oxidative protection were also reported for this species (Munné-Bosh and Peñuelas, 2004). In spite of this, Ogaya et al. (2003) and Ogaya and Peñuelas (2004) reported a higher growth reduction and phenophase delay in *A. unedo* than in other co-existent species, such as *Phillyrea latifolia* and *Quercus ilex*. Gratani and Varoni (2004) also compared these species and found a higher reduction of photosynthesis and stomatal conductance in *A. unedo* after drought. These results suggest a higher sensitivity to drought in *A. unedo* than in other pre-mediterranean species and therefore enhance its potential as an indicator of early drought effects following ecological restoration actions.

The main objective of the present study was to analyze the effect of seed provenance on the short-term development in *Arbutus unedo* seedlings. The hypothesis tested is that seedlings from drier provenances would be better adapted to low water content conditions. With this objective, seeds were harvested in three distant provenances in mainland Portugal, representing different climates. Two different water regimes were simulated and plant response during early stages of development was assessed

through several eco-physiological and morphological traits, using ten mother plants from each provenance.

6.2. Material and Methods

6.2.1. Seed harvesting: sites and procedure

Seeds were harvested at three different locations in mainland Portugal, at distances of at least 150 km, with latitudinal-related climate differences and also between coastland and inland provenance regions (Table 4). The aridity index of Martonne (De Martonne, 1926) however distinguishes the Aljezur location as semi-dry from the humid Gerês and Pesqueira locations. This reflects both the higher annual temperature and lower annual rainfall at Aljezur than the other two locations.


Between October and December 2010, the three different locations were visited to harvest ripe fruits of *A. unedo*. In each site, 30 plants with ripe fruits were selected randomly, according to the nearest neighbour method, ensuring a minimum distance of at least 5-10 meters between plants. At the mother-plant level, 30 fruits were harvested, ensuring its even distribution over the canopy. Fruits were then depulped and seeds were washed, dried and stored in glass bottles with silica. Seeds were then placed in the fridge at 4-5°C for approximately 14 months before the beginning of the experiment.

6.2.2. Experimental setup: germination and growth system

Seeds were soaked in distilled water for 2 hours and, after excluding those that floated, were set to germinate on Petri dishes over moist filter paper. Three Petri dishes were used as replicates and a total of approximately 30 seeds of one mother-plant were sown on each per Petri-dish. Seeds were then placed in a germination chamber with 12:12 hour's photoperiod at a constant temperature of 15°C. Germination was monitored two times per week and recorded when the radicle emerged from the testa. The bulk of germination was observed after 3 weeks when germination attained in average 70-90%. Seedlings were transferred to forest trays using 42 seedlings from 10 mother plants per provenance, randomly selected from the mother-plants with higher and more complete germination at that time. The experimental setup comprised 14 forest trays (96 cells and 75 cm³ /cell) that were filled with substrate (a mixture of limed sphagnum peat moss and coconut peat 1:1

v/v). In order to allow a progressive dehydration of substrate during an imposed drought period, the substrate was mixed with hydrogel at a dose of 1 g dry weight in 1L of substrate (Chirino et al., 2011). Seedlings were distributed randomly inside each forest tray using 3 seedlings per each mother plant and provenance. The same distribution was repeated for all the 14 forest trays, in a randomised block design, with 7 trays randomly assigned to each of the two water content treatments. The high and low water content treatments were designated as HWC and LWC, respectively. In resume, the experimental setup consisted in 1344 seedlings in total, comprising 10 mother-plants per each of 3 provenances exposed to 2 different water regimes.

Table 4 –Köppen climate classification, Aridity Index of Martonne and long term (Meteorological data from 1981-2010) mean annual temperature and mean total precipitation for the three provenances.



| District | Site | Climate classification (Köppen) | Aridity Index of Martonne | Mean annual temperature (°C) | Mean total precipitation (mm) |
|-----------|-----------|-----------------------------------------------------|---------------------------|------------------------------|-------------------------------|
| Braga | Gerês | Temperate Mediterranean with dry, mild summer (Csa) | 57.89 | 15.0 | 1449 |
| Vila Real | Pesqueira | Temperate Mediterranean with dry hot summer (Csb) | 43.36 | 13.6 | 1023 |
| Faro | Aljezur | Temperate Mediterranean with dry, mild summer (Csa) | 18.23 | 17.9 | 509 |

The seedlings grew in a walk-in phytotron (10000 EHVP, Aralab, Portugal) for approximately three months in total, two months of growth under optimal soil moisture conditions followed by one month of differential watering (LWC and HWC). At the end of the experiment, seedlings were watered to field capacity and the physiological measurements were undertaken. The photoperiod was of 12:12 hours of light and dark and the photon flux density (PPFD) during daylight was adjusted to $449.52 \pm 0.28 \mu\text{mol m}^{-2} \text{s}^{-1}$. Seedlings were fertilized once, after one month of growth throughout irrigation using a mixture of 18-11-18 (NPK) at a dose of 0.28 g.L^{-1} .

The environmental conditions of the phytotron (air temperature and relative humidity) were adjusted over the experiment to ensure a gradual transition to a progressively drier environment. Thus, at the beginning of the experiment, the average indoor air temperature was programmed to 15°C and air humidity to 80%. After approximately one month, when the first leaves emerged, the temperature was set to 20°C. The air relative humidity was programmed to 60:80% (day/night) for one more month and then reduced to 50:70%. These conditions were maintained until the end of the experiment.

6.2.3. High and Low Water Content treatments

The water content treatments were defined by a differential water supply according to the following: for the HWC treatment, trays were irrigated until field capacity (receiving ~ 2 L of water per tray) and for the LWC treatment trays were irrigated with the same frequency at a sub-optimal level (~1 L of water per tray). A sub-optimal level of irrigation was established for the LWC treatment in order to ensure that the trays would be slightly above the wilting point until the next irrigation. To accomplish this, trays were monitored through weight every 1-2 days and the water volume for irrigation was calculated based on the weight loss of the trays. The final weight of the HWC was calculated based on average tray weight at field capacity and the LWC target weight corresponded to 70% of the HWC. The volumetric soil water content (VWCs) was calculated by means of the gravimetric method. The correspondent water potentials were calculated based on the theoretic water liberation curve for this substrate.

During the growth period, the HWC and LWC treatments presented similar volumetric water contents with an average of 21.6 ± 0.7 % (-0.03 MPa; $F= 0.41$; $p=0.54$). After that, treatments were conducted to differential watering presenting in average double the water content in the HWC treatment (21.9 ± 3.2 %; -0.03 MPa) than in the LWC (10.6 ± 4.2 %; -0.1 MPa; $F=490.13$; $p<0.001$). At the end of the experiment and before the morphological and physiological characterization, all the trays were irrigated to field capacity, resulting in similar water content for both treatments ($F= 0.31$; $p= 0.59$; 26.5% and 26.2%, respectively; -0.02 MPa). Consistently the leaf water potential measured at pre-dawn at the end of the experiment was similar for both treatments (-0.63 MPa and -0.65 MPa for the HWC and LWC, respectively; $F= 0.43$; $p= 0.52$).

6.2.4. Morphological and physiological measurements

As mentioned before, at the end of the experiment both treatments were watered to field capacity and a set of physiological and morphological measurements was carried out. This irrigation was justified by difficulties in taken the physiological measurements during the differentiated watering period. A high stress level in most of the plants and consequent stomata closure could be in the origin of these difficulties.

For the morphological measurements, 5 plants per mother-plant and treatment were selected. Shoot height (H_s), root collar diameter (RCD) and number of leaves (NL) were measured. Afterwards, seedlings were cut at the cotyledons insertion and separated into three fractions: leaves, stem and roots. The dry weight of each fraction was determined after oven drying at 70°C for 48 h. The biomass relative variables were then defined as following: leaves dry weight (LDW) stem dry weight (SDW), root dry weight (RDW) and total dry weight (TDW). Root Weight Ratio ($RWR = RDW/TDW$) and Leaf weight Ratio ($LWR = LDW /TDW$) were determined. Specific leaf area (SLA) was calculated through the analysis of the dry weight of three circles of 0.27 cm² per leaf and the Total Leaf Area (TLA) of the plant was projected through the SLA value using LDW. Sapwood to leaf area ratio (SW:LA) was calculated by dividing the sectional area of xylem per total leaf area ($SW:LA= (Pi.(RCD/2)^2)/TLA$). Relative Growth Rate (RGR) was calculated by the differences in average shoot height per mother-plant (H_s) between the end of the experiment (t_2) and the initial time (t_1) of the differential irrigation ($RGR= \ln (H_s t_2) - \ln (H_s t_1)/ (t_2-t_1)$).

The physiological measurements were taken at midday during 4 consecutive days using an Infra Red Gas Analyser (IRGA, Licor 6400 XT). Photosynthesis (A , $\mu\text{mol CO}_2\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), Stomatal conductance (G_s , $\text{mol H}_2\text{O}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), Transpiration (E , $\text{mmol H}_2\text{O}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), Effective Quantum Yield of Photosystem II (Φ PS II) and C_i ($\mu\text{mol CO}_2$ mol air^{-1}) were measured with a saturated irradiance of 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (10% blue light) and a molar flow rate of 200 $\mu\text{mol s}^{-1}$. The Intrinsic Water Use Efficiency (IWUE, $\mu\text{mol CO}_2\cdot \text{mol H}_2\text{O}^{-1}$) was also calculated ($IWUE= A / G_s$). Plant water potential was measured at predawn in the same plants using a Scholander pressure chamber (Soil moisture Equipment Corp., Santa Barbara, CA, USA). Three from the 5 plants per mother plant used for the morphological measurements were randomly selected for the physiological measurements.

6.2.5. Data analysis

Dynamics of soil water content between treatments were compared by ANOVA for Repeated Measurements (ANOVAR) using the different dates as factors.

RGR was analysed through 2-Way ANOVA (non-nested) using provenance and substrate water content as factors. All other variables were analysed through 2-Way ANOVA using a nested design, i.e. mother-plant as the random nested factor within provenance and provenance and treatments as fixed factors. When differences between treatments were significant, 1-way ANOVA using treatment as factor were performed for each provenance separately. Independently from the overall absence of significant differences and because of the observed differences in TDW, the same procedure was performed to test differences in RGR. ANOVA assumptions were considered by analysing the distribution of the residuals and homoscedasticity through the Levene's test. When the assumptions were not met, standard transformations, such as log (x), square root (x), sine (x) and cosine (x), were carried out. Tukey's HDS post-hoc tests were done when significant provenance differences were detected ($\alpha \leq 0.05$). The SPSS v.18 software was used for all statistical analyses.

6.3. Results

6.3.1. Seedling development

From the onset of the differential watering regime to the end of the experiment (1 month later), seedlings grew about 15 mm in height and develop five new leaves, in average. Hence, both treatments and provenances presented a similar RGR in height ($F = 0.75$ $p = 0.39$ and $F = 0.1$; $p = 0.38$ for water treatment and provenance effect, respectively). Likewise, there were no significant differences in Hs or number of Leaves between water content treatments at the end of the experiment (Table 5; $F = 1.40$; $p = 0.25$ and $F = 0$; $p = 0.98$, respectively). The RGR was however significantly different between water content treatments for Gerês that grew significantly more under HWC (1-Way ANOVA: $F = 3.77$; $p < 0.05$) but the same was not observed for the other two provenances (1 Way ANOVA: $F = 0.20-0.72$; p 's = 0.67-0.68; Fig.17).

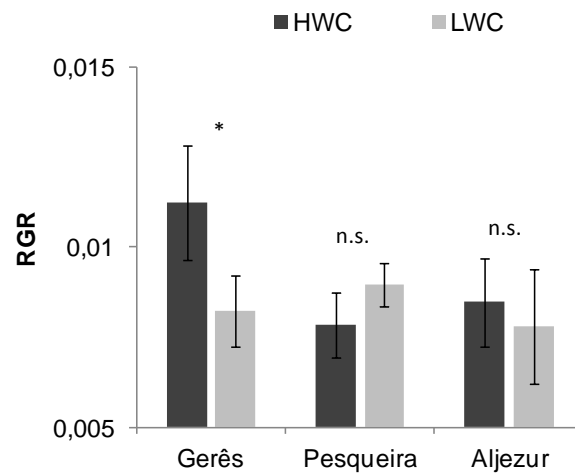


Figure 17 – Mean and standard error of the Relative Growth Rate (RGR) calculated through the values of shoot height (H_s ; mm) during the differential watering period for the three studied provenances (Gerês, Pesqueira and Aljezur) under high and low water content conditions (HWC and LWC, respectively). Significant differences between water content treatments within the different provenances are indicated by the notation: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ and n.s. – not significant.

6.3.1.1. Drought effect

At the end of the growing period, the different watering treatments had a significant effect on most of the morphological characteristics of the seedlings. In general, high water availability (HWC) promoted greater seedling development, and TDW, RCD, TLA, LDW and LWR were significantly higher in HWC than in LWC seedlings (Table 4). Interestingly, though the number of leaves was similar for both treatments, leaf size differed between treatments, as reflected by LDW and TLA. On the contrary, there was more investment in belowground biomass proportionally to the total dry weight, i.e. RWR was significantly lower for the HWC than for the LWC treatment and the same tendency was observed for root to shoot ratio (Table 5; Fig.18).

After irrigation, the average of net photosynthesis was higher for LWC seedlings than for HWC seedlings (Table 5; Fig.19). The quantum yield of the photosystem (Φ_{PSII}) under steady-state conditions followed the same tendency. Contrarily, stomatal conductance and transpiration were similar for both treatments.

Table 5 – Main effects in morphological and physiological parameters tested through 2-Way Analysis of Variance (ANOVA) using water content (WC) and provenance (Prov) as fixed factors and mother-plant (MP) as a random nested factor in provenance. Statistically significant values ($p < 0.05$) are in bold. Abbreviations: shoot height (Hs), total dry weight (TDW), root collar diameter (RCD), total leaf area (TLA), specific leaf area (SLA), leaves dry weigh (LDW), stem dry weigh (SDW), root dry weight (RDW), leaf weight ratio (LWR), root weight ratio (RWR), root to shoot ratio (R:S), sapwood to leaf area ratio (SW:LA), Net photosynthesis (A), Stomatal conductance (Gs), Transpiration (E), Intrinsic Water Use Efficiency (IWUE), Yield Photosystem II (Φ PS II) and substomatal CO_2 concentration (Ci).

| | | Water content treatment | | | Provenance | | Main effects of factors and interactions | | | | |
|---------------------------------|-------------------------------------------------------|-------------------------|-------------|-------------|-------------|-------------|------------------------------------------|--------------|--------------|--------------|--------------|
| <i>Morphological traits</i> | | HWC | LWC | Gerês | Pesqueira | Aljezur | WC | Prov | WC*Prov | MP (Prov) | WC*MP (Prov) |
| Hs | mm | 59.33±0.91 | 58.24±0.87 | 59.33±0.90 | 58.08±1.07 | 58.91±1.28 | 0.246 | 0.857 | 0.395 | 0.005 | 0.711 |
| Nº Leaves | | 15.28±0.28 | 14.56±0.27 | 15.06±0.35 | 15.09±0.35 | 14.60±0.32 | 0.984 | 0.389 | 0.199 | 0.080 | 0.950 |
| TDW | g | 0.30±0.01 | 0.28±0.01 | 0.28±0.01 | 0.30±0.01 | 0.28±0.01 | 0.025 | 0.205 | 0.495 | 0.129 | 0.625 |
| RCD | mm | 1.33±0.02 | 1.25±0.02 | 1.31±0.02 | 1.30±0.02 | 1.26±0.02 | 0.008 | 0.318 | 0.191 | 0.432 | 0.227 |
| TLA | cm ² | 25.21±0.80 | 22.55±0.63 | 27.11±1.04 | 21.85±0.62 | 22.57±0.87 | 0.000 | 0.000 | 0.172 | 0.133 | 0.997 |
| SLA | mm ² .g ⁻¹ | 147.30±5.80 | 149.93±5.13 | 173.82±8.39 | 126.60±3.94 | 146.20±6.06 | 0.452 | 0.039 | 0.457 | 0.261 | 0.799 |
| LDW | g | 0.18±0.005 | 0.16±0.004 | 0.17±0.006 | 0.18±0.005 | 0.17±0.006 | 0.001 | 0.292 | 0.352 | 0.017 | 0.938 |
| SDW | g | 0.039±0.001 | 0.036±0.002 | 0.038±0.002 | 0.036±0.001 | 0.038±0.001 | 0.230 | 0.768 | 0.619 | 0.701 | 0.502 |
| RDW | g | 0.08±0.002 | 0.08±0.002 | 0.07±0.003 | 0.09±0.002 | 0.08±0.002 | 0.911 | 0.016 | 0.574 | 0.246 | 0.128 |
| LWR | | 0.61±0.004 | 0.58±0.004 | 0.60±0.006 | 0.59±0.005 | 0.59±0.005 | 0.000 | 0.132 | 0.805 | 0.331 | 0.602 |
| RWR | | 0.26±0.005 | 0.29±0.004 | 0.26±0.006 | 0.28±0.005 | 0.28±0.005 | 0.000 | 0.088 | 0.186 | 0.003 | 0.977 |
| R:S | | 0.37±0.01 | 0.41±0.01 | 0.37±0.01 | 0.40±0.01 | 0.40±0.01 | 0.001 | 0.200 | 0.123 | 0.011 | 0.942 |
| SW:LA | | 0.06±0.002 | 0.06±0.002 | 0.05±0.002 | 0.07±0.002 | 0.06±0.002 | 0.704 | 0.003 | 0.693 | 0.787 | 0.206 |
| <i>Eco-physiological traits</i> | | | | | | | | | | | |
| A | $\mu\text{mol CO}_2.\text{m}^{-2}.\text{s}^{-1}$ | 2.08±0.11 | 2.63±0.13 | 2.27±0.14 | 2.25±0.15 | 2.58±0.16 | 0.019 | 0.268 | 0.272 | 0.625 | 0.153 |
| Gs | $\text{mol H}_2\text{O}.\text{m}^{-2}.\text{s}^{-1}$ | 0.06±0.003 | 0.07±0.003 | 0.06±0.004 | 0.06±0.003 | 0.07±0.003 | 0.277 | 0.351 | 0.384 | 0.549 | 0.377 |
| E | $\text{mmol H}_2\text{O}.\text{m}^{-2}.\text{s}^{-1}$ | 1.58±0.08 | 1.69±0.06 | 1.66±0.087 | 1.50±0.083 | 1.74±0.083 | 0.166 | 0.329 | 0.661 | 0.451 | 0.375 |
| IWUE | $\mu\text{mol CO}_2.\text{mol H}_2\text{O}^{-1}$ | 35.65±1.47 | 41.69±1.60 | 37.22±1.99 | 39.37±1.78 | 40.11±1.96 | 0.137 | 0.119 | 0.039 | 0.972 | 0.138 |
| Φ PS II | | 0.19±0.007 | 0.22±0.007 | 0.21±0.008 | 0.20±0.010 | 0.21±0.008 | 0.053 | 0.852 | 0.666 | 0.337 | 0.170 |
| Ci | $\mu\text{mol CO}_2.\text{mol air}^{-1}$ | 315.96±2.39 | 305.93±2.59 | 313.31±3.22 | 309.90±2.90 | 308.47±3.19 | 0.090 | 0.146 | 0.021 | 0.978 | 0.195 |

6.3.1.2. Provenance differences

The provenance effect was statistically significant for specific leaf area, total leaf area, root dry weight and sapwood to leaf area. Seedlings from Gerês showed higher values of TLA and less sclerophyllous leaves (Table 5; Fig.20). On the other hand, root development (RDW) was higher for Pesqueira than for Aljezur and Gerês. Accordingly, SW:LA was significantly different among provenances with lower values for Gerês than for Pesqueira ($F=7.12$; $p<0.01$; Tukey: $p<0.01$).

No differences were observed in any physiological variable but significant interactions were observed for IWUE and C_i , reflecting that different provenances respond differently to LWC (Table 5; Fig 19).

The subsequent tests for differences between treatments within each provenance (1-way ANOVA) revealed that Pesqueira, contrarily to the other provenances, was not significantly negatively affected by the LWC (Fig.20). In spite that Gerês and Aljezur had lower root collar diameter and leaf dry weight under LWC, Pesqueira showed similar values between treatments for the aforementioned traits. All provenances presented significantly higher LWR for the HWC. On the other hand and contrarily to what was observed for Pesqueira and Aljezur, Gerês did not show higher RWR under LWC. Moreover, Gerês was also the only provenance in which total leaf area was negatively affected by LWC (Fig.20).

None of the morphological variables presented interactions between treatment and provenance or treatment and mother-plant, however, families varied significantly in H_s , LDW and RWR (Table 5).

On the other hand, the mother-plant random factor did not contribute significantly for the overall variability in any of the physiological variables. Additionally, no significant interactions were found between mother-plant and watering treatment (Table 5).

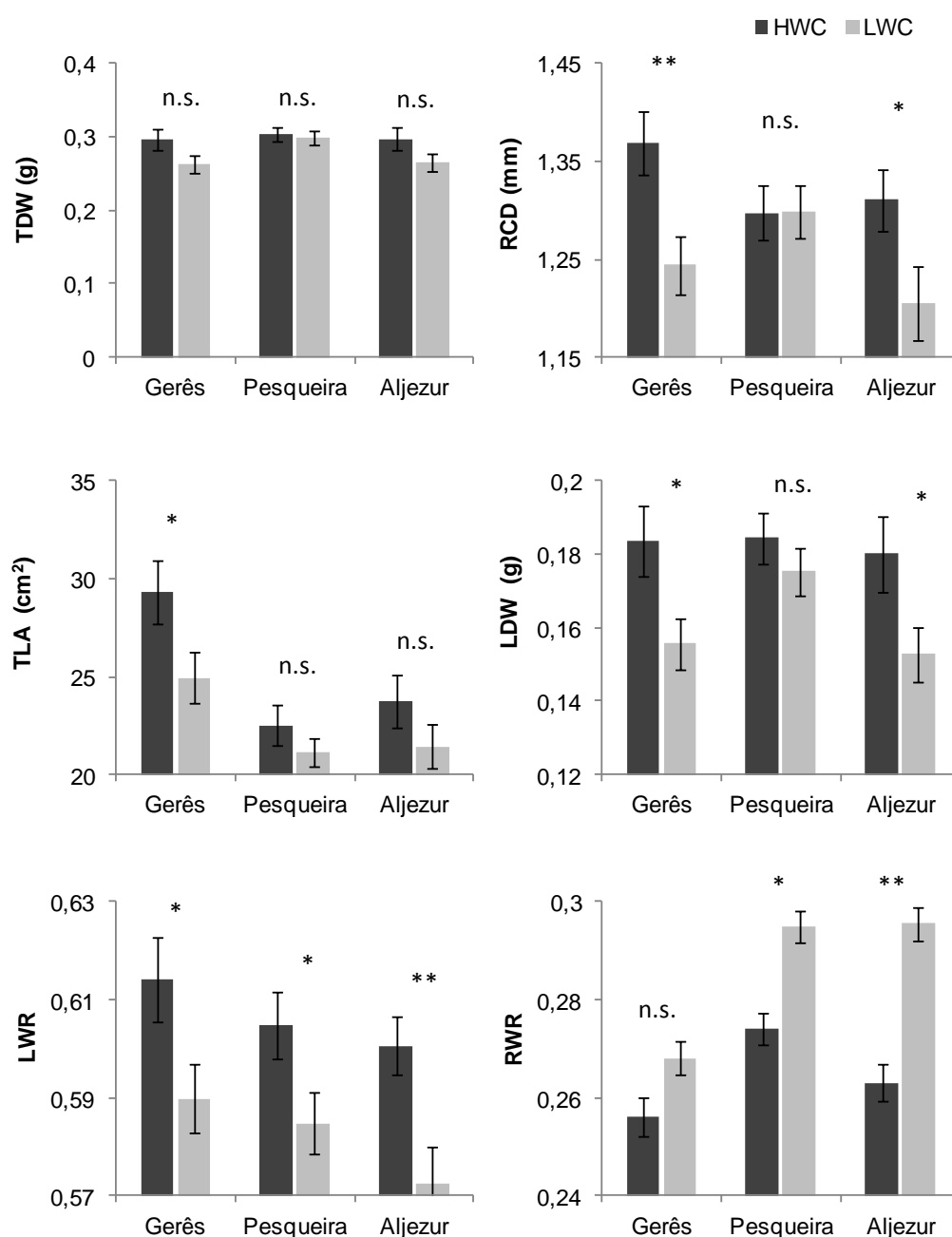


Figure 18 - Mean and standard error of total dry weight (TDW), root collar diameter (RCD), total leaf area (TLA), leaf dry weight (LDW), leaf weight ratio (LWR) and root weight ratio (RWR) for the three studied provenances (Gerês, Pesqueira and Aljezur) under high and low water content conditions (HWC and LWC, respectively). Significant differences between water content treatments within the different provenances are indicated by the notation: * p<0.05; **p<0.01; *** p<0.001 and n.s. – not significant.

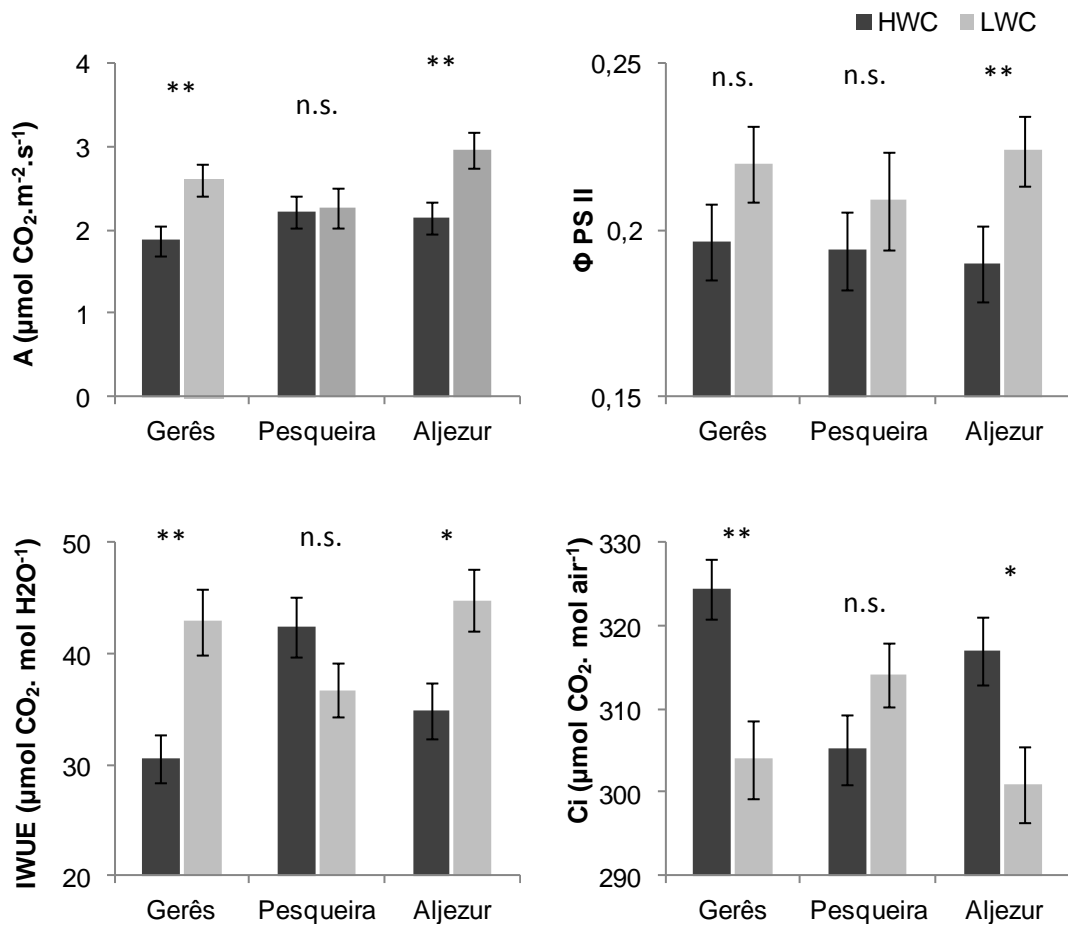


Figure 19 – Mean and standard error of net photosynthesis (A), effective quantum yield (Φ PSII) intrinsic water use efficiency (IWUE), and substomatal concentration of CO_2 (Ci) for the three studied provenances (Gerês, Pesqueira and Aljezur) under high and low water content conditions (HWC and LWC, respectively). Significant differences between water content treatments within the different provenances are indicated by the notation: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ and n.s. – not significant.

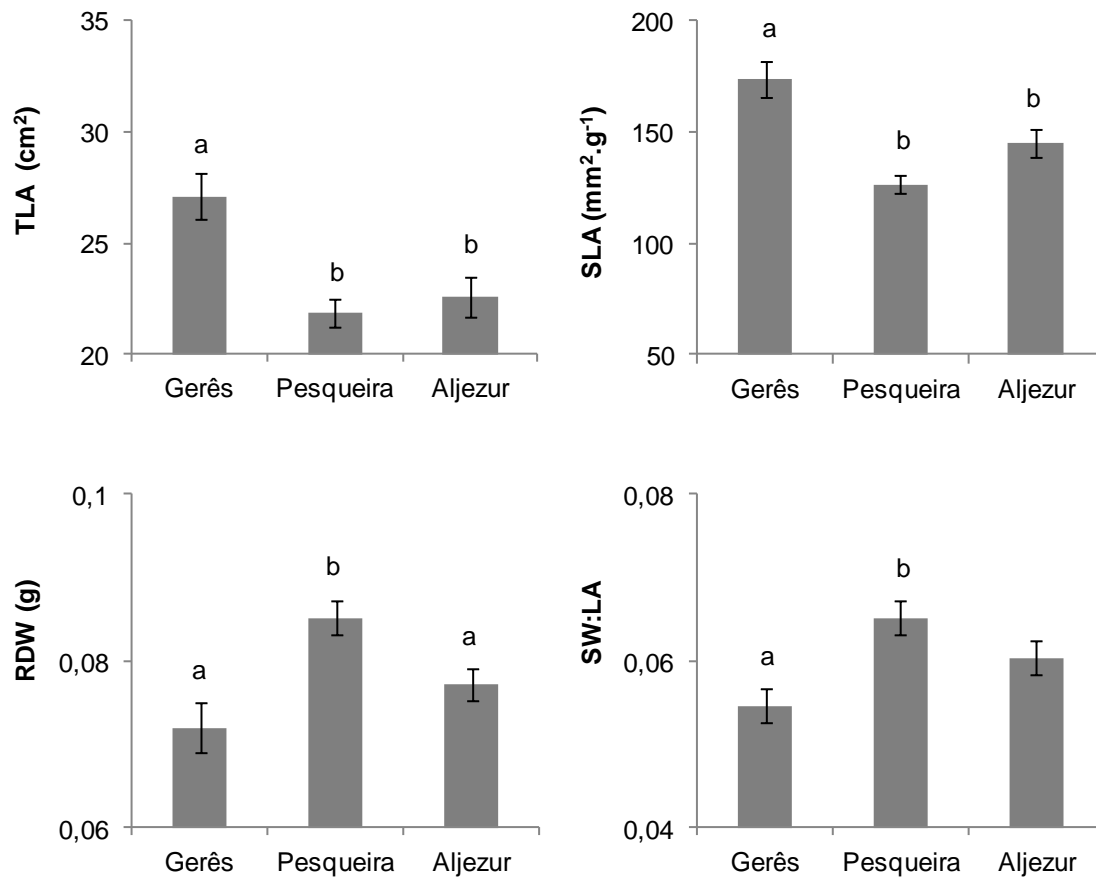


Figure 20 – Mean and standard error of total leaf area (TLA), specific leaf area (SLA), root dry weight (RDW) and sapwood to leaf area (SW:LA). Different letters indicate differences between provenances.

6.4. Discussion

6.4.1. Drought effect

In the present study, seedlings of *A. unedo* showed significant morphological and physiological adjustments after only one month of differential irrigation, re-affirming the relevance of water availability and the sensitivity of this species to water limitations in early developmental stages.

In general, seedlings that grew under higher water content invested more in aboveground biomass (leaf biomass, leaf area and stem diameter) whilst those that were subjected to lower water content developed relatively more belowground biomass (root biomass). Similar morphological adjustments were previously reported

in *A. unedo* by Ogaya et al. (2003) and Navarro-García et al. (2011). Additionally, seedlings subjected to water limitation showed an increase in the photosynthetic rate and in the photochemical efficiency of PSII after irrigation to field capacity (recovery). Both values of photosynthesis obtained in this study were within the range of previously observed values for *A. unedo* (Harley et al., 1986) and other Mediterranean shrub species (Hernández et al., 2011). The photosynthesis enhancement observed in the LWC treatment could be related to a higher nutrient accumulation in the substrate after this treatment. This accumulation could be, in its turn, caused by the lower plant development observed in two out of the three studied provenances during differential irrigation.

The three provenances did not show the same morphological adjustments to drought (described below).

6.4.2. Provenance differences

Both aboveground (TLA, SLA, SW:LA) and belowground adaptations (RDW) revealed a significant provenance effect. The wettest provenance (Gerês) was the provenance with highest RGR under HWC and the only provenance that was significantly and negatively affected by LWC in this trait. This result suggests a higher growth potential of this provenance under high water content on the one hand, and on the other a higher sensitivity to drought.

The wettest provenance showed the highest values of TLA and SLA and poor belowground biomass allocation under low water regimes, suggesting a poorer adaptation to drought conditions. The low sclerophylly (high SLA) and consequent bigger leaf area (TLA) agrees well with the climate of Gerês (high rainfall and low aridity). In this region, because of being hardly subjected to low water availability, plants can develop bigger transpiration surfaces without desiccation risk. Additionally, in this wet ecosystems by having higher water availability, vegetation density can become higher. In this sense, one of the main limitations to plant performance will be light capture for photosynthesis and plants develop strategies to increase light capture through bigger and thinner leaves (Evans and Poorter, 2001).

Previous studies revealed a high heritability of SLA and a clear relationship between the degree of sclerophylly and intrinsic water use efficiency (de Miguel et al., 2011).

This relation was observed for the high water content treatment but not for the low water treatment. The observed discrepancy could be therefore caused by the aforementioned differences in plant photosynthetic performance. *Pesqueira* had, on one hand, higher root dry weight and sapwood to leaf area and, on the other, a similar root collar diameter and leaf dry weight for both water regimes. A higher root investment under drought was also observed in other species for provenances from drier sites (Li and Wang, 2003; Zhang et al., 2004). Thus, the aforementioned higher adaptation efficiency to low water contents in *Pesqueira* could be explained by an adaptation of this provenance to a drier summer.

The provenance related differences in seedlings' response to drought, that were not significantly influenced by the mother-plant, could either be originated in stress memory inheritance through epigenetic mechanisms or in genetic differences at the provenance level (Chinusamy and Zhu, 2009). However, Gomes et al. (2010) did not find genetic differences between Portuguese provenances of *Arbutus unedo* which highlights the importance of further research in plant short term heritage under contrasting environmental conditions. This knowledge could be relevant to unravel the intensity and speed of plant adaptation to climate change, also contributing to the adjustment of future species' distribution predictions.

Zhang et al. (2004) pointed the possibility of the use of water use efficiency over growth parameters an easier- non destructive measurable indicator of drought adaptation in different populations. Contrarily, a higher expression of morphological adaptations in provenance response to drought was reported previously (García-Plazaola and Becerril, 2000). In the present study, the morphological traits were better indicators of differences in drought adaptations between provenances than the physiological variables and therefore the interpretation of physiological results should be, when possible, assisted by morphological traits.

6.5. Conclusions

Provenance played a significant role in the early development of *A. unedo* under different water stress regimes, reinforcing the importance of seed source when planning ecological restoration actions. The wettest provenance showed a poorer drought adjustment than the other two provenances. In addition, the provenance from the driest summer site developed seedlings with higher root dry weight which were

also not significantly affected by drought in 4 out of the 5 aboveground affected traits. The observed seed source climate-related adaptations to water regimes raise the importance of further research to unravel the speed and nature of water scarcity adaptations in Mediterranean species. This knowledge could improve germination and early establishment success predictions for different plant populations, allowing more reliable and cost-effective management decisions under climate change scenarios. The observed drought-related adaptations reinforce the potential of the use of *A. unedo* as a model species for the study of drought-associated mechanisms in Mediterranean species.

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Chapter 7

Discussion and Conclusions

The present study aimed at improving the knowledge base for seeding shrub native species after major disturbance, such as high intensity and/or frequency wildfires in Mediterranean woodlands.

The first part of the thesis (chapters 2, 3, 4) aimed at deepen the knowledge on the germination ecology of 8 of the most cover-representative shrub species in Portugal mainland, representing different post-fire regeneration strategies, stages of succession and seed dispersal phenology. The second part of the thesis (chapters 5 and 6) was comprised by two cases of study, using two different species and aimed at addressing the role of low water availability in germination and early seedling development, respectively. All chapters focused on the role of provenance in the germination or early developmental response. The specific objectives were to: i) address the role of pre-germination treatments and/or low water content in germination and/or early establishment response; ii) test the role of provenance in such responses and iii) investigate possible associations between the response observed and the climate conditions at the seed source.

7.1. Towards a better understanding of germination ecology in Mediterranean species

Plant plasticity is a key factor in adaptation to environmental changes and an essential element in plant evolution (Valladares et al. 2007). Hence, the effect of provenance could be the result of environmental conditioning during seed development and/or of long term adaptation of the species (genetics). The present conceptual framework (chapter 2) explores the influence of seed source in dormancy intensity through the influence of different environmental conditions during seed development.

The presented review addressed aspects such as seasonality, seed dormancy breaking mechanisms and dormancy intensity. A deeper knowledge of the influence of those aspects in germination was used to create a structured framework to assist seed handling protocols as a general decision tool for storage, pre-treatment and

sowing temperature requirements. The possible consequences of the selected harvesting sites in the aforementioned handling protocols are currently considered as a source of variation but further understanding of the environmental influences in dormancy intensity could be used in improving germination predictions (Ch. 2). In this context the further development of a mathematical model for germination prediction following seeding under ecological restoration would be an important step to improve the understanding of the germination ecology of these species. The development of a reliable tool for germination prediction in Mediterranean species could lead to an increase of the use of native seeds in ecological restoration actions and ultimately amplify the use of native seeds also to other purposes, enhancing ecosystem sustainability (Simmons 2007).

The main effects on the role of provenance in the germination of shrub species with contrasting seed dispersal phenology were further analysed in chapters 3 and 4. The patterns observed in provenance response to different conditions and their possible link with adaptation to local climate, according to the main germination strategies present in Mediterranean species are discussed.

In fire-prone shrub species with common and widespread occurrence at the understory of Portuguese forests (Ch. 3) the heat pre-treatments induced germination enhancement in 4 out of the 5 studied species.

The species studied here, although not being considered as suitable for post-fire restoration, are an important element for biodiversity improvement of forest commercial stands and have also a cultural value, namely honey production (*Erica* spp.), potential heavy metal depuration (*Cistus ladanifer*; Kidd et al. 2003) and animal feeding (Leguminosae shrubs). These cultural values could also be considered while evaluating the potential of their use in ecological restoration in non-fire prone areas. In this context, the highest dependence on fire of certain species and populations can threatened them in northern ecosystems, where their introduction through restoration actions might be considered. *Erica umbellata* was the only species whose germination was not enhanced by, at least, one of the heat pre-treatments. This result was in line with those reported by literature, suggesting that heat per-se is not enough to stimulate germination in this species (Gonzalez-Rabanal and Casal 1995; Moreira et al. 2010).

There were differences in germination-enhancement following heat between different provenances, with the southern seeds presenting higher germination following heat treatments. This could be a result of higher selection pressure of fire and/or heat in south provenances, resulting in higher adaptation of these populations.

There was an apparent relation at the provenance level between seed size, seed source and germination potential (Ch. 3). Seed size has been previously reported as a non-hereditary and highly phenotypic plastic trait (Galloway et al. 2009). This assumption is mainly based on the observation of high variability in seed size within the same individual, which indicates environmental rather than genetic differences (Leishman et al. 2000). Previous studies have reported the influence of local climatic conditions in seed size, but the mechanisms underlying seed-size variations among communities remain unclear (Leishman et al. 2000).

The commonly accepted premise used at the inter-specific level, that bigger seeds germinate better is not always true within a species. On one hand bigger seeds can indicate the presence of more reserves and bigger embryo but, on the other if dormancy is present, the role of seed size can become less intuitive. In the present study, the contrary, i.e. smaller seeds germinate better was observed at the provenance level for the fire-prone species, *Cistus ladanifer*, *Erica australis*, *Pterospartum tridentatum* and *Phillyrea angustifolia*. This relation could be related with dormancy; however, the mechanisms involved in this process remain unclear. It could either mean that north (cooler) provenances have bigger seeds which are less dormant and therefore die after heat treatments or that they present a stronger or other type of dormancy that is not stimulated by the set of pre-treatments tested.

In the Leguminosae species, when considering non treated seeds, there was a tendency for higher germination in the northern provenances. This could be a cue in favour of the first hypothesis, i.e. northern seeds which are bigger present less dormancy. For the Ericaceae species, however, the second hypothesis is more likely to be true (i.e. provenances from warmer places are less dormant) since in this species the northern non-treated seeds presented similar germination than those of other provenances.

The presence of a higher percentage of non-dormant seeds in cooler and/or wetter provenances which are, at the same time bigger, could be related with the selection pressure exerted upon this trait. In wetter provenances, the competition for light is

higher and plants can develop bigger seeds to guarantee that the seedling offspring is competitive.

The relation between seed size and germination response following heat should be further researched to a better understanding of population germination differences in relation to fire adaptation mechanisms. Especially since fire is considered as an important driven force in shaping physical dormancy in fire-prone species (Moreira and Pausas 2012).

As to the autumn-winter dispersed species (Ch.4), the effect of high moisture storage varied according to the species studied. This treatment affected negatively the amount or speed of germination in *Phillyrea angustifolia* (physical dormancy) and *Pistacia lentiscus* (no-dormancy) whereas the opposite was observed for germination speed in *Arbutus unedo* (physiological dormancy). The provenance role in this response was, once more, significant. Germination was significantly higher in warmer provenances (south) of both species presenting dormancy, where the enhancement treatments and/or adequate germination temperatures had less expression in germination enhancement. This result suggests that warm temperatures and or drought during develop lead to weaker dormancy. Though previous studies reported that warmer temperatures during seed development can lead to weaker dormancy, this relation was not straightforward and depended from the species studied (Guttermann 2000; Murdoch and Ellis 2000). The effect of water stress during seed maturation can affect dormancy intensity, by for e.g. decreasing ABA sensitivity, enhancing therefore germinability (Fenner and Thompson 2005). The direction of this influence remains, however, poorly understood as it seems to also vary according to the species studied (Guttermann 2000).

Differences in germination results between laboratorial and field experiments were reported previously (Bischoff et al. 2006; Oliveira et al. 2012). The non-constant stimuli that is often present in the field could be involved in this response however the use of standardized temperatures in laboratory essays (20°C) could also be the cause of poor germination predictions in Mediterranean species. This could be caused by the presence of a Mediterranean adaptation syndrome that lowers the maximum temperature threshold favourable for germination (Fenner and Thompson 2005). This syndrome could explain the germination response observed in *Phillyrea angustifolia* that, failing to germinate under constant temperatures, experienced a dramatic increase in germination under a lower thermo-period (Ch. 4). A lower maximum

temperature threshold in *Arbutus unedo* was also recently reported and could be in the origin of the low germination percentages observed in chapter 4 (Bertsouklis and Papafotiou 2013). Finally, differences in temperature thresholds for germination could be caused by different dormant intensities, which could be in its turned caused by differences in seed development or post-harvesting conditions (Vergis 1964; Fenner and Thompson 2005). This would explain the higher germination percentages in seeds from warmer provenances observed for *P. angustifolia* and *A. unedo* under standard temperatures (20°C; Ch.4).

Previously published studies addressing germination in Mediterranean species with contrasting dispersal phonologies and dormancy types in both field and laboratory conditions can be used to estimate temperature thresholds, species seasonality, dormancy breaking conditions and, finally, dormancy intensity cues. in order to define the best variables and treatments to be researched for model building. In spite of the higher realism of field studies, the use of laboratorial studies to address specific questions, such as germination and water potential thresholds in key species are highly recommended in further research. Based on the tendencies observed under controlled conditions, pre-treatments, storage and sowing conditions can be chosen with more confidence for follow up studies in the field to validate the model predictions under variable conditions.

Only after all these steps, the use of seed prediction models in ecological restoration can be done with a certain degree of confidence for a broad number of species. This process should consider different seed provenances disentangling the intensity and duration of seed developmental conditions in dormancy intensity in order to have a better prediction of species adaptation success following ecological restoration actions through seeding.

Finally, the influence of other relevant factors such as nutrients and light conditions should also be tested to enhance germination and early establishment predictions. Some environmental conditions, such as light during seed development were proven to have important inter-generational epigenetic effects in plant adaptation (Galloway and Etterson 2007). In spite of the logistical challenges that the inclusion of all this factors in seed provenance studies could represent, their study would allow disentangling the extent of the effects of the various environmental conditions in germination success, enhancing reliable perditions.

The exact influences of micro-conditions of seed development could be disentangled through more detailed research by for e.g. using seeds from various sites within each provenance and/or assessing germination in different generations under contrasting pre-treatments and germination conditions. Species with relatively short generations and broad distribution that present, at the same time, fire and cold related adaptations, such as *Calluna vulgaris*, could be used in these studies.

7.2. Effects of contrasting watering conditions

7.2.1. Germination

Previous studies on *Pistacia lentiscus* revealed a poor resistance to aging processes after seed storage in the soil seed bank (García-Fayos and Verdú 1998). There was no clear role of provenance in the germination of this species under standard conditions (Ch. 5). Besides that, cold under high moisture (mimicking over winter soil storage) did not affect significantly germination in the northern provenance (cooler and wetter) in contrast with the negative effect observed for the other two studied provenances (Ch. 4). Even then, the germination response in this species was mainly determined by other factors, since most of the seeds sowed did not germinate. As only filled fruits were used in the present study (and also non-empty seeds) the origins of this germination failure could not be due to the embryo malformations observed previously in this species, needing further research (Verdu and Garcia-Fayos 1998).

Osmotic stress significantly hampered germination in *Pistacia lentiscus*. The germination attained under osmotic stress was consistently correlated with the germination potential under control conditions, independently of the provenance studied. Though provenance does not seem to have a role in seed water relations in a non-dormant species this result should not be extended to species presenting dormancy. This is because if different provenances present different intensities could also vary in water potential thresholds (Alvarado and Bradford 2005).

Another factor that proved to influence germination significantly was the intra-population variability, related to mother-plant. Although that only for the north provenance, there were found correlations between seed and maternal plant morphology and germination in *Pistacia lentiscus* (Ch. 5). Seed density positively

influenced germination and mother-plant height was negatively correlated with initial seedling growth.

These findings do not imply that seed size has a genetic determined base, as it could be the result of different environment pressures and life stories at the mother-plant level. Hence, the cumulative effect of environmental factors could be inherited through epigenetic mechanisms raising the evidence of the observed associations (Galloway and Etterson 2007; Navie et al. 2007).

The negative relation between mother-plant size and seedling growth could be related with the spatial germination patter of higher germination under the canopy than in open sites described for this species (Garcia-Fayos and Verdu 1998). Thus, bigger plants could provide a better nursery effect and therefore the offspring seedling could survive with less initial growth investment. Similar adaptation mechanisms have been described previously as changing inter-generations in response to different light environments in plants (Galloway and Etterson 2007).

The observed associations between maternal traits and germination deserve further research. The efforts to understand the causes and possible influence of these associations would be plenty justified as they could contribute to the adjustment of seed harvesting protocols to optimize germination potential. Furthermore, this knowledge could contribute to a broader sensing of germination potential in natural populations, supporting species in situ conservation.

7.2.2. Seedling development

The provenance role in early establishment was addressed, under contrasting watering conditions, for *Arbutus unedo* using a series of morphological and physiological traits (Ch. 6). The provenances studied here do not represent a purely latitudinal gradient but this gradient plus a more continental and hot summer provenance. The provenance role in adaptation to low water content was again significant and seedlings from the wetter provenance seem to be less adapted to low water contents.

Plant adaptations to water stress are complex and can be studied at various levels and scales, from cell to community performance. The nature of phenotypic plasticity influences the role of water stress in species acclimatization and adaptation, i.e. by

the inhibition or enhancement of certain functions to face environmental changes (Ch. 6). Depending on the intensity and duration of the stress period, the reduction of certain functions by, for e.g. transpiration and/or leaf area reduction or osmotic adjustments to increase water uptake can represent successful adaptation mechanisms to cope with drought.

It is challenging thought to point out a series of morphological or eco-physiological traits to assess plant performance under drought in the early establishment. From a broader perspective, if the plant is able to invest in water uptake (roots) create cover, grow and, at the same time, protect the photosynthetic apparatus from damage under drought conditions then it is likely that it will have a good degree of resilience to summer periods.

The non-destructive character and rapid response of eco-physiological measurements represent many advantages for monitoring plant performance, over the more labour, often destructive morphological measurements. Eco-physiological variables can be of particular interest to measure the effects and acclimatization to water stress in plants. However, the sensitivity of photosynthesis and other eco-physiological indicators to variable conditions, adds extra methodological challenges for their use under field conditions. Aspects such as time of the day should be carefully controlled when considering these measurements. In spite of this, the use of eco-physiological tools to monitor plant performance in ecological restoration actions is strongly advisable (Cooke and Suski 2008). *Arbutus unedo* species presented clear morphological responses to contrasting watering conditions after only a few months of growth. This result highlights the potential use of *A. unedo* as a model species to study drought adaptation in late successional Mediterranean species.

Finally, the studies developed here using Portuguese provenances distant from 200 - 600 km were a first step to elucidate the influence of seed sources from different climate regions in the germination and early establishment in Mediterranean shrub species. In order to infer on the consequences of the observed adaptations under climate changes, broader studies including more distant populations at the limits of species distribution would be desirable.

7.3. Management-related implications for ecological restoration actions

Planning of ecological restoration actions should consider future scenarios to ensure the provision by ecosystem goods and services under global change (Jackson and Hobbs 2009). The actual species distribution limits, for e.g., can be inadequate under future conditions. Furthermore, the inclusion of local adaptation measurements in climate change predictions could increase the reliability of future species' distribution scenarios (Morin and Thuiller 2009). Thus, to deepen the knowledge on the ability, degree, speed and persistence of different reproductive adaptation mechanisms in plants is fundamental to improve the reliability of ecological restoration actions under climate change scenarios.

Many external threats can act as hardening factors and the speed and degree of species adaptation could determine its survival and reproductive success (Chambel et al. 2005). Epigenetic modifications also play a crucial role in the adaptive evolution as stress exposure can lead to genome instability, which can have trans-generational effects (Hauser et al. 2011).

The knowledge on the combination of the persistence of a species through its reproductive and individual resilience will allow a better understanding of how climate changes can influence the persistence of different species in highly disturbed habitats.

The tendency for lower germination observed in the northern provenances (cooler and wetter areas; Ch. 3 and 4) together with a lower adaptation of the same provenance to drought in the early establishment (Ch. 6) could have implications in their successful use in ecological restoration actions in Mediterranean areas. This could be particularly important under extreme drought and or heat events, likely to happen according to the foreseen climate change scenarios (Vallejo et al. 2012). The degree and speed of adaptation of different provenances to extreme conditions should, as mentioned above, be disentangled through more detailed studies, given two or more generations breed under different conditions. By understanding species' acclimatization and adaptation mechanisms, reliable steps can be made in order to understand the effects of climate changes in species' distribution limits (Valladares et al. 2007) and more accurate ecological restoration-related decisions can be made.

The present study revealed important findings in provenance-related adaptation of Mediterranean species that seem to be related, at least partly to the climate of the

seed source. Further research steps should unravel the origin and nature of these differences, by studying in particular, acclimatization and adaptation in different generations exposed to extreme scenarios. In particular, the speed and persistence of the species' ability to cope with climate change should be tested disentangling if the observed responses are due to single generational short-term effects (direct environmental effects in seed development), two or more generational reversible effects (epigenetics) or long-term adaptation (genetics).

Both life-time and intergenerational effects should be further included in ecological restoration success-related predictions under climate changes conditions.

The study of possible genetic relatedness between the studied populations, disentangling the role of genetic, epigenetics and environment in seed development and germination could also use the application of modern techniques such as microsatellites and PCA. The high costs associated with the use of these techniques should however be considered when designing the experiments.

The knowledge on the performance, acclimatization and adaptation potential of different provenances, could finally support the selection of better drought-adapted provenances, reducing other ecological restoration related costs, such as post-seeding or planting irrigation (Chirino et al. 2010). The results of such studies should however be carefully interpreted as the introduction of alien genes is not exempted of causing negative impacts in the genetic expression of local populations (Mijnsbrugge et al. 2010). This could imply that the introduction of provenances that are better adapted to high disturbed conditions (as advised in Lesica and Allendorf 1999) could compromise the future resilience of plant communities. For that reason, the impacts of the promotion of population migrations and/or the use of commercial seeds of doubtful origin must be always carefully considered before planning ecological restoration actions (Tishew et al. 2011).

Regarding the complex task of assisting ecosystem resilience and evolution, interdisciplinary and integrated research aiming at supporting management should be a priority for ecological restoration. In the US, research projects applied to ecological restoration management are already being developed for species with high expression in dry lands (USDA, online). The inclusion of ecological research in forestry programs should also promote effective communication between researchers

and managers which should be further considered when planning ecological restoration actions in the Mediterranean.

7.4. Conclusions

The main conclusions of this thesis regarding the assessment of higher plant species suitability for ecological restoration following major disturbance are the subsequent:

- 1- The knowledge on seed dispersion phenology and dormancy type plus provenance could be used to create a conceptual framework for germination prediction in the Mediterranean. This management tool could improve the success of seed handling protocols and eventually contribute to decrease the use of commercial seeds in ecological restoration actions;
- 2- Both fire-prone (spring-summer dispersed) and mid to late succesional species (autumn-winter dispersed) revealed a significant role of provenance in germination response. Therefore the germination obtained for a certain population should not be generalized to the species level. The observed differences could be related with differences in dormancy intensity caused by different seed developmental conditions; however the role of epigenetic and/or genetic adaptations should be included in further research;
- 3- The present findings also revealed a tendency for climate-related adaptations during early development in *Arbutus unedo*, especially in relation to the wettest provenance. This species presented high potential to be used as a model species in further studies to predict eco-physiological responses to drought in late successional Mediterranean species;
- 4- There were significant associations between germination or seedling emergence and seed or mother-plant morphological characteristics; however these relations were not clear enough to contribute to the improvement of seed harvesting protocols. Further research on the mechanisms underlying this relations still need to be disentangled.

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