Università degli Studi di Cagliari

DOTTORATO DI RICERCA
BOTANICA AMBIENTALE E APPLICATA
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Germination niche of Sardinian endemic species in mountain riparian deciduous forests

BIO/03

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A sa famíglia e sa isposa mia
cun tottu su coru


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Summary

The Supramontes region and Gennargentu massif are two of the most interesting territories of Central Eastern Northern Sardinia. Riparian vegetation among mountainous waterways of these territories are mainly constituted by *Alnus glutinosa* with other associated *taxa* such as *Taxus baccata*, *Ilex aquifolium* and *Rhamnus persicifolia*. Rare and threatened Sardinian endemic species such as *Ribes multiflorum* subsp. *sandalioticum, Aquilegia barbaricina, Rhamnus persicifolia* and *Paeonia corsica* grow under and close to the canopy of such riparian woods.

Temperature is considered one of the major environmental factor governing seed germination in moist soil and, also is responsible for changes in dormancy states of seeds. Seed dormancy prevents germination in a specified period of time, under any combination of environmental factors that otherwise favour germination and it is mediated, at least in part, by the plant hormones abscisic acid and gibberellins. Dormancy can be broken by some environmental stimuli, such as a cold and/or warm stratification and gibberellic acid (GA) treatment. As dormancy is present throughout the higher plants in all major climatic regions, adaptation has resulted in divergent responses to the environment. Through this adaptation, germination is timed to avoid unfavourable weather for subsequent plant establishment and reproductive growth. In non-dormant seeds, the germination response to accumulated temperature could be modelled by a thermal time (θ) approach; in this model, seeds accumulate units of thermal time (°Cd) to germinate for a percentile g of the population. When seeds are subjected to temperatures (T) above a base temperature for germination (T_b), germination rate increases linearly with temperature to an optimum temperature (T_o), above which germination rate starts to decrease. In the sub-optimal range ($T_o - T_b$), germination occurs in the time t_g, when the thermal time accumulated has reached the critical value (θ_g) for a percentile g of the population, and can be described as $\theta_g = (T - T_b)t_g$.

In this work, the class of dormancy and thermal requirements for seed dormancy release and germination were investigate and/or confirmed for *R. persicifolia*, *A. barbaricina, P. corsica* and *R. multiflorum* subsp. *sandalioticum*; a thermal-time model, based on a soil heat sum approach, was developed in order to characterize its thermal niche for germination and predict its seed germination phenology in the field.

R. persicifolia showed physiological dormancy (PD), while the other three species highlighted morphophysiological dormancy (MPD); in particular, epicotyls MPD was found in *P. corsica* and confirmed in *R. multiflorum* subsp. *sandalioticum*. Thermal thresholds (T_b and θ_{50}) requirements of seed germination were identified for all these species; in addition, the thermal thresholds for embryo growth was detected for *A. barbaricina*. The soil heat sum model developed in this work may have applicability to predictions of *in situ* regeneration of other species growing on Mediterranean mountain waterways. This work could confirmed that the studied species, belonging to different families placed in different phylogenetic clades, could have experienced a convergent evolution on their seed morphology and type of seed dormancy, as a response to similar environmental and climatic conditions due by the same habitat and ecosystem.

Seed structure

Seeds are the dispersal and propagation units of the Spermatophyta (seed plants): Gymnosperms (conifers and related clades) and Angiosperms (flowering plants). A seed contains an embryo consisting of a simple axis (radicle/hypocotyl) with cotyledons (seed leaves) attached. Plants in the classes Monocotyledons and Dicotyledons of the flowering plants (angiosperms) have respectively one and two cotyledons, and some gymnosperms have several cotyledons. A typical seed includes three basic parts: an embryo, a supply of nutrients for the embryo, and a seed coat. In many angiosperms, the mature embryo is surrounded by the endosperm (endospermic seed), whereas in others the endosperm is absent or reduced to a few cell layers (non-endospermic seed; see Fig 1). The shape of embryos and their sizes are variable. In angiosperm seeds with a well-developed endosperm, the embryo occupies a smaller fraction of the seeds than in other non-endospermic species, where the growing embryo completely resorbs the endosperm into the cotyledons (e.g. many legumes). The exterior covering structure of the seed is called the seed coat or testa. (Bewley and Black, 1985). The endosperm in an angiosperm is the product of a fusion of a second male gamete and two nuclei in the embryo sac of the ovule and is therefore triploid. The perisperm in other angiosperms is from maternal origin (diploid), and the tissue surrounding the embryo in gymnosperms is also maternal (haploid). The testa in all seeds originates from the boundary layers of the ovule.

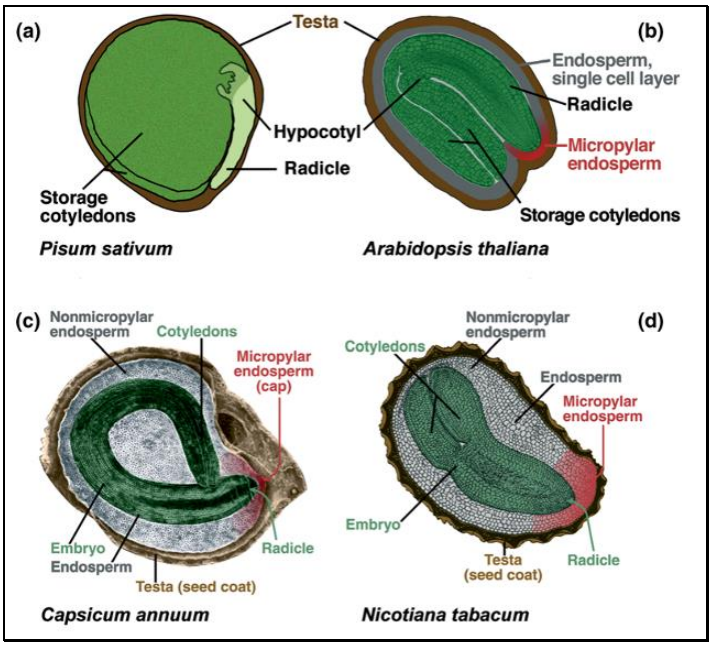

Figure 1 - Biodiversity of the structure of mature seeds of angiosperms. In several species the endosperm is completely obliterated during seed development and the nutrients are translocated to storage cotyledons. Mature seeds of (a) *Pisum sativum*, (b) *Arabidopsis thaliana*, (c) *Capsicum annuum* and (d) *Nicotina tabacum* (from Finch-Savage and Leubner-Metzger, 2006).

In his classic paper on the comparative internal morphology of seeds, Martin (1946) examined seeds of 1287 genera of plant, and created a system of seed classification in which divided seed embryos into three primary types: basal, peripheral, and axile. Basal is further divided into four subtypes and axile into seven (Fig. 2). In the "basal" seed types, embryos are usually small, non-peripheral, and restricted to the inferior half of the seed, except in the lateral type. The seeds are medium to large, with abundant endosperm, starchy or oily. The rudimentary and broad subtypes are found in the monocotyledons and dicotyledons; the capitate and lateral subtypes are typical of the monocotyledons (Fig. 2). In the "pheripheral" seed type, embryos are usually elongated and large. The embryo occupies one-quarter to three-fourths of the seed. It is partially contiguous to the seed coat and often curved, central or lateral, with cotyledons narrow or expanded. The endosperm or perisperm is starchy. As is typical in dicotyledons (Fig. 2). In the "axile" seed types, embryos range from small (occupying only part of the seed's lumen) to large (occupying the whole lumen), central (axile), straight, curved, coiled, bent, or folded. The endosperm can be oily or starchy. Found in gymnosperms, dicotyledons and monocotyledons (Fig. 2).

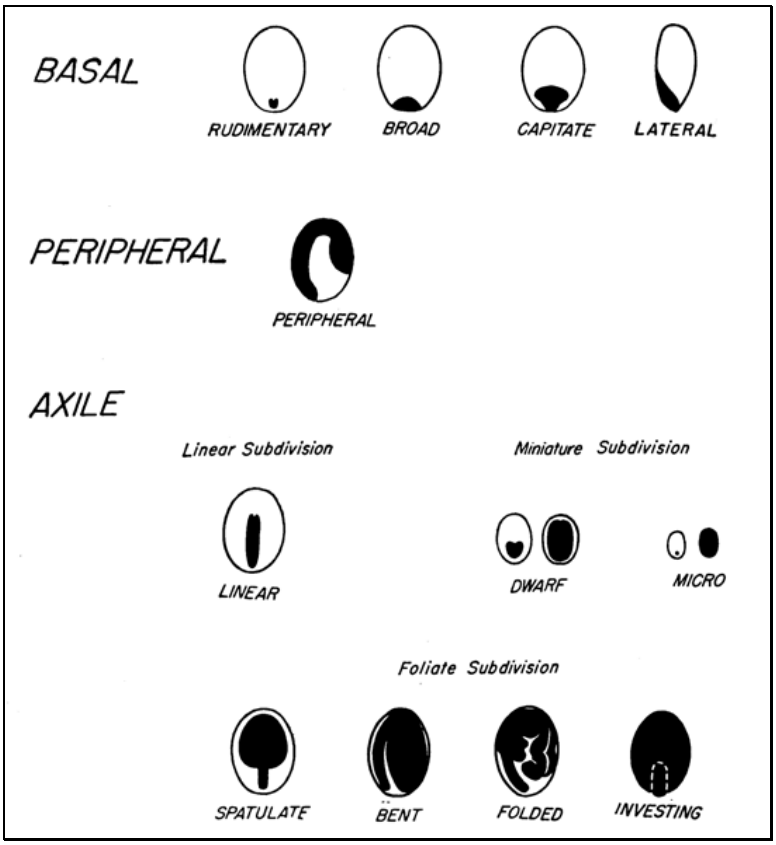

Figure 2 – The seed type identified by Martin (1946). Image from Martin (1946).

Recently, a revision of Martin's seed classification system, with particular reference to his dwarf-seed type was proposed by Baskin and Baskin (2007). According to this revision, the dwarf category has been removed and the micro category replaced by "undifferentiated" to reflect the state of the embryo in fresh seeds. Seed size is not longer a criterium for the assignment to a specific seed type; in particular, the "micro seed type" is replaced by a "undifferentiated seed type", indicating that the embryo in fresh seeds lacks organs and that most of these seeds are of micro-size, but there are also relatively large seeds whose embryos lack organs at the to time of maturity.

Seed germination

Germination involves the imbibition of water, a rapid increase in respiratory activity, the mobilization of nutrient reserves and the initiation of growth in the embryo. It is an irreversible process; once germination has started the embryo is committed irrevocably to growth or death (Fenner and Thompson, 2005). The visible sign that germination is

complete is usually the penetration of the structures surrounding the embryo by the radicle; the result is often called visible germination. Subsequent events, including the mobilization of the major storage reserves, are associated with growth of the seedling. Virtually all of the cellular and metabolic events that are known to occur before the completion of germination of non-dormant seeds also occur in imbibed dormant seeds; indeed, the metabolic activities of the latter are frequently only subtly different from those of the former. Hence, a dormant seed may achieve virtually all of the metabolic steps required to complete germination, yet for some unknown reason, the embryonic axis (i.e. radicle) fails to elongate (Bewley, 1997). Uptake of water by a mature dry seed is triphasic, with a rapid initial uptake (phase I) followed by a plateau phase (phase II). A further increase in water uptake occurs only after germination is completed, as the embryonic axes elongate. Because dormant seeds do not complete germination, they cannot enter phase III (Fig. 3).

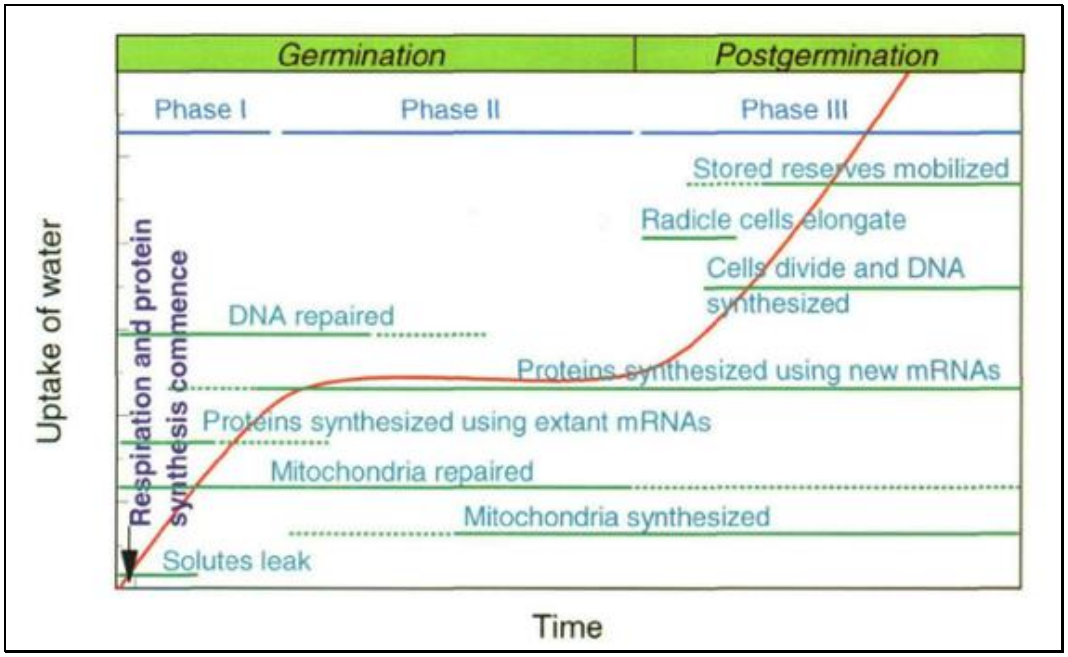

Figure 3 - Time course of major events associated with germination and subsequent post-germinative growth (from Bewley, 1997).

Among environmental factors, temperature is considered one of the major environmental factor governing seed germination in moist soil and, also is major responsible for changes in dormancy states of seeds (Baskin and Baskin, 1998). In a series of studies on geographical variation in germination temperature in Europe, P. A. Thompson (cited in Probert, 2000) concluded that both minimum and maximum

temperatures for germination varied consistently along a north-south gradient; both were lower in Mediterranean species compared with those from northern Europe. Indeed, some workers have identified a typical 'Mediterranean' germination syndrome, a key feature of which is a rather low optimal temperature (typically 5-15◦C) for germination (Thanos *et al.*, 1989). In northern Europe, the priority is to avoid germinating during or immediately before the severe winter, which often seems to be best arranged by needing relatively high temperatures for germination (Fenner and Thompson, 2005). Spring germination is typical of temperate and alpine plants, where in this season the germination prevails due to temperatures being too low to stimulate emergence following autumn dispersal or due to a requirement for cold stratification over winter (Baskin and Baskin, 1998; Walck *et al.*, 2011; Mondoni *et al.*, 2012).

Seed dormancy

Seed dormancy is an innate seed property that defines the environmental conditions in which the seed is able to germinate. It is determined by genetics with a substantial environmental influence (Donohue, 2005) which is mediated, at least in part, by the plant hormones abscisic acid and gibberellins. Not only is the dormancy status influenced by the seed maturation environment, it is also continuously changing with time following shedding in a manner determined by the ambient environment. As dormancy is present throughout the higher plants in all major climatic regions, adaptation has resulted in divergent responses to the environment. Through this adaptation, germination is timed to avoid unfavourable weather for subsequent plant establishment and reproductive growth (Finch-Savage and Leubner-Metzger, 2006). A more sophisticated and experimentally useful definition of dormancy has been proposed by Baskin and Baskin (2004): a dormant seed does not have the capacity to germinate in a specified period of time under any combination of normal physical environmental factors that are otherwise favourable for its germination, i.e. after the seed becomes non-dormant (Baskin and Baskin, 1998).

Nikolaeva (1977) devised a dormancy classification system reflecting the fact that dormancy is determined by both morphological and physiological properties of the seed. Based on this scheme, Baskin and Baskin (1998, 2004) have proposed a comprehensive classification system which includes different classes of seed dormancy: physiological (PD), morphological (MD), morphophysiological (MPD), physical (PY)

and combinational (PY + PD). The system of dormancy classification is hierarchical, and consists of five classes, further divided into levels and types:

1) Physiological dormancy (PD) is the most abundant form and is found in seeds of gymnosperms and all major angiosperm clades (Fig. 5). It is the most prevalent dormancy form in temperate seed banks and the most abundant dormancy class 'in the field'. PD is also the major form of dormancy in most seed model species 'in the laboratory'. PD can be divided into three levels: deep, intermediate and non-deep (Baskin and Baskin, 2004). Baskin and Baskin (2004) considered the ability of exogenous GA to overcome dormancy a decisive element in distinguishing among levels of PD in their dormancy classification systems. In fact, the balance between abscisic acid (ABA) and GA and sensitivity to these hormones regulates the onset, maintenance and termination of dormancy. ABA synthesis and signalling (GA catabolism) dominate the dormant state, whereas GA synthesis and signalling (ABA catabolism) dominate the transition to germination (Finch-Savage and Leubner-Metzger, 2006). Table 1 reported the characteristics of dormancy of the three PD levels (Baskin and Baskin, 2004).

Table 1- Characteristics of dormancy in seed with deep, intermediate and non-deep PD (from Baskin and Baskin, 2004).

Deep
Excised embryo produces abnormal seedling
GA does not promote germination
Seeds require *c.* 3–4 months of cold stratification to germinate
Intermediate
Excised embryo produces normal seedling
GA promotes germination in some (but not all) species
Seeds require 2–3 months of cold stratification for dormancy break
Dry storage can shorten the cold stratification period
Non-deep
Excised embryo produces normal seedling
GA promotes germination
Depending on species, cold (*c.* 0–10°C) or warm (≥15°C) stratification breaks dormancy
Seeds may after-ripen in dry storage
Scarification may promote germination

Based on patterns of change in physiological responses to temperature, five types of non-deep PD can be distinguished (Baskin and Baskin, 2004). Most seeds belong to type 1 or 2, in which the temperature range at which seed germination can occur increases gradually during the progression of non-deep dormancy release from low to higher (type 1; Fig. 4) or from high to lower temperature (type 2; Fig. 4). In type 3 (Fig.

4), temperature range for germination increases during dormancy release as a continuum from medium to high and low, while in type 4 and 5 (Fig. 4), dormant seed not germinate at any temperature, but non-dormant seeds germinate only at high temperature (type 4) or at low temperature (type 5).

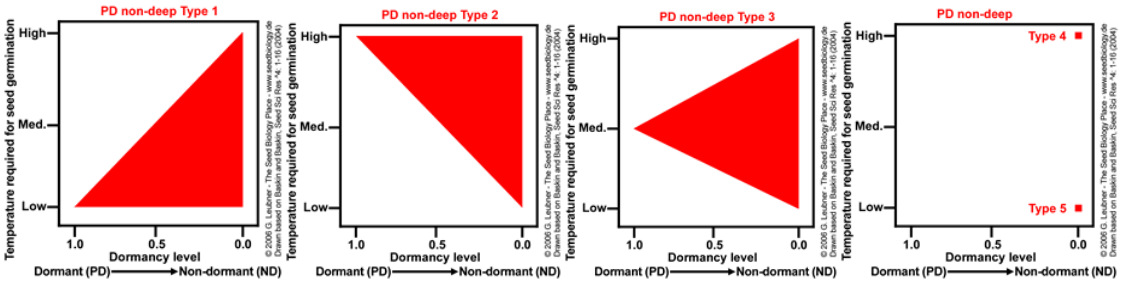

Figure 4 - Five types of non-deep Physiological seed dormancy (PD) according to Baskin and Baskin (2004). Modified from Leubner (2014; http://www.seedbiology.de).

2) In seeds with Morphological dormancy (MD), the embryo is either small (underdeveloped) and undifferentiated or small (underdeveloped) and differentiated, i.e. cotyledon(s) and radicle can be distinguished. In seeds with non-dormant, underdeveloped, differentiated embryos, the embryos simply need time to grow to full size and then germinate (radicle protrusion). The dormancy period is the time required for completion of embryo growth, after which the radicle emerges, and arbitrary cut-off time for assigning seeds to MD is about 30 days. Thus, seeds that take significantly longer than 30 days to germinate are considered to have MPD. Seeds with this latter kind of dormancy (MPD) have an underdeveloped embryo with a physiological component of dormancy. Thus, in order to germinate they require a dormancy-breaking pre-treatment. 3) In seeds with morphophysiological dormancy (MPD), embryo growth/radicle emergence requires a considerably longer period of time than in seeds with MD. There are eight known levels of MPD, based on the protocol for seed dormancy break and germination, and temperature or temperature sequence required to break them is show in Table 2.

Table 2 - Eight levels of morphophysiological dormancy (from Baskin and Baskin, 2004).

	Temperature required[a]		
Type of MPD[b]	To break seed dormancy	At time of embryo growth	GA_3 overcomes dormancy
Non-deep simple	W or C	W	+[c]
Intermediate simple	W + C	W	+
Deep simple	W + C	W	+/–
Deep simple epicotyl	W + C	W	+/–
Deep simple double	C + W + C	W	?
Non-deep complex	C	C	+
Intermediate complex	C	C	+
Deep complex	C	C	–

[a]W, warm stratification; C, cold stratification.
[b]MPD, morphophysiological dormancy.
[c] +, yes; +/–, yes/no; –, no.

In addition, Baskin *et al.* (2008) reported a new level of morphophysiological dormancy (i.e. non-deep simple epicotyl) in *Viburnum odoratissimum* seeds, and more recently Jayasuriya *et al.* (2010) reported a new kind of epicotyl PD in a tropical Fabaceae species, where the delay in plumule emergence is not correlated to an underdeveloped embryo.

4) Physical dormancy (PY) is caused by water impermeable layers of palisade cells in the seed or fruit coat that control water movement. Mechanical or chemical scarification can break PY dormancy (Baskin and Baskin, 1998); 5) Combinational dormancy (PY + PD) is evident in seeds with water impermeable coats (as in PY) combined with physiological embryo dormancy (Baskin and Baskin, 2004).

Evolutionary trends of seeds structure and dormancy

The most obvious morphological difference in mature angiosperm seeds is their 'embryo to seed' size ratios resulting from the extent to which the endosperm is obliterated during seed development by incorporating the nutrients into the storage cotyledons. Based on the internal morphology of mature seeds, Martin (1946) defined seed types with distinct embryo to endosperm ratios, arranged them in a seed phylogenetic tree and proposed evolutionary seed trends (see Fig. 5 that shows the distribution of Martin's seed types in the modern angiosperm phylogenetic tree). Forbis *et al.* (2002) calculated 'embryo to seed' (E:S) values for the different seed types. These E:S values show a clear trend increasing from low E:S values up the phylogenetic tree to high E:S values. Finch-Savage and Leubner-Metzger (2006) reported their general evolutionary seed trends. Their supported that: (a) in mature seeds of primitive angiosperms a small embryo is embedded in abundant endosperm tissue and this

characteristics prevail among basal angiosperms; (b) the general evolutionary trend within the higher angiosperms is via the linear axile seed type (embryo linear axile and developed, endosperm abundance medium to high) towards the foliate axile seed types (embryo foliate axile and developed, often storage cotyledons, endosperm abundance low or endosperm obliterated) with storage cotyledons. In addition, embryo dominance and endosperm reduction lead via the foliate axile seed type with spatulate axile embryo to the diverted seed types bent, folded and investing axile embryo; (c) the general seed trends there are clade-specific seed type differences, with some exceptions; (d) a small embryo is also found in primitive gymnosperms and an increase in the E:S values is also evident within the gymnosperms (Fig. 5).

An increase in relative embryo size appears therefore to be a general evolutionary trend within the angiosperms and the gymnosperms. MD and MPD are present in basal type seeds, but can also be typical of some specialized species (Fig. 5). MD is thought to be the ancestral dormancy type among seed plants and is the most primitive dormancy class. MD and MPD are typical in primitive angiosperms and gymnosperms (Fig. 5). PD and ND are distributed over the entire phylogenetic tree. The most phylogenetically restricted and derived dormancy classes are PY and PY + PD (Fig. 5).

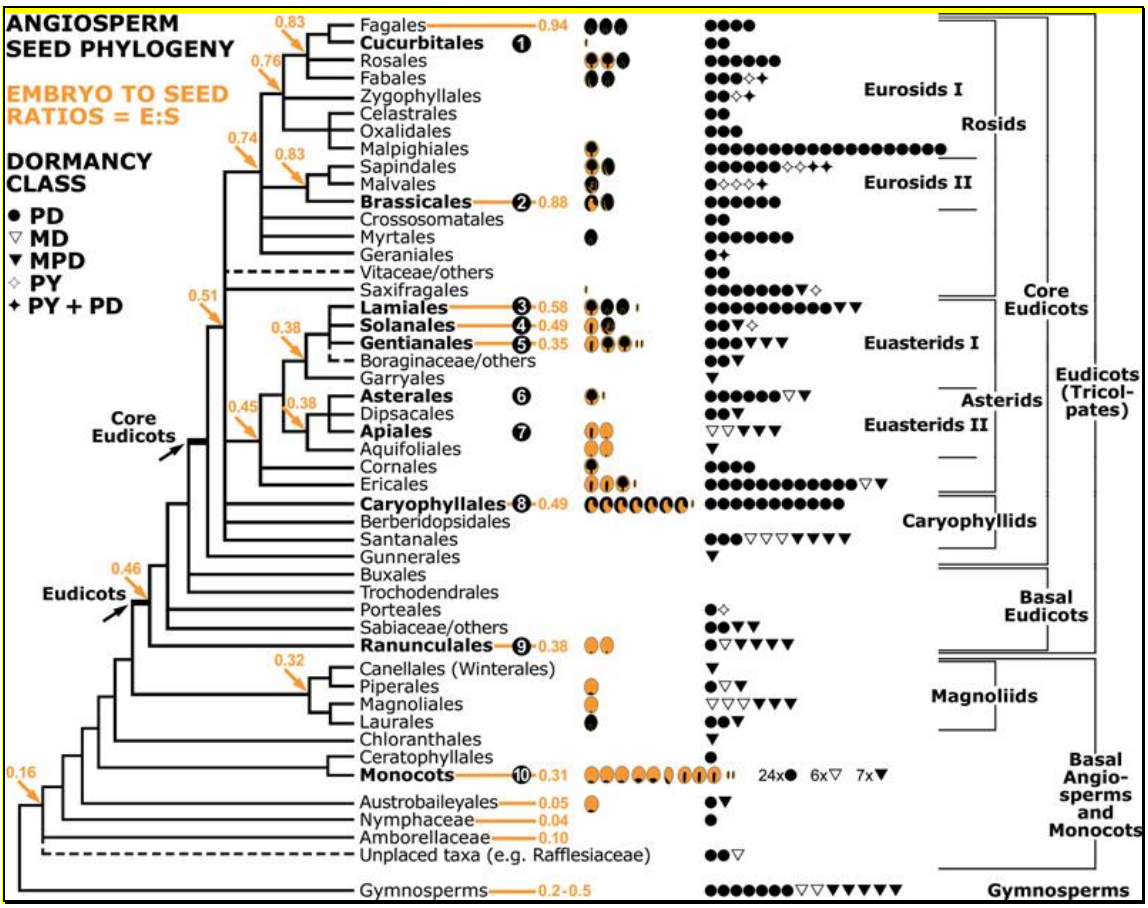

Figure 5 - Angiosperm seed evolution depicted in a phylogenetic tree constructed by the Angiosperm Phylogeny Group II (2003). Image from Finch-Savage and Leubner-Metzger (2006).

Thermal time model

Three cardinal temperatures (base, optimum and ceiling) describe the range of temperature over which seeds of a particular species can germinate (Bewley and Black, 1994). The minimum or base temperature (T_b) is the lowest of temperature at which germination can occur, while the optimum temperature (T_o) is the temperature at which germination is most rapid, and the maximum or ceiling temperature (T_c) is the highest temperature at which seeds can germinate (Fig. 6).

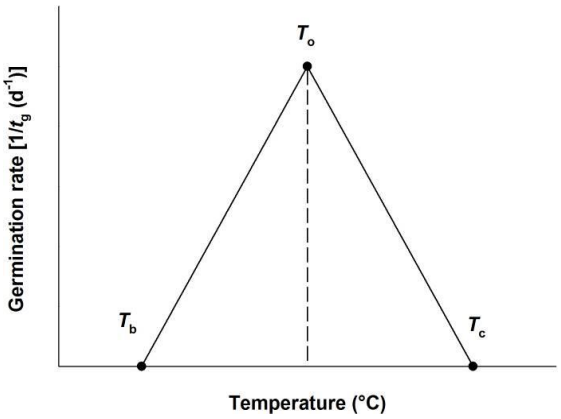

Figure 6 - Schematic illustration of cardinal temperatures: base temperature (T_b), optimum temperature (T_o) and ceiling temperature (T_c).

Many mathematical models have been developed to describe germination patterns in response to temperature (e.g, Garcia-Huidobro *et al.*, 1982; Covell *et al.*, 1986; Ellis *et al.*, 1986; Pritchard and Manger, 1990; Hardegree, 2006). In these models, seeds accumulate units of thermal time (°Cd) to germinate for a percentile *g* of the population. When seeds are subjected to temperatures (T) above a base temperature for germination (T_b), germination rate increases linearly with temperature to an optimum temperature (T_o), above which germination rate starts to decrease (Garcia-Huidobro *et al.*, 1982). In the sub-optimal range ($T_o - T_b$), germination occurs in the time t_g, when the thermal time accumulated has reached the critical value (θ_g) for a percentile *g* of the population. The germination response of the seeds to constant temperature in the suboptimal range has been modelled using the equation (Garcia-Huidobro *et al.*, 1982):

$$1/t_g \text{ (days}^{-1}) = (T_g - T_b) / \theta$$

where $1/t_g$ is the rate of germination of different percentiles of a seed population at sub-optimal constant temperatures, t_g is the time taken for cumulative germination to reach percentile *g*, T_g is the temperature (°C), T_b is the base temperature for subset *g* of the population, and θ is the thermal time (°Cd above T_b) required for cumulative germination to achieve percentile *g*.

Linear regression has been used to express probit(*g*) as a function of thermal time (θ_g) and the form of cumulative germination response of seeds has been described by the equation (Covell *et al.*, 1986):

$$\text{probit}(g) = K + \theta_g / \sigma,$$

where K is an intercept constant when θ_g is zero, θ_g may be normal or log-normal distributed (and the best model evaluated on the basis of the r^2 values; Hardegree, 2006), and σ is the standard deviation of the response to θ_g (i.e. the reciprocal of the slope), and represents the sensitivity of the population to θ_g (Covell *et al.*, 1986).

The models have been used to predict seed germination of non-dormant seeds (and subsequent seedling emergence) in the field from these simple parameters describing the seed response to ambient soil conditions (e.g. Finch-Savage and Phelps, 1993; Hardegree and Van Vactor, 2000; Steadman *et al.*, 2003; Chantre *et al.*, 2009), and more recently, to assess the impact of different simulated climate change scenarios on seed dormancy release and germination timing (Orrù *et al.*, 2012).

Mediterranean climate

The Mediterranean climate is characterized by its seasonality in temperature and rainfall, which leads to a hot drought in summer and a cool wet winter (Joffre *et al.*, 1999). This peculiarity has important implications for plant germination physiology, since dry summer conditions limit water availability and thus germination and growth, while cool winter temperatures can limit germination during the season with high water availability (Rundel, 1996). Under a Mediterranean climate, plant growth and reproduction must occur in a window of favourable conditions that may vary in length and in which environmental cues and constraints play a central role (Debussche *et al.*, 2004). Maximum germinability of Mediterranean species occurs at temperatures between 15 and 20°C, and usually decreases above 20°C, but remains high at temperatures below 15°C (Thanos *et al.*, 1995; Galmés *et al.*, 2006; Luna *et al.*, 2012). Among species there are considerable variation in the germination-temperature relationship and, while some species have a narrow range of temperature requirements, others exhibit reduced variability when tested across a range of temperatures. The germination temperature-niche may interact with other factors controlling germination, such as soil moisture or dormancy break. Seeds of some species may remain dormant until they are exposed to cold period and after germinate, but in other species the cold period can impose dormancy (Baskin and Baskin, 1998; Probert, 2000).

Mediterranean basin

The Mediterranean basin has been recognised as one of the 34 most important "biodiversity hotspots" (Fig. 7), considering its high number of endemic plant species (Mittermeier *et al.*, 2004). This area not only constitutes a refuge for many relic species, but the relatively short distance of many islands and peninsulas promotes floristic exchanges and active plant speciation. The western Mediterranean basis includes the Tyrrhenian islands (Balearic Islands, Corsica, Sicily and Sardinia).

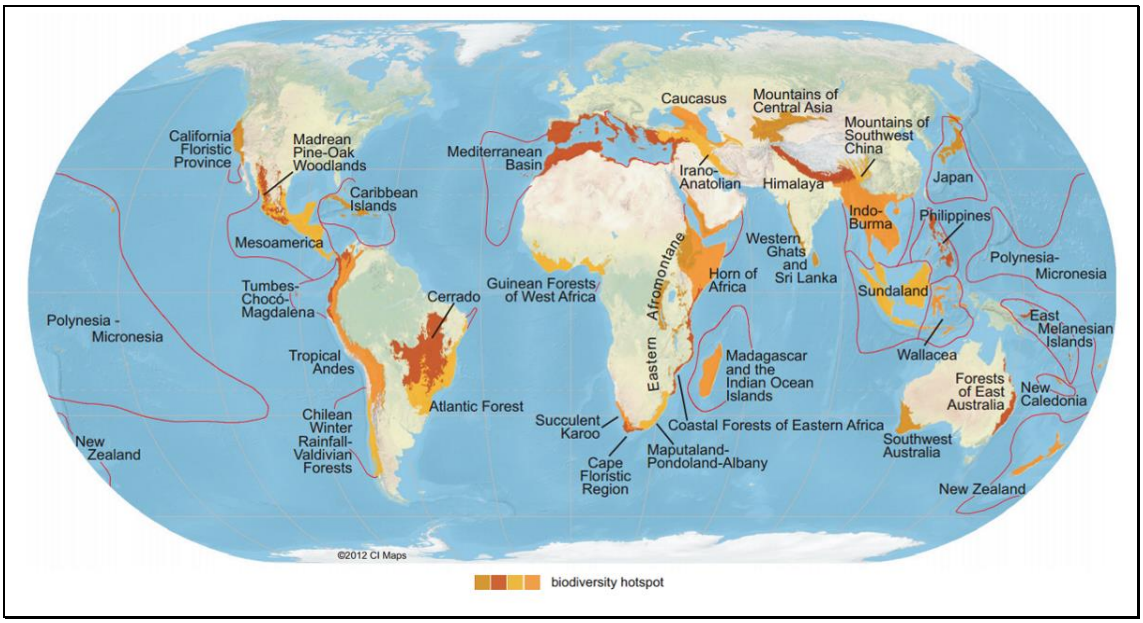

Figure 7 - Biodiversity Hotspots Map from http://www.conservation.org.

Sardinia (Fig. 8), with its 24,090 km2, is the second-largest island in the Mediterranean Sea. The prolonged isolation and high geological diversity created a wide range of habitats rich in endemic species, particularly on its mountain massifs, where the insularity is strengthened by the altitude and diversity of terrains (Médail and Quézel, 1997). The Sardinian flora consists of 2408 taxa including 2295 species (Conti et al., 2005) 168 of which are exclusive endemics (Bacchetta et al., 2012). Médail and Diadema (2009) identified the Central- Northern Sardinia as one of the 52 putative floristic refugia within the Mediterranean, i.e. places facilitating the long-term persistence of a species (one or more glacial-interglacial cycles) or of one or more of its meta-populations in a well-defined geographical area (e.g. mountain range, gorge). This region represents also a Southern European refugium (sensu Tzedakis et al., 2002) for

some temperate tree species, as detected for the Supramontes biogeographic sector (CE-Sardinia; Fenu et al., 2010). The Supramontes region and Gennargentu massif are two of the most interesting territories of Central Eastern Northern Sardinia (Fig. 7). Cañadas et al. (2014), on the basis of the distribution of endemic-vascular-plant richness, identified the Supramontes and Gennargentu as micro-hotspots in Sardinian region; these micro-hotspots hosting more than the 20% of the 171 Sardinian endemic taxa.

Figure 8 – Sardinia (yellow square) with the Gennargentu (red square) and Supramontes massif (orange square).

Riparian vegetation among mountainous waterways of these territories are mainly constituted by *Alnus glutinosa* (L.) Gaertn. with other associated *taxa* such as *Taxus baccata* L., *Ilex aquifolium* L. and *Rhamnus persicifolia* Moris. Threatened Sardinian endemic species such as *Ribes multiflorum* Kit *ex* Roem *et* Schult. subsp. *sandalioticum* Arrigoni, *Aquilegia barbaricina* Arrigoni *et* E.Nardi, *Rhamnus persicifolia* Moris and *Paeonia corsica* Sieber ex Tausch (Fig. 9) grow under and close to the canopy of such riparian woods.

Figure 9 - Sardinian endemic species.

Climate change

The Intergovernmental Panel on Climate Change (IPCC) reports the definition of "Climate change" as a change in the state of the climate that can be identified (e.g. using statistical tests) by changes in the mean and/or the variability of its properties, and that persists for an extended period, typically decades or longer. It refers to any change in climate over time, whether due to natural variability or as a result of human activity (IPCC, 2007). IPCC has predicted temperature increases of approx. 2 – 4°C by 2090 – 2099 according to different emission scenarios and in particular, in the Mediterranean region, a declining trend of precipitation was observed from 1900 to 2005 (IPCC, 2007). Large increases in temperature have been predicted and reported for the Mediterranean mountain ranges (Bravo *et al.*, 2008). Predictions have been grouped into four families of scenarios (A1, A2, B1 and B2) that explore alternative development pathways, covering a wide range of demographic, economic and technological driving forces and resulting greenhouse gas emissions (IPCC, 2007). The

A1 storyline assumes a world of very rapid economic growth, a global population that peaks in mid-century and rapid introduction of new and more efficient technologies; B1 describes a convergent world, with the same global population as A1, but with more rapid changes in economic structures toward a service and information economy; B2 describes a world with intermediate population and economic growth, emphasising local solutions to economic, social, and environmental sustainability; A2 describes a very heterogeneous world with high population growth, slow economic development and slow technological change. Climate-change impacts on biodiversity, both positive and negative, are already manifest in recent widespread shifts in species ranges and phenological responses. The climate in high mountains is warming up at an exceptionally high rate (Bravo *et al.*, 2008). In response to the climate change, plants can adapt to the new environmental conditions or, when possible, migrate to track their climatic niches (Meineri *et al.*, 2013).

Aims

The aim of this work is to:

i. investigate the seed dormancy breaking treatments and germination requirements in freshly matured seeds of *R. persicifolia*, *A. barbaricina*, *P. corsica*, and *R. multiflorum* subsp. *sandalioticum*, both in controlled condition and in the field.

ii. evaluate if these endemic species, mainly growing among mountain Mediterranean streams, show the same patterns of seed dormancy and germination, and if these species are adapted to these particular habitats and ecological conditions.

iii. investigate the thermal requirements for seed dormancy release and germination of the targeted *taxa*, and develop a thermal-time model based on a soil heat sum approach to predict their seed germination in the field.

iv. evaluate if the projected climate change scenarios likely threaten the natural regeneration of mountain species.

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Chapter I - Thermal niche for in situ seed germination by Mediterranean mountain streams: model prediction and validation for Rhamnus persicifolia seeds[1]

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Abstract

- *Background and Aims*: Mediterranean mountains species face exacting ecological conditions of rainy, cold winters and arid, hot summers, which affects seed germination phenology. In this study, a soil heat sum model was used to predict field emergence of *Rhamnus persicifolia,* an endemic tree species living at the edge of mountain streams of central eastern Sardinia.
- *Methods*: Seeds were incubated in the light at a range of temperatures (10 to 25 and 25/10°C) after different periods (up to 3 months) of cold stratification at 5°C. Base temperatures (T_b), and thermal times for 50% of germination (θ_{50}) were calculated. Seeds were also buried in the soil in two natural populations (Rio Correboi and Rio Olai), both underneath and outside the tree canopy, and exhumed at regular intervals. Soil temperatures were recorded using data loggers and soil heat sum (°Cd) calculated on the basis of the estimated T_b and soil temperatures.
- *Key Results*: Cold stratification released physiological dormancy (PD), increasing final germination and widening the range of germination temperatures, indicative of a type 2 non deep PD. T_b was reduced from 10.5°C for non-stratified seeds to 2.7°C for 3-months cold stratified seeds. The best thermal time model was obtained by fitting probit germination against log °Cd. θ_{50} was 2.6 log °Cd for untreated seeds and 2.17 - 2.19 log °Cd for stratified seeds. When θ_{50} values were integrated with soil heat sum estimates, field emergence was predicted from March to April and confirmed through field observations.
- *Conclusions*: T_b and θ_{50} values facilitated model development of thermal niche for *in situ* germination of *R. persicifolia*. These experimental approaches may be applied to model the natural regeneration patterns of other species growing on Mediterranean mountain waterways and of physiologically dormant species, with overwintering cold stratification requirement and spring germination.

Keywords: base temperature, climate change, cold stratification, physiological dormancy, Rhamnaceae, *Rhamnus persicifolia,* soil heat sum, thermal time.

Introduction

Seed dormancy prevents germination in a specified period of time, under any combination of environmental factors that otherwise favour germination (Baskin and Baskin, 2004). Thus, dormancy is an adaptive trait that optimizes the distribution of germination over time in a population of seeds (Copete *et al.*, 2011). In seasonal climates, temperature is usually the main environmental factor governing seed germination in moist soil (Fenner and Thompson, 2005). Seeds of many temperate plant species are dormant at the time of dispersal, and specific temperature requirements must be met before dormancy is lost and germination is possible (Baskin and Baskin, 1998). Depending on the species and timing of dispersal, seeds may experience a warm period before autumn and winter begin, or be subjected to cold stratification during winter immediately after autumn shedding (Baskin and Baskin, 1989; Noronha *et al.*, 1997). The requirement for chilling, widespread amongst temperate species, represents a natural mechanism which ensures that germination occurs in the spring (Probert, 2000). During exposure to low temperatures, the range of temperatures over which seeds will germinate, as well as germination percentages, increases (Baskin and Baskin, 1988).

The Mediterranean climate is characterized by its seasonality in temperature and precipitation, which leads to a hot drought in summer and a cool wet winter (Joffre *et al.*, 1999). This peculiarity has important implications for plant germination physiology, since dry summer conditions limit water availability and thus germination and growth, while cool winter temperatures can limit germination during the season with high water availability (Rundel, 1996).

The canopies of woody plants modify the microclimate beneath and around them through interception of precipitation and by shading, which influence maximum soil temperature and the amount of soil moisture available to plants (Breshears *et al.*, 1998). As the course of action and relative importance of factors regulating germination in the laboratory may be quite different from those occurring under field conditions (Thompson, 1973), linkage between field, garden and laboratory studies is crucial (Brändel and Schütz, 2005).

As reproduction niche and reproductive success are related to temperature, all aspects of the plant reproductive cycle are potentially sensitive to climate change (Bykova *et al.*, 2012). The Intergovernmental Panel on Climate Change (IPCC) has predicted temperature increases of approx. 2 – 4°C by 2090 – 2099 and in particular, in

the Mediterranean region, a declining trend of precipitation was observed from 1900 to 2005 (IPCC, 2007). In response to the climate change, plants can adapt to the new environmental conditions or, when possible, migrate to track their climatic niches (Meineri *et al.*, 2013).

In non-dormant seeds, the germination response to accumulated temperature has been modelled by a thermal time (θ) approach (Garcia-Huidobro *et al.*, 1982; Covell *et al.*, 1986; Ellis *et al.*, 1986; Ellis *et al.*, 1987; Pritchard and Manger, 1990; Hardegree, 2006). In this model, seeds accumulate units of thermal time (°Cd) to germinate for a percentile g of the population. When seeds are subjected to temperatures (T) above a base temperature for germination (T_b), germination rate increases linearly with temperature to an optimum temperature (T_o), above which germination rate starts to decrease (Garcia-Huidobro *et al.*, 1982). Thus, in this sub-optimal range ($T_o - T_b$), germination occurs in the time t_g, when the thermal time accumulated has reached the critical value (θ_g) for a percentile g of the population, and can be described as $\theta_g = (T - T_b)t_g$.

Intra-specific variation in T_b among populations of the same species may be due to different environmental conditions during seed development (Daws *et al.*, 2004). However, T_b has been found to change also with dormancy status. In particular, Pritchard *et al.* (1999) found that T_b decreased by 1°C every 6 days of pre-chilling at 6°C, in *Aesculus hippocastanum* L. seeds. Thus seed dormancy release in this species could be described simply in terms of T_b reduction, gradually allowing germination to occur at progressively lower temperatures (Pritchard *et al.*, 1999). In addition, seed germination may be predicted in relation to thermal time accumulation (heat sum, °Cd) above a gradually reducing T_b (Steadman and Pritchard, 2004). This approach has been used to predict seed germination in the field (i.e. Hardegree and Van Vactor, 2000; Steadman *et al.*, 2003; Chantre *et al.*, 2009) and, more recently, to assess the impact of different simulated climate change scenarios on seed dormancy release and germination timing in *Vitis vinifera* L. subsp. *sylvestris* Hegi (Orrù *et al.*, 2012).

Sardinian massifs represent a Southern European refugium for some temperate tree species *sensu* Tzedakis *et al.* (2002). In this region, vegetation among mountain waterways is mainly constituted by *Alnus glutinosa* (L.) Gaertn. woods, where also the rare Sardinian endemic *Rhamnus persicifolia* Moris may be found. Seeds of the Rhamnaceae have an investing embryo (Martin, 1946) and can be non-dormant or, following the dormancy classification system (Baskin and Baskin, 1998; Baskin and

Baskin, 2004), show physiological (PD), physical (PY) or combinational (physical and physiological; PY+PD) dormancy. PY is the most represented class in this family (61% of the investigated species), followed by PY+PD (22%), PD (12%) and ND (6%; Walck *et al.*, 2012). Mattana *et al.* (2009) reported that germination of *R. persicifolia* seeds could be achieved, without any scarification, at warm temperatures (≥ 20°C), excluding the presence of PY. Whilst there was no obligate requirement for alternating temperature or light, pre-chilling had a positive effect on germination rate, reducing T_{50} by more than 50%, and indicating the presence of PD in this species. However, the effect of pre-chilling on seed germination over a wide range of temperatures, and the identification of the type of PD according to the seed dormancy classification system (Baskin and Baskin, 2004), remain to be investigated.

The aims of this work were to (1) investigate the thermal requirements for seed dormancy release and germination of the rare *R. persicifolia* and (2) develop a thermal-time model, based on a soil heat sum approach, in order to characterize its thermal niche for seed germination and predict its seed germination phenology in the field.

Material and Methods

Study species

Rhamnus persicifolia is a small dioecious tree or shrub. It is closely related to *R. cathartica*, but with elliptic-lanceolate leaves and reddish ripe drupes. It is endemic to Central-Eastern Sardinia (Italy) occurring at 600 - 1500 m a.s.l. on both limestone and siliceous substrata. This species grows in scattered groups or as single trees, in riparian woods or hygrophilous scrubs along mountainous waterways (Mattana *et al.*, 2009). *R. persicifolia* is included in the Italian Red Book as vulnerable (Conti *et al.*, 1992; Conti *et al.*, 1997), because of its narrow distribution and population decline, induced by human activities and by climate change (Arrigoni, 1977). To date, only six populations are known; half of these are threatened by low plant numbers or unbalanced sex ratio (Bacchetta *et al.*, 2011).

Seed lot details

Fruits of *R. persicifolia* were collected directly from 15 plants on 16 September 2011 along the Rio Correboi (Villagrande Strisaili, OG) and from five plants on 30 September 2011 along the Rio Olai (Orgosolo, NU) streams in the Central-Eastern Sardinia (see Table 1). The low number of sampled plants was due to the few female individuals found on these two populations (see Bacchetta *et al.*, 2011). Seeds were immediately separated from the pulp by rubbing the fruits through sieves under running water. The cleaned seeds were then spread out and left to dry at room temperature, until the experiments started as specified below.

Table 1 - Locations, habitat characteristics and dates of experimental trials carried out in each site (Rio Correboi: RC; Rio Olai: RO) of the two natural populations of *R. persicifolia.*

Population	Soil substrate type	Experimental sites	Habitat	Altitude (m a.s.l.)	Aspect	Date of field sowing	Dates of exhumation and days after sowing
Rio Correboi (Villagrande Strisaili, OG), RC	Metamorphytes	RC1 IN	Riparian wood with *Alnus glutinosa* – Mantle shrubs with *Rubus ulmifolius*	1209	0	30/09/11	26/04/12 (209 days) 25/06/12 (269 days)
		RC1 OUT	Open grassland of *Carici-Genistetea lobelioidis.*		NE		
		RC2 IN	Riparian wood with *A. glutinosa* – Mantle shrubs with *R. ulmifolius*	1267	0	30/09/11	09/12/11 (70 days) 29/03/12 (181 days) 26/04/12 (209 days) 25/06/12 (269 days)
		RC2 OUT	Open grassland of *Carici-Genistetea lobelioidis.*		NE		
		RC3 IN	Shady rocky outcrop with *Ribes multiflorum* subsp. *sandalioticum* and *Rubus ulmifolius*	1347	NE	30/09/11	26/04/12 (209 days) 25/06/12 (269 days)
		RC3 OUT	Open grassland of *Carici-Genistetea lobelioidis.*		NE		
Rio Olai (Orgosolo, NU), RO	Metamorphytes	RO IN	Riparian wood with *A. glutinosa* – Mantle shrubs with *R. ulmifolius*.	970	0	05/10/11	09/12/11 (65 days) 29/03/12 (176 days) 26/04/12 (204 days) 25/06/12 (264 days)
		RO OUT	Open grassland of *Carici-Genistetea lobelioidis*.		0		

For each experimental site, IN and OUT differentiate between underneath and outside the canopy, respectively.

Germination tests under controlled conditions

For the Rio Correboi provenance collection, three replicates of 20 seeds were sown on the surface of 1% agar water in 90 mm diameter plastic Petri dishes and incubated in the light (12 h light / 12 h darkness), for 1 to 4 months at a range of constant temperatures (10, 15, 20, and 25°C) and at an alternating temperature regime (25/10°C). In the alternating temperature regime, the 12 h light period coincided with the elevated temperature period. At the same time, three different cold stratification periods were started (5°C in 1% agar water in 90 mm diameter plastic Petri dishes for 1, 2 and 3 months: C1, C2 and C3 treatments, respectively) and, at the end of each pre-treatment, seeds were incubated, as above detailed.

Due to the low availability of seeds collected in the Rio Olai (see Table 1), these seeds were only stratified for 3 months at 5°C and then incubated at 25°C (12 h light / 12 h darkness). These conditions were chosen on the basis of earlier findings (Mattana *et al.*, 2009).

Germination was defined as visible radicle emergence (> 1 mm). Germinated seeds were scored three times a week. At the end of the germination tests, when no additional germination had occurred for two weeks, a cut test was carried out to determine the firmness of remaining seeds and the number of empty seeds. Firm seeds were considered to be viable. This methodology was chosen on the basis of previous findings on seeds of this species, which highlighted a very high seed viability, with 100% of filled seeds staining uniformly red in 1% solution of 2,3,5-triphenyl-tetrazolium chloride (Mattana *et al.*, 2009).

Field experiments

Within 15-20 days of collecting, seeds were placed in fine-mesh polyester envelopes (3 replicates of 25 seeds) and buried in soil at a depth of 2-3 cm. Envelopes were buried both underneath (IN) and outside (OUT) the canopy, with a distance between them of ca. 6 m, at each experimental site of the 2 natural populations, for a total of 6 experimental sites for Rio Correboi (RC), in order to cover the whole altitudinal range of the population and two for Rio Olai (RO; Table 1). Envelopes buried in experimental sites RC2 and RO were exhumed at about 3-months intervals from September 2011 to June 2012 (with an intermediate exhumation also in April 2012; Table 1). Alternatively,

those buried in experimental sites RC1 and RC3 were exhumed only in April and June 2012. Retrieved envelopes were analysed in the laboratory, where they were washed under running water and opened. The number of germinated seeds was recorded, and a cut test carried out to check the viability of any remaining non-germinated seeds, as described above.

Soil temperatures at the level of the envelopes were recorded IN and OUT the canopy at 90-minutes intervals, using data loggers (TidbiT® v2 Temp logger, Onset Computer Corporation, Cape Cod, Massachusetts, U.S.).

Data analysis

The final germination percentage was calculated as the mean of the three replicates ± standard deviation (± 1SD), on the basis of the total number of filled seeds. Generalized Linear Models (GLMs) were used to compare: 1) final germination percentages and T_b achieved under controlled conditions for seed collected in Rio Correboi, followed by a *post-hoc* pairwise comparisons *t*-test (with Bonferroni adjustment), and 2) the field germination percentages at each experimental site (RC1, RC2, RC3 and RO) at different exhumation dates (Dec. 2011, Mar. 2012, Apr. 12 and Jun. 12), both underneath (IN) and outside (OUT) the canopy (see Table 1). GLMs, with a logit link function and quasibinomial error structure, were used when analysing germination percentages, while a GLM with a log link function and quasipoisson error structure was used for analysing T_b values. Quasibinomial and quasipoisson error structures and *F* tests with an empirical scale parameter instead of chi-squared on the subsequent ANOVA were used in order to overcome residual overdispersion (Crawley, 2007).

Thermal time analyses were carried out for Rio Correboi seeds germinating at constant temperatures for untreated seeds (0, control) and after each cold pre-treatment (C1, C2 and C3). Estimates of time (t_g, days) taken for cumulative germination to reach different percentiles (g) for successive increments of 10% germination were interpolated from the germination progress curves (Covell *et al.*, 1986). The germination rate ($1/t_g$) was regressed, using a linear model, as a function of temperature according to the following equation (Garcia-Huidobro *et al.*, 1982):

$$1/t_g \text{ (days}^{-1}) = (T_g - T_b) / \theta \qquad \text{(Eq. 1)}$$

An average (± 1SD) of the *x*-intercept among percentiles was calculated for the sub-optimal temperature range (10–20°C) to establish the T_b for each treatment (Ellis *et al.*, 1986; Pritchard and Manger, 1990). Linear regression equations were then recalculated for each percentile, but constrained to pass through T_b (Hardegree, 2006). A comparison of regressions was then made between this model and one in which the T_b were allowed to vary for all the percentiles and the best estimate was considered to be that which resulted in the smallest residual variance (Covell *et al.*, 1986). Thermal time (θ, °Cd) estimates for each treatment were then calculated separately as the inverse of the sub-optimal regression equations (Covell *et al.*, 1986; see Eq. 1).

T_b values were fitted as a function of the length of the stratification period using a linear model. Generally, θ did not accumulate during pre-treatments because the stratification temperature (5°C) was lower than T_b. However, in seeds stratified at 5°C for 120 days (C3), T_b reduced during stratification to below the stratification temperature itself. Using the relationship between rate of decline of T_b and temperature, and assuming that the rate of reduction of T_b continued unchanged, according to Steadman and Pritchard (2004), θ accumulated during the C3 stratification phase was calculated.

Germination percentages were transformed to probits using tabular data from Finney (1971). Linear regression was used to express probit(g) as a function of thermal time (θ_g) and the form of cumulative germination response of seeds described by the equation (Covell *et al.*, 1986):

$$\text{probit}(g) = K + \theta_g / \sigma \quad \text{(Eq. 2)},$$

where K is an intercept constant when θ_g is zero, θ_g may be normal or log-normal distributed (and the best model evaluated on the basis of the r^2 values; Hardegree, 2006), and σ is the standard deviation of the response to θ_g (i.e. the reciprocal of the slope), and represents the sensitivity of the population to θ_g (Covell *et al.*, 1986). Thus the flatter the slope of the fitted line, the greater the variation in response to thermal time between individual seeds (Daws *et al.*, 2004).

A heat sum approach was used to predict seed germination in the field, according to Orrù *et al.* (2012). These authors used environmental temperatures of the natural populations above T_b to assess the temperature accumulation till the achievement of θ_{50} (Orrù *et al.*, 2012). In this study, soil heat sum was calculated,

starting from the date of sowing, according to the following equation, modified from Daws and Jensen (2011):

$$\textit{Soil heat sum}\ (°\text{Cd}) = \{\sum [(T_S - T_b) \times t]\}/18 \qquad \text{(Eq. 3)},$$

where T_S is the temperature at each logging interval recorded by data loggers, T_b is the base temperature for germination calculated day by day, according to the length of stratification experienced in the field, t is the length of logging interval expressed in hours and 18 is the number of logging records per day. All statistical analyses were carried out using R v. 2.14.0 (R Development Core Team 2011).

Pluviometric data for Rio Correboi (monthly averages of rainfall from 1922 to 2009 from the nearby climatic station of Fonni, NU) and Rio Olai (monthly averages of rainfall from 1936 to 2009 from the nearby climatic station of Montes, Orgosolo, NU), were acquired from Regione Autonoma della Sardegna (http://www.regione.sardegna.it/j/v/25?s=131338&v=2&c=5650&t=1). The presence / absence of the tree canopy of riparian wood with *A. glutinosa* was observed at each field excursion realized during this study.

Results

Seed germination under controlled conditions

The fitted GLM highlighted a statistically significant effect ($P < 0.001$) on germination of temperature (*T*) and treatment (*S*) factors and of their interaction (*T* x *S*; Fig. 1) for seeds collected in Rio Correboi (see Table 1). Untreated seeds (0) germinated at percentages ranging from ca. 50% to ca. 87% at all the tested temperatures, except at 10°C where germination was less than 15% (Fig. 1). The applied cold stratification treatments increased seed germination percentages and widened the range of germination temperatures (Fig. 1). In particular, the effect of cold stratification was positive and statistically significant ($P < 0.001$) at 10°C, with germination increasing with the length of stratification from 12 ± 8% (0) to 92 ± 8% (C3) and at 15°C, with percentages increasing from 61 ± 5% (0) to 87 ± 3% (C3). Untreated and cold-stratified seeds reached high germination when incubated at the alternating temperature regime (25/10°C), with percentages > 80% for 0, C1 and C2 treatments, without statistically

significant differences ($P > 0.05$); whereas after C3, germination significantly ($P < 0.05$) decreased to 68 ± 11% (Fig. 1).

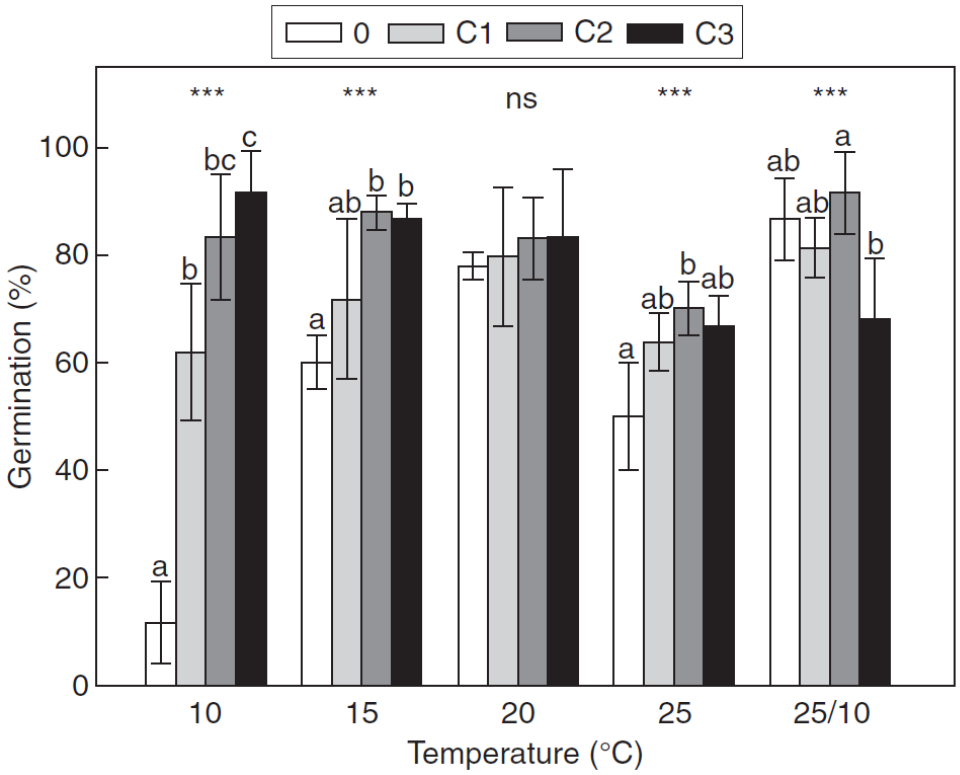

Figure 1 - Effects of temperatures and cold treatments (0, control; C1, C2 and C3 cold stratification at 5°C for 1, 2 and 3 months, respectively) on final germination for *Rhamnus persicifolia* seeds collected in Rio Correboi. Data are the mean of three replicates (1 ± s.d.). Temperatures, treatments and their interaction are statistically significant ($P < 0.001$ by GLM). Post hoc pairwise *t*-test comparisons (with Bonferroni adjustment) were carried out for each germination temperature and bars with different letters indicate significant ($P < 0.05$) variation.

Final germination for seeds collected in Rio Olai incubated at 25°C after 3 months at 5°C was 60 ± 7%.

Thermal requirement for germination

Goodness of fit (r^2) for the linear regressions of $1/t$ against temperature for Rio Correboi collections showed that the best sub-optimal model included data only up to 20°C (i.e. excluding 25°C; Fig. 2A). Based on germination rate responses for each 10th percentile from 10% to 80% germination, it was possible to estimate the mean base temperature for germination (T_b) in the sub-optimal temperature range for each treatment (Fig. 2A). Average T_b were 10.5 ± 0.6, 8.5 ± 0.9, 6.1 ± 1.4 and 2.7 ± 0.8°C, for 0, C1, C2 and C3 treatments, respectively. For the different treatments, linear regressions were re-

calculated for each percentile, constraining them to pass through the mean T_b. This model led to no differences on residual sum of squares and showed higher values of r^2 for all of the linear regression equations ($r^2 > 0.75$ for 0, $r^2 > 0.93$ for C1, $r^2 > 0.81$ for C2 and $r^2 > 0.81$ for C3), than the model where T_b varied for each percentile ($r^2 > 0.73$ for 0, $r^2 > 0.87$ for C1, $r^2 > 0.73$ for C2 and $r^2 > 0.54$ for C3). T_b values were statistically different ($P < 0.001$) by GLM and the *post hoc* pairwise *t*-test comparison (with Bonferroni adjustment) highlighted significant differences among all treatments (Fig. 2B). The relationship between T_b and the length of the stratification period at 5°C is shown in Fig. 2B. The linear regression highlighted that this negative relationship was statistically significant ($r^2 = 0.91$, $P < 0.0001$; Fig. 2B), with T_b decreasing by 0.09°C per day of stratification or by 1°C for every 11 d of chilling. After 68 days of stratification, T_b decreased below 5°C, and seeds accumulated 1.36 log °Cd (θ_s) in the next 22 days until the end of the C3 treatment at 90 days.

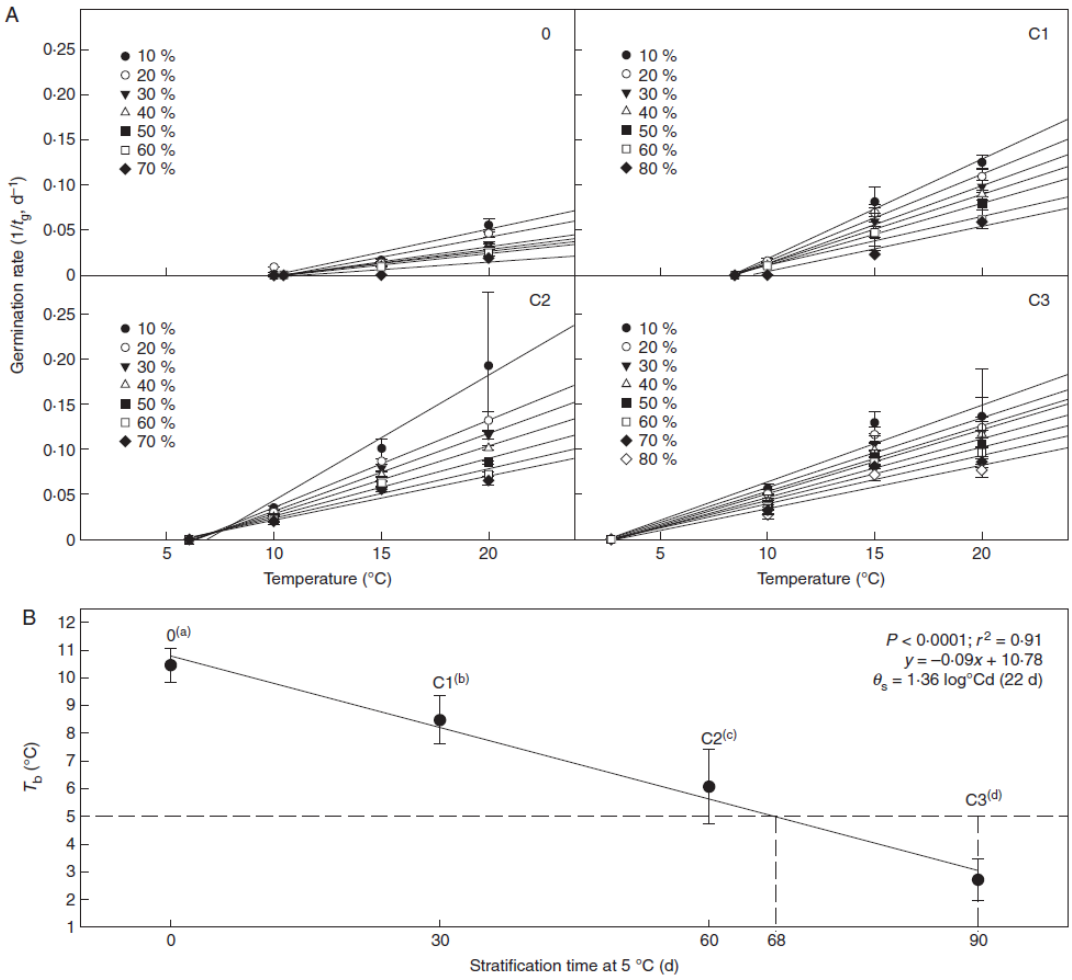

Figure 2 - (A) Base temperatures (T_b), calculated for different germination percentiles of *Rhamnus persicifolia* seeds, after each pre-treatment (0, control; C1, C2 and C3 cold stratifications at 5°C for 1, 2 and 3 months, respectively) and incubation in the suboptimal temperatures (10-20°C). Within each pre-treatment, the linear regressions for the different percentiles were constrained to the common value of T_b. Linear regressions of percentiles with $P > 0.05$ were not included. (B) Relationship between T_b and stratification time at 5°C. Data are the mean ± s.d. of T_b of each percentile. Statistical differences among pre-treatments were analyzed by GLM followed by post hoc pairwise *t*-test comparisons (with Bonferroni adjustment). Mean T_b values with different letters are significantly different at $P < 0.05$.

Figure 3 shows the relationship between log thermal time (θ) and germination expressed in probits, calculated according to Eq. 2. The relationship between log θ and probit germination had better residual sums of square (0.1091, 0.0961, 0.0228 and 0.1366 for 0, C1, C2 and C3, respectively) and r^2 (0.95, 0.97, 0.99 and 0.96 for 0, C1, C2 and C3, respectively) than when expressed on a linear scale (data not shown). Thermal time for 50% of germination (θ_{50}) was greater for the control (2.59 log °Cd) compared to the cold treated seeds (from 2.17 to 2.19 log °Cd; Fig. 3). Seeds of 0 and C2 had a greater σ value (0.26 and 0.25 log °Cd, respectively) compared to C1 and C3 (0.18 and 0.12 log °Cd, respectively; Fig. 3).

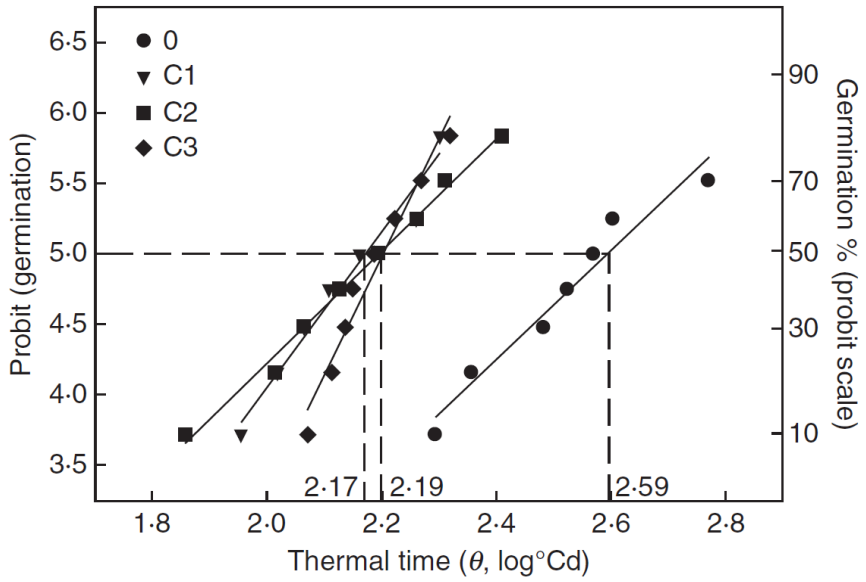

Figure 3 - Probit germination after each pre-treatment (0, control; C1, C2 and C3 cold stratification at 5°C for 1, 2 and 3 months, respectively) as a function of log-thermal time requirement. Thermal times were calculated from germination time-courses from estimated T_b of 10.5, 8.5, 6.1 and 2.7°C for 0, C1, C2 and C3, respectively. Points are the mean of three replicates. Thermal times to reach θ_{50} are also shown (dashed lines).

Seed germination in the field

In December 2011, the great majority of seeds (> 85%) were dormant (Table 2), although a few seeds (< 3%) had started to germinate in RO. In March 2012, seeds started germinating also in Rio Correboi, while the majority of the remaining seeds were still dormant, and the level of dead seeds always < 7% (Table 2). In Rio Olai, the majority of the seeds germinated, reaching values of ca. 70% both IN and OUT and the remaining seeds were mainly dead (Table 2). By April 2012, germination in RC1 was ca. 60% with ca. 25% of seeds remaining dormant and 15 % dead, for both IN and OUT. In RC2 IN and OUT, ca. 75 and 35% of the seeds, respectively, had germinated; ca. 14 and 45% of seeds were dormant and ca. 11 and 20% of seeds dead. For RC3 OUT, germination reached ca. 43%, with ca. 10 and 47% being dormant or dead respectively. No germination data were available for RC3 IN due to predation by animals (Table 2).

At the last exhumation, in June 2012, the percentage of dead seeds was high for all the experimental sites in both populations, ranging from 24 ± 9% for RC1 OUT to 91 ± 4 % for RC2 OUT, and all the remaining seeds germinated (Table 2). The bag in RC1 IN was not retrieved, as it was likely washed away, while seeds in that of RC3 IN were predated by animals (Table 2).

Table 2 - Evaluation categories of the exhumed seeds (%), recorded soil temperatures (°C), calculated soil heat sum (log °Cd), and field germination percentages (mean ± s.d.) for each experimental site (Rio Correboi: RC; Rio Olai: RO) underneath (IN) and outside (OUT) the canopy at the different exhumation dates.

Date of exhumation	**Experimental site**	**Evaluation categories of the exhumed seeds (%, mean ± 1SD)**										**Period**	**Recorded mean soil temperature (°C)**		**Calculated soil heat sum (log °Cd)**		**Predicted soil heat sum (log °Cd)**	
		IN					OUT						IN	OUT	IN	OUT	IN	OUT
		G	V	D	NT	P	G	V	D	NT	P							
09/12/2011	RC2	0 ± 0	95 ± 2	5 ± 2	-	-	0 ± 0	98 ± 2	1 ± 2	-	-	II	4.9	3.0	1.54	0.95	-	-
	RO	1 ± 2	83 ± 8	16 ± 11	-	-	3 ± 2	87 ± 6	11 ± 4	-	-	II	7.5	6.6	1.68	1.88	-	-
29/03/2012	RC2	32 ± 18	61 ± 19	7 ± 2	-	-	2 ± 3	95 ± 5	5 ± 5	-	-	IV	6.3	3.5	1.96	1.43	2.15	-
	RO	73 ± 12	6 ± 7	21 ± 16	-	-	75 ± 3	8 ± 7	17 ± 7	-	-	IV	8.2	11.0	2.16	2.43	2.29	2.28
26/04/2012	RC1	57 ± 24	28 ± 13	15 ± 15	-	-	61 ± 12	25 ± 13	13 ± 2	-	-	IV	8.9	10.4	2.44	2.37	2.22	2.23
	RC2	74 ± 5	14 ± 5	11 ± 2	-	-	35 ± 12	45 ± 8	20 ± 7	-	-	IV	9.5	9.7	2.34	2.28	2.28	2.16
	RC3	-	-	-	-	100	43 ± 25	10 ± 9	47 ± 33	-	-	III	6.7	16.2	1.94	2.69	-	2.18
	RO	55 ± 11	7 ± 4	38 ± 11	-	-	73 ± 3	14 ± 11	13 ± 8	-	-	IV	11.7	13.7	2.49	2.68	2.21	2.27
25/06/2012	RC1	-	-	-	100	-	76 ± 9	0 ± 0	24 ± 9	-	-	V	17.4	27.5	3.01	3.15	-	2.29
	RC2	71 ± 21	0 ± 0	29 ± 21	-	-	9 ± 4	0 ± 0	91 ± 4	-	-	V	19.3	27.0	3.02	3.11	2.26	-
	RC3	-	-	-	-	100	4 ± 4	0 ± 0	96 ± 4	-	-	III	16.6	27.6	2.90	3.22	-	-
	RO	45 ± 24	0 ± 0	55 ± 24	-	-	57 ± 11	0 ± 0	43 ± 11	-	-	V	17.1	27.1	3.02	3.19	2.18	2.22

The soil heat sum values, predicted on the basis of the thermal time (θ) model (expressed as probit germination and log °Cd; see Fig. 3) are also reported for the different germination percentages for values comprised between 10 and 80% (see Fig. 3).G = germinated seeds; V = viable dormant seeds; D = dead seeds; P = preyed seeds; NT = not retrieved envelopes.

Periods, identified according to the presence/absence of the canopy and to the seasons for all the experimental sites for RC1, RC2 and RO, correspond to: (I) from the sowing to the disappearance of the tree canopy; (II) from the disappearance of the canopy to the start of the stratification period; (III) the stratification period; (IV) from the end of the stratification period to the appearance of the canopy; (V) from the appearance of the canopy to the start of the summer droughts; (VI) the summer drought period. For RC3 they correspond to: (I) from the sowing to the start of the stratification period; (II) the stratification period; (III) from the end of the stratification period to the start of the summer droughts; (IV) the summer drought period.

GLM highlighted a statistically significant ($P < 0.001$) effect for all the factors (Date, D; Position, P; Site S) as well as for their interactions, except for the two way interaction D x P and the three way interaction D x P x S for which $P > 0.05$ (Table 3).

Table 3 - GLM results for the effect on seed germination in the field of the following factors: "Date" (D: Dec. 11, Mar. 12, Apr. 12 and Jun. 12), "Position" (P: IN and OUT) and "Experimental site" (S: RC1, RC2, RC3 and RO).

	Df	**Deviance**	**Resid. df**	**Resid. dev**	***F***	***P* (>F)**
NULL			62	3105.85		
Date (D)	3	1371.24	59	1734.61	59.3520	< 0.001
Position (P)	1	98.21	58	1636.40	12.7530	< 0.001
Site (S)	3	456.67	55	1179.73	19.7661	< 0.001
D x P	3	34.36	52	1145.37	1.4872	> 0.05
D x S	5	408.83	47	736.54	10.6173	< 0.001
P x S	2	385.86	45	350.68	25.0519	< 0.001
D x P x S	3	10.34	42	340.34	0.4474	> 0.05

Soil heat sum and thermal niche for in situ seed germination

The establishment of the tree canopy of *A. glutinosa* woods was very similar in the two streams (Rio Correboi and Rio Olai), starting at the end of April and disappearing in mid-October (Fig. 4). In detail, the annual trend of soil temperatures could be divided into six periods, according to the presence/absence of the canopy and to the seasons, for RC1, RC2 and RO experimental sites: (I) from the sowing at the end of September/early October to the disappearance of the tree canopy in mid-October; (II) from the disappearance of the canopy in mid-October to the start of the stratification period, when mean daily temperatures fell to 5°C in December; (III) the main stratification period, from December to March, when mean daily temperatures are close to 5°C; (IV) from the end of the stratification period in March to the appearance of the canopy in April; (V) from the appearance of the canopy in April to the start of the summer droughts in June/July; (VI) the summer drought period when rainfall drastically reduces (Fig. 4 and Table 2). The absence of a riparian wood in RC3 (see Table 1) lead to only four environmental periods: (I) from the sowing to the start of the stratification period in December; (II) the stratification period until March; (III) from the end of the stratification period to the start of the summer droughts in June/July; (IV) the summer drought period.

By combining Eq. 3 and the equation in Fig. 2B, where T_b was calculated day by day, for Rio Correboi seeds, according to the length of stratification experienced in the field, it was possible to calculate the soil heat sum reached by the seeds at the different exhumation times for each experimental site of both populations (Table 2). The values calculated for RC2 and RO (for which there was a complete temporal sequence) were compared with those estimated using the thermal time (θ) model, expressed as probit germination and log °Cd (for germination values comprised between 10 and 80%; see Fig. 3). The linear regression highlighted a statistical significant relationship between calculated and estimated data (n = 5; P = 0.0018; r^2 = 0.97; y = 1.0992x – 0.1739).

In RC2 (Fig. 4A) the length of the effective stratification periods was 92 days for IN and 98 days for OUT (with 41 and 47 days of snow cover, respectively), leading to T_b values at the end of the stratification period of 2.9°C and 2.5°C for IN and OUT, respectively. Before (periods I-II) and during stratification (period III), mean soil temperatures were similar or lower than T_b (10.2°C), preventing the soil heat sum accumulation for germination. However, after stratification (period IV) the lower T_b values and the increasing soil temperatures allowed the threshold of 2.19 log °Cd (which corresponds to the value to achieve 50% of germination in laboratory, θ_{50}) to be reached 194 (IN) and 211 (OUT) d from sowing (Fig. 4B). This estimated time was confirmed by the germination recorded in the field (Table 2; Fig. 4C).

In RC1, the length of the stratification period was 90 days for IN and 104 days for OUT environmental conditions, leading to T_b values at the end of the stratification period of 2.9°C and 1.9°C for IN and OUT, respectively. After stratification, the threshold for θ_{50} was reached 186 and 200 d after sowing for IN and OUT, respectively, consistent with the field values presented in Table 2. In RC3, the effective stratification period was 116 d for IN and 93 days for OUT, leading to T_b values at the end of the stratification period of 2.5°C and 2.9°C for IN and OUT, respectively. Therefore, the threshold for θ_{50} was reached in period III, 171(OUT) and 219 (IN) d after sowing. Although few field germination data were available for this experimental site, the highest germination (43.0 ± 25.2% for OUT) was recorded in April (Table 2).

In RO, the length of stratification period was 75 d for both IN and OUT (with 15 and 20 days of snow cover, respectively), leading to T_b values at the end of the stratification period of 4.4°C for each site (Fig. 4). Before (periods I-II) and during stratification (period III), mean soil temperatures were similar or lower than T_b (10.2°C), leading to a slow accumulation of heat sum (1.73 log °Cd for IN and 1.97 log

°Cd for OUT) by the end of the period III (Fig. 4B). After stratification (period IV), the lower T_b values and the increasing soil temperatures enabled θ_{50} for RC seeds to be reached 164 (OUT) and 178 (IN) d after sowing (Fig. 4B). Although these times were estimated using data from seeds belonging to a different population (RC), the estimated dates were confirmed by the high germination percentages recorded in the field from March to April (Fig. 4C).

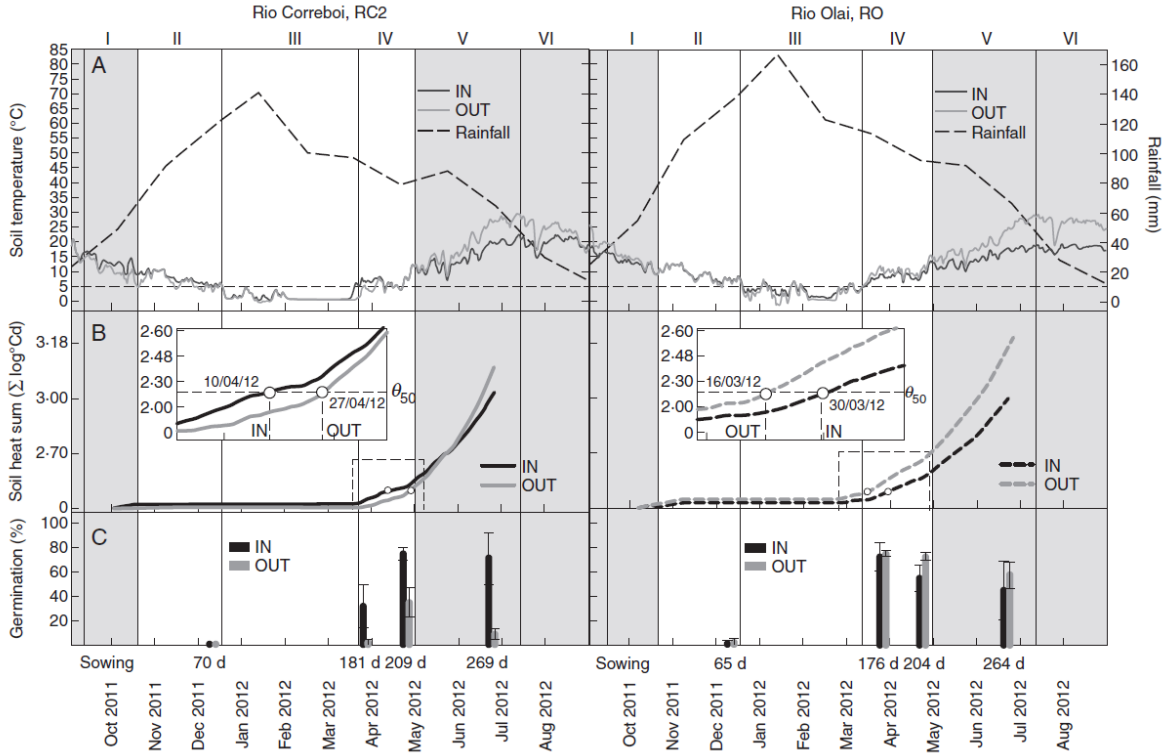

Figure 4 - Soil temperatures, soil heat sum and field germination for Rio Correboi (RC2) and Rio Olai (RO). (A) Annual trends of mean daily temperatures recorded in the soil both underneath (IN) and outside (OUT) the tree canopy and mean monthly rainfall (data from the nearby weather stations of Fonni and Montes for RC2 and RO, respectively); (B) soil heat sum (expressed in log °Cd); and (D) field germination (3 replicates of 25 seeds each) IN and OUT at each time of exhumation, expressed in days (d) after the sowing. The inset plot (C) shows the detail of the achievement of the θ_{50} threshold value (2.19 log °Cd). The background grey squares correspond to the presence of the tree canopy. The details of periods I, II, III, IV, V and VI are as given for Table 2.

Discussion

Type of dormancy

Final germination of *R. persicifolia* seeds was significantly improved by cold stratification (5°C) at intermediate and low temperatures, confirming the presence of physiological dormancy (PD) and supporting earlier observations (Mattana *et al.*, 2009).

PD is also known in seeds of *R. cathartica*, *R. caroliniana*, *R. frangula* and *R. purshiana* (Baskin and Baskin, 1998), *R. alaternus* and *R. cathartica* (Dupont *et al.*, 1997; García-Fayos *et al.*, 2001), and *R. alnifolia* and *R. lanceolata* (Sharma and Graves, 2005). As just 1 month of cold stratification is sufficient to break *R. persicifolia* seed dormancy, the seeds appear to have non-deep PD (Baskin and Baskin, 2004). Further, as the temperature range at which the *R. persicifolia* seeds could germinate widened from higher to lower, the seeds have type 2 non-deep PD (Baskin and Baskin, 2004).

Thermal requirements for germination

The optimal temperature for germination of non-dormant seeds of *R. persicifolia* is presumed to be around 20°C, as the best fit of the germination rate data in the sub-optimal temperature range excluded 25°C, which fell in the supra-optimal temperature range. T_b in seeds of *R. persicifolia* varied from ca. 10°C for non-treated seeds to ca. 3°C for 3-months cold stratified seeds. To our knowledge this is the first report of T_b for a member of the Rhamnaceae. As constraining the linear regressions of each percentile for germination through the mean T_b improved the residual sum of squares and r^2 values, T_b can be used to describe the whole population response in *R. persicifolia* seeds, as previously reported for other species (e.g. Covell *et al.*, 1986; Ellis *et al.*, 1987; Pritchard and Manger, 1990; Orrù *et al.*, 2012).

Treatments for dormancy release clearly modified T_b in *R. persicifolia* seeds and the widening of the range of temperatures for germination can be used as a surrogate for the efficient removal of dormancy. Chilling at 5°C reduced T_b in *R. persicifolia* seeds by ca. 0.09°C per day of chilling, such that T_b reached the chilling temperature after 68 days of stratification. A similar trend has been observed in *A. hippocastanum* seeds with T_b reducing by 0.17°C per day of chilling at 6°C (Pritchard *et al.*, 1999). In both these species, the sequential removal of dormancy lowers T_b until the stratification temperature becomes permissive for germination growth *per se* (Pritchard *et al.*, 1999). However, the process is nearly twice as rapid in *A. hippocastanum* seeds, with T_b reducing by 1°C for every 5.9 days of chilling compared with 11.1 days of chilling in *R. persicifolia.* Consequently, it is clear that the quantitative impacts of a shortened cold season as a result of climate change will be highly species-specific in respect to the efficiency of dormancy loss and timing of the germination.

The best model was obtained by fitting germination expressed in probit and log-normal (log °Cd) rather than normal distributed thermal times (°Cd), as previously reported for other herbaceous (Covell *et al*., 1986; Ellis and Butcher, 1988) and tree species (Pritchard and Manger, 1990). Seeds of *R. persicifolia* vary in their thermal time estimates to reach θ_{50}, depending on treatment. Chilling increased the rate of accumulation of thermal units (°Cd) at any temperature in the sub-optimal range, leading to a reduction in θ_{50} values from 2.59 log °Cd (385°Cd) for untreated seeds to about 2.18 log °Cd (150°Cd) for cold stratified seeds. Batlla and Benech-Arnold (2003) also detected a cold-induced decrease in θ_{50}, from 80°Cd to 56°Cd, for seeds of *Polygonum aviculare* stratified at 12 and 1.6°C, respectively. Similarly, the thermal history of *V. vinifiera* subsp. *sylvestris* seed lots varying with maternal environment is known to affect θ_{50} (33.6°Cd to 68.6°Cd) for non-dormant, cold-stratified seeds (Orrù *et al.*, 2012).

Soil heat sum and thermal niche for in situ seed germination

Maximum germination of Mediterranean species is typically in the range 5-15°C and is limited in autumn and winter, and usually decreases markedly above 20°C (Thanos *et al.*, 1995; Luna *et al.*, 2012). *R. persicifolia* showed a typical germination phenology of temperate and alpine plants, where spring germination prevails due to temperatures being too low to stimulate emergence following autumn dispersal or due to a requirement for cold stratification over winter (Baskin and Baskin, 1998; Walck *et al.*, 2011; Mondoni *et al.*, 2012). However, under harsh Mediterranean climatic conditions, the topsoil in the mountains remains moistened for only few weeks after snow-melt, such that adaptation for fast germination in the early spring is an advantage (Giménez-Benavides *et al.*, 2005; Mattana *et al.*, 2010). The dormancy breaking and thermal time requirements identified in this study, together with the recorded annual trends in soil temperature, allowed a model for thermal niche of seed germination to be constructed and spring emergence to be predicted for *R. persicifolia* seeds. Soil temperatures around 5°C (i.e. the stratification temperature tested in the controlled conditions) from December to February for Rio Olai (ca. 75 days) and from December to March (ca. 95 days) for Rio Correboi facilitate both a fall in T_b to ca. 3°C and efficient germination of the seeds in March and April when the mean soil temperatures are of ca. 10°C.

Plant distribution and competitiveness are highly dependent on environmental envelopes or niches (Walck *et al.*, 2011; Bykova *et al.*, 2012). For *R. persicifolia* habitat, up to six temperature periods were identified throughout the year, which contribute to a better understanding of the field germination period in this and other species growing along Mediterranean mountain waterways; especially as there have, hitherto, been no historical series of monthly averages of temperatures and rainfall at altitudes higher than ca. 1100 m a.s.l. on Sardinia. In each investigated site, seed germination of *R. persicifolia* was obtained after cold stratification, when the canopy was absent. Tree canopy seems therefore to have no influence on seed germination *sensu stricto*, but closure of canopy could influence survival of new established seedlings due to microclimate amelioration (moister and cooler) during the dry and hot Mediterranean summers (Valiente-Banuet *et al.*, 1991; Greenlee and Callaway, 1996; Gómez-Aparicio *et al.*, 2005). This was confirmed by the high germination percentages reached under controlled conditions by untreated and cold-stratified seeds (> 80%) when incubated in the alternating temperature regime (25/10°C). The ecological significance of germination stimulation by alternating temperature can be interpreted as a season-sensing system for temperate plants because the diurnal fluctuation of the soil surface temperature is large in the spring before dense vegetation covers the ground of deciduous forest or grassland (Shimono and Kudo, 2003).

The ecology of germination identified in this study for *R. persicifolia*, explains the present distribution of this species which is mainly limited to small "temperate" refuge areas along mountain waterways (Mattana *et al.*, 2009), where the general lack of rainfall during summer is overcome by the moisture of the soil. These findings confirm the identification of *R. persicifolia* as a species with a relic distribution, as previously reported by Arrigoni (1977) and Bacchetta *et al.* (2011).

The quantification of thermal time for germination has been used in different studies to characterise changes in seed dormancy and subsequent germination in the field (i.e. Forcella *et al.*, 2000; Hardegree and Van Vactor, 2000; Steadman *et al.*, 2003; Chantre *et al.*, 2009). Recently, Orrù *et al.* (2012) used an environmental heat sum approach (using mean monthly temperatures) to predict germination timing under two simulated IPCC scenarios (+1.8°C for B1 and + 3.4°C for A2; IPCC, 2007) for *V. vinifera* subsp. *sylvestris* seeds. The B1 scenario of +1.8°C would still adequately overcome dormancy for all the investigated populations, whereas under the A2 scenario with +3.4°C the higher winter temperature would not allow seed dormancy loss in the

lowest investigated population (Orrù *et al.*, 2012). The same altitude related pattern of seed dormancy release and germination in response to global warming can be assumed for *R. persicifolia* seeds. An increase of +1.8°C (B1) would not reduce the stratification period at 5°C for the high Rio Correboi population (ca. 90 days, leading to a T_b of ca. 3°C), whereas it could affect that of the low Rio Olai population (ca. 21 days; T_b of ca. 9°C). However, an increase of +3.4°C (A2) would reduce the cumulative stratification time at 5°C to only 50 (T_b of ca. 6.5°C) and 17 days (T_b of ca. 9°C) for Rio Correboi and Rio Olai, respectively. According to the B1 scenario, these changes on T_b and the increased soil temperatures would affect the germination time, by anticipating field germination to February-March and March-April, for Rio Olai and Rio Correboi, respectively. An increase of + 3.4°C (A2) could lead to germination in the field in Autumn (November) in both sites. This phenological shift to germination in autumn is possible as seeds of this species may germinate also at temperatures ≥ 15°C without any cold stratification. Therefore, warmer temperatures and a consequent reduction of the stratification period would not be detrimental *per se* for seed germination. However, seedling survival over winter might then become the limiting event for the natural regeneration of the species. Moreover, projections for Mediterranean mountains predict lower precipitations mainly during spring (Nogués-Bravo *et al.*, 2008) and the seedling growing season could be shortened also by a reduction in soil moisture and water availability.

Conclusions

In conclusion, type 2 non deep physiological dormancy (PD) was identified for *R. persicifolia* seeds, the thermal niche requirements for dormancy release and germination were quantified and predictions for germination validated through field observations of emergence. Overall, the results confirm the value of using a soil heat sum approach to predict the effects of subtle changes in field temperature on germination performance. The soil heat sum model developed for seed germination in this species may have applicability to predictions of *in situ* regeneration of other species growing on Mediterranean mountain waterways and of physiologically dormant species of temperate and alpine regions, where spring germination prevails due to a requirement for cold stratification over winter.

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Chapter II - Multiphasic thermal parameters for embryo growth, seed dormancy loss and germination in Aquilegia barbaricina

Abstract

- *Background and Aims*: The threshold-based thermal time models have been used to investigate seed dormancy loss and germination. In this study, this approach was applied to characterize thermal requirements for embryo growth as well as dormancy release and germination of *Aquilegia barbaricina* seeds.
- *Methods*: Seeds of two different populations of *A. barbaricina* were incubated in the light at a range of temperatures (10 – 25 and 25/10 °C), without any pre-treatment, after W+C stratification (3 months at 25 °C followed by 3 months at 5 °C), and a GA_3 treatment (250 mg/L in the germination substrate). During germination tests, the time of seed coat and endosperm rupture were scored and embryo growth assessed. Base temperatures (T_b) and thermal times for 50% (θ_{50}) of embryo growth and seed germination were calculated.
- *Key Results*: The species showed an intermediate morphophysiological dormancy (MPD) and warm followed by cold stratification and GA_3 treatment promoted embryo growth and subsequent seed germination. Embryo growth did not differ among populations, while differences were found on germination. T_b for embryo growth was approximately 5 °C both in W+C stratified and GA_3 treated seeds. For W+C pre-treated seeds the optimal temperature for embryo growth (T_{oe}) was ca. 15 °C and the ceiling temperature (T_{ce}) ca. 29 °C. T_b for germination varied from ca. 5 to 7 °C in W+C stratified seeds, and from 5 to 8 °C for GA_3 treated seeds. θ_{50} for embryo growth reduced from 2.64 log °Cd for GA_3 treated seeds to 2.10 log °Cd for W+C stratified seeds. Same trend was detected also for germination, with a reduction in θ_{50} values from ca. 2.80 log °Cd to 2.03 log °Cd for GA_3 treated and W+C stratified seeds, respectively.
- *Conclusions*: The modelling of the thermal time approach applied on embryo growth, associated to results obtained on seed germination, is an important first study that correlates the thermal threshold with seed morphology. Multi-step seed germination detected in this study identified embryo growth phase as the riskiest step for the germination process of *A. barbaricina* seeds.

Keywords: *Aquilegia barbaricina*, columbine, morphophysiological dormancy, multi-step germination, Ranunculaceae, thermal threshold, thermal time.

Introduction

Two-step germination, in which testa and endosperm rupture are sequential events controlled by phyto-hormone balance, is widespread over the entire phylogenetic tree and has been described for many families, e.g. Amaranthaceae (Karssen, 1976), Solanaceae, (Krock *et al.*, 2002; Petruzzelli *et al.*, 2003), and Brassicaceae (Liu *et al.*, 2005; Müller *et al.*, 2006), as well as in Ranunculaceae (Hepher and Roberts, 1985). In many plant species the seed-covering layers impose a physical constraint to radicle protrusion, which has to be overcome by the growth potential of the embryo (Kucera *et al.*, 2005; Müller *et al.*, 2006). Abscisic acid (ABA) and gibberellic acid (GA) play an important role in a number of physiological processes of seed germination. ABA induces dormancy during maturation, and GA plays a key role on dormancy release and in the promotion of germination, and can act on testa and endosperm rupture (Finch-Savage and Leubner-Metzger, 2006). Müller *et al.* (2006) reported that, in *Lepidium sativum* L. and *Arabidopsis thaliana* (L.) Heynh. seeds, endosperm rupture is promoted by GA and inhibited by ABA.

It is known that temperature is one of the most important environmental factors controlling germination (Probert, 2000), determining both the fraction of seeds in a population that germinate and the rate at which they emerge (Heydecker, 1977). In non-dormant seeds, the germination response to accumulated temperature has been modelled by a thermal time (θ) approach (Covell *et al.*, 1986; Ellis *et al.*, 1986; Ellis *et al.*, 1987; Garcia-Huidobro *et al.*, 1982; Hardegree, 2006; Pritchard and Manger, 1990). In this model, seeds accumulate units of thermal time (°Cd) to germinate for a percentile g of the population. When seeds are subjected to temperatures (T) above a base temperature for germination (T_b), germination rate increases linearly with temperature to an optimum temperature (T_o), above which germination rate starts to decrease (Garcia-Huidobro *et al.*, 1982). Thus, in this sub-optimal range ($T_o - T_b$), germination occurs in the time t_g, when the thermal time accumulated has reached the critical value (θ_g) for a percentile g of the population, and can be described as $\theta_g = (T - T_b)t_g$. Intra-specific variation in T_b among populations may be due to different environmental conditions during seed development (Daws *et al.*, 2004). Thermal time approach has been used to predict seed germination in the field (i.e. Hardegree and Van Vactor, 2000; Steadman *et al.*, 2003; Chantre *et al.*, 2009), and recently, to assess the impact of different simulated climate change scenarios on seed dormancy release and germination timing in *Vitis*

vinifera L. subsp. *sylvestris* Hegi (Orrù *et al.*, 2012), and to model the *in situ* natural regeneration patterns of *Rhamnus persicifolia* Moris (Porceddu *et al.*, 2013). However, to date there are no specific studies on the threshold temperatures and thermal time requirements on embryo growth.

Seeds of Ranunculaceae species contain rudimentary or linear underdeveloped embryos (Martin, 1946; Baskin and Baskin, 2007) and can exhibit both morphological (MD) and morphophysiological (MPD) dormancy (Baskin and Baskin, 1994, 1998; Walck *et al.*, 1999). In particular, *Aquilegia* sp. pl. seeds have linear underdeveloped embryos (*sensu* Baskin and Baskin, 2007) and stratification of the seeds at 3–5°C for 2–4 weeks is recommended before sowing for germination (Ellis *et al*., 1985). Mattana *et al*. (2012) reported MPD for *Aquilegia barbaricina* Arrigoni *et* E.Nardi and *A. nugorensis* Arrigoni *et* E.Nardi, where the combination of both warm and cold pre-treatment was needed to break dormancy. However, the results shown by these authors were not exhaustive, highlighting, in particular for *A. barbaricina*, low final percentages of seed germination.

Two-step germination has already documented in Ranunculaceae (Hepher and Roberts, 1985), leads us to hypothesize that such event could be occur also in members of *Aquilegia*. Furthermore, linear underdeveloped embryos present in *Aquilegia* spp. suggests that the thermal time approach would be applied to other phases of seed germination that from imbibition ends with the radicle protrusion. Therefore, the aims of this work were to: (1) identify the phases of seeds germination of *Aquilegia barbaricina* and (2) investigate the thermal requirements for embryo growth, dormancy release and seeds germination of this threatened species.

Materials and Methods

Study species

Aquilegia barbaricina (Ranunculaceae) is a rhizomatous perennial herb underground branched with stems 30-60 cm high (Arrigoni *et* Nardi, 1977; Fenu *et al.*, 2011). The fruits are erect capsules which produce dark trigonal seeds with a rudimentary, underdeveloped embryo (Mattana *et al.*, 2012). Phenological data indicate a flowering period from May to June and a fruiting period from June to July (Mattana *et al.*, 2012). *A. barbaricina* is an exclusive endemic to the Gennargentu and Supramontes regions (CE-Sardinia), growing from 800 to 1,400 m a.s.l. in wet woodlands, meadows and

stream margins, mainly occurring on siliceous substrates and secondarily on limestone ones (Fenu *et al.*, 2011; Garrido *et al.*, 2012). This species is included in the IUCN Red Lists (http://www.iucnredlist.org), and it is classified as "Critically Endangered" (Fenu *et al.*, 2011), and also as one of the 50 most endangered plants of the Mediterranean islands (de Montmollin and Strahm, 2005).

Seed lot details

Seeds of *A. barbaricina* were collected directly from plants in riparian woods of *Alnus glutinosa* (L.) Gaertn. at the time of natural dispersal in early summer 2011 in two different populations in CE-Sardinia (Table 1).

Table 1 – Population data and seed lot details.

Locality	Population code	Region	Geographical coordinates (UTM - Datum WGS84)	Elevation range (m a.s.l.)	Aspect	Date of collecting	Mean seed mass (mg ± SD)
Rio Correboi (Villagrande Strisaili, OG)	RC	Gennargentu	N 40°03' E 09°20'	1190 - 1300	E - NE	29/06/2011	1.26 ± 0.06
Rio Olai (Orgosolo, NU)	RO	Supramontes	N 40°07' E 09°22'	948 - 970	NE	28/06/2011	1.40 ± 0.05

Germination tests

3 replicates of 20 seeds each per condition, belonging to each investigated population (see Table 1), were sown in July 2011, on the surface of 1% agar water in 60-mm diameter plastic Petri dishes. Dishes were incubated in the light (12 h of irradiance) at different range of germination temperatures (10, 15, 20, 25 and 25/10°C). In the alternating temperature regime, the light period coincided with the elevated temperature. Further replicates were given a warm (W = 25°C for 3 months) followed by a cold stratification (C = 5°C for 3 months), before being incubated at the range of germination temperatures (Table 2). This pre-treatment was chosen on the basis of the findings of a previous study on seed germination of this species (Mattana *et al.*, 2012). 3 extra replicates of 20 seeds each were also sown on the surface of 1% agar water with 250 $mg \cdot l^{-1}$ GA_3 and incubated in the light (12 h light / 12 h dark) at the range of germination temperatures.

Germination was defined as visible radicle emergence. Germinated seeds were scored 3 times a week. During germination tests, seeds with split seed coat were scored and the time from seed coat splitting to endosperm rupture was estimated by daily monitoring the time from seed coat splitting to radicle emergence in 15 seeds for each condition, belonging to each investigated population. Germination tests lasted for a minimum of 1 month and a maximum of 4 months. When no additional germination had occurred for 2 weeks, a cut-test was carried out to determine the viability of the remaining seeds. The final germination percentage was calculated as the mean of 3 replicates (± 1 SD), on the basis of the total number of filled seeds.

Table 2 - Experimental design.

Condition		**Embryo growth measurements**	
Code	**Description**	**Number of measurements**	**Timing**
0	Control	5	After 15, 30, 60, 90 and 120 days.
W + C	3 months, 25°C (W) → 3 months, 5°C (C)	13	After 15, 30, 60 and 90 days during warm (W), 15, 30, 60 and 90 days during cold (C), and 15, 30, 60 and 90 and 120 days after sowing for germination.
GA_3	GA_3 (250 $mg \cdot l^{-1}$) in the germination medium	5	After 15, 30, 60, 90 and 120 days.

Embryo measurements

Embryo growth was assessed at different times, during the above described conditions and germination temperatures by measuring 10 seeds for each sample interval (see Table 2). Seeds were cut in half under a dissecting microscope and images of embryos acquired using a Zeiss SteREO Discovery.V8, with an objective Achromat S 0.63x, FWD 107mm (Carl Zeiss MicroImaging GmbH) at a 6.3x magnification, coupled to a Canon (Power shot G11) digital camera. Embryo and seed lengths were measured using the image analysis software ImageJ 1.41o (National Institutes of Health, Bethesda, MA, USA). Seed length was measured ignoring the seed coat. The initial embryo length was calculated by measuring 20 randomly selected seeds before the start of the experiments. The embryo length of seeds with a split seed coat but no radicle protrusion (i.e. critical embryo length) was determined for 20 randomly selected seeds and used for seeds that had germinated before measurements (Vandelook *et al.*, 2007).

Thermal time analyses

Thermal time analyses were carried out for non-dormant seeds of both populations, germinating at constant temperatures after W+C pre-treatment (W = 25°C for 3 months followed C = 5°C for 3 months) and with GA_3 treatment (250 mg l^{-1} in the germination substrate). Estimates of time (t_g, days) taken for cumulative germination to reach different percentiles (g) for successive increments of 10% germination were interpolated from the germination progress curves (Covell *et al.*, 1986). Germination rate ($1/t_g$) was regressed, using a linear model, as a function of temperature according to the following equation (Garcia-Huidobro *et al.*, 1982):

$$1/t_g \text{ (days}^{-1}) = (T - T_{bg}) / \theta_g \qquad \text{(Eq. 1).}$$

An average (± 1SD) of the x-intercept among percentiles was calculated for the sub-optimal temperature range (10–20°C) to establish the base temperature for germination (T_{bg}) for each treatment (Ellis *et al.*, 1986; Pritchard and Manger, 1990). Linear regression equations were recalculated for each percentile, but constrained to pass through T_{bg} (Hardegree, 2006). A comparison of regressions was then made between this model and one in which the T_{bg} were allowed to vary for all the percentiles and the best estimate was considered to be that which resulted in the smallest residual variance (Covell *et al.*, 1986). Thermal time (θ_g, °Cd) estimates were then calculated separately as the inverse of the sub-optimal regression equations (Covell *et al.*, 1986; see Eq. 1).

Germination percentages were transformed to probits using tabular data from Finney (1971). Linear regression was used to express probit(g) as a function of both θ_g and log θ_g for the suboptimal temperature range for each seed lot and the best model evaluated on the basis of the r^2 values (Hardegree, 2006). The following equation was used to describe the form of cumulative germination response of seeds (Pritchard and Manger, 1990):

$$\text{probit}(g) = K + \log \theta_g / \sigma \qquad \text{(Eq. 2),}$$

where K is an intercept constant when thermal time (θ_g) is zero and σ is the standard deviation of the response to log θ_g (i.e. the reciprocal of the slope), and represents the

sensitivity of the population to θ_g (Covell *et al.*, 1986). Thus, the flatter the slope of the fitted line the greater the variation in response to thermal time between individual seeds (Daws *et al.*, 2004).

Thermal time approach as above described for seed germination was also used for analysing embryo growth rate. Estimates of time (t_e, days) taken for different percentiles of seeds (e) to reach the critical embryo length were interpolated from the embryo growth progress curves. Embryo growth rate ($1/t_e$) was regressed, using a linear model, as a function of temperature according to the modified equation 1:

for the sub-optimal range, $1/t_e \text{ (days}^{-1}) = (T - T_{be}) / \theta_{e1}$ (Eq. 3),

while for the supra-optimal range, $1/t_e \text{ (days}^{-1}) = (T_{ce} - T) / \theta_{e2}$ (Eq. 4).

An average (± 1SD) of the x-intercept among percentiles was calculated for both sub-optimal and supra-optimal temperature ranges, to establish the base temperature (T_{be}) and, when possible, the ceiling temperature (T_{ce}) for embryo growth, respectively. The optimum temperature for embryo growth (T_{oe}) was calculated as the intercept of sub- and supra-optimal temperatures response functions. Thermal time (θ_e, °Cd) estimates were calculated separately as the inverse of the regression equations. Linear regression equations were recalculated for each percentile, but constrained to pass through T_{be}. Linear regression was used to express probit cumulative percentiles of embryo growth (e) as a function of both θ_e and log θ_e and the best model evaluated on the basis of the r^2. The equation 5 was used to describe the form of cumulative percentiles response of seeds to reach the critical embryo length for the sub-optimal temperature range:

$$\text{probit } (e) = K_1 + \log \theta_{e1} / \sigma_1 \quad \text{(Eq. 5)},$$

where K_1 is an intercept constant when thermal time (θ_{e1}) is zero and σ_1 is the standard deviation of the response to log θ_{e1} (i.e. the reciprocal of the slope), and represents the sensitivity of the population to θ_{e1}.

Statistical analysis

Generalized Linear Models (GLMs) were used to compare embryo length, rate of endosperm rupture event, final germination percentages among tested temperatures and base temperature (T_b) among percentiles for seed collected both in Rio Correboi (RC) and Rio Olai (RO). Then, significant differences within each condition were analysed by a *post-hoc* pairwise comparisons *t*-test (with Bonferroni adjustment). GLMs with a log link function and quasipoisson error structure were used for analysing embryo length, rate of endosperm rupture and T_b values, while a GLM with a logit link function and quasibinomial error structure was used for analysing germination percentages. Quasipoisson and quasibinomial error structures and *F* tests with an empirical scale parameter instead of chi-squared on the subsequent ANOVA were used in order to overcome residual overdispersion (Crawley, 2007). All statistical analyses were carried out with R v. 2.14.0 (R Development Core Team, 2011).

Results

Embryo growth, endosperm rupture and seed germination

The mean initial embryo length was 0.029 ± 0.006 mm for seeds of both populations (Fig. 1). During the W pre-treatment, embryo increased very slightly, and the mean embryo lengths after 90 days were 0.042 ± 0.005 mm for RO and 0.037 ± 0.003 for RC, without statistical significant differences ($P > 0.05$) respect to initial value (Fig. 1). Similar trend was observed during the subsequent C pre-treatment, and the final embryo lengths measured after 180 days were 0.047 ± 0.007 and 0.044 ± 0.011 mm for RO and RC, respectively (Fig. 1). After 150 days of pre-treatment (W for 90 days and then C for 60 days) the embryo, in each population, did not growth further. GLM analysis detected no statistical significant differences ($P > 0.05$) between initial embryo length and final embryo length measured at the end of W+C, and highlighted no statistical differences also among populations.

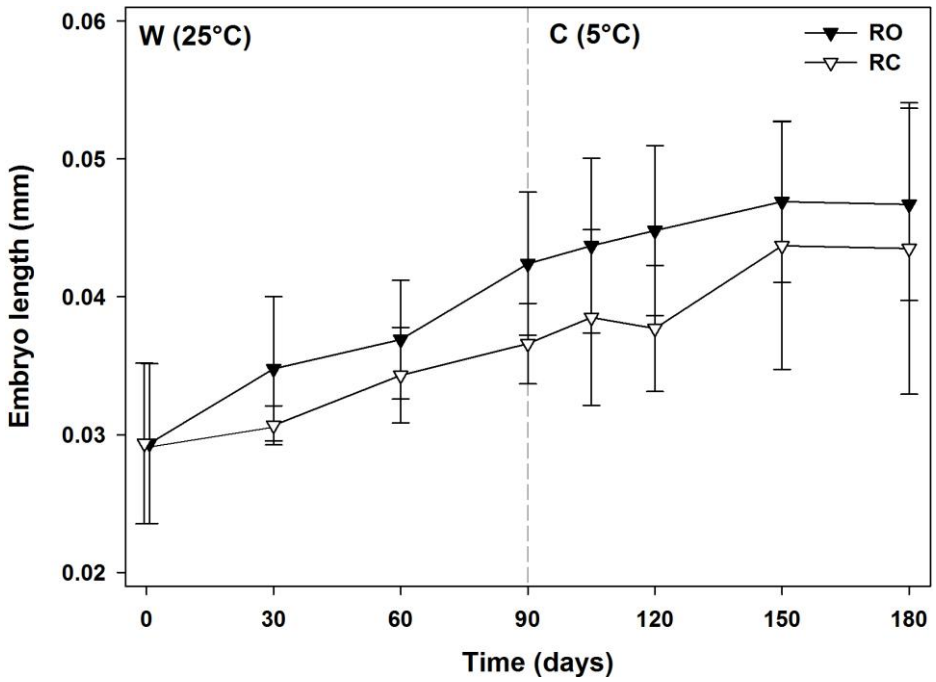

Figure 1 – Embryo growth during stratification at 25°C for 3 months (W) and then at 5°C for another 3 months (C) for seeds collected in Rio Correboi (RC) and Rio Olai (RO) populations. Initial and final embryo lengths measured at the end of W and C pre-treatments, are not significantly different at $P > 0.05$ with GLM, as well as between populations. Data are the mean (± SD) of 20 seeds for initial embryo length and of 10 seeds for each subsequent measurement.

GLM analysis highlighted a statistically significant differences ($P < 0.001$) on embryo lengths for the "treatment" factor, while no statistical differences were found for one way analysis of "population" and "temperature" factors and for all their interactions (Table 3).

Statistically significant differences ($P < 0.001$) were found on estimate rate of endosperm rupture, for "treatment" and "temperature" factors, while no statistical difference ($P > 0.05$) was detected for the "population" factor. A statistically significant difference ($P < 0.001$) was found for the interactions "treatment" × "population" and "treatment" × "temperature" and no statistical significant differences were detected for the interactions "population" × "temperature" and "treatment" × "temperature" × "population" (Table 3).

GLM highlighted a statistical differences ($P < 0.05$) on percentages of seed germination, for all factors, as well as for all their interactions (Table 3).

Table 3 - GLMs results of the following factors: "Treatment" (0, control; W+C, 25°C for 3 months and then 5°C for another 3 months; GA_3, 250 mg l^{-1} in the germination substrate), "Temperature" (10, 15, 20, 25 and 25⁄10°C),"Population" (RO, Rio Olai; RC, Rio Correboi) and interaction of them for embryo length (mm), rate of endosperm rupture (days-^{1}) and seed germination (%).

Embryo length (mm)	**Df**	**Deviance**	**Resid. Df**	**Resid. Dev**	***F***	***P* (>F)**
NULL			298	5.2642		
Treatment	2	3.1617	296	2.1025	244.1995	<2e-16 ***
Population	1	0.0010	295	2.1015	0.1549	0.6942
Temperature	4	0.0445	291	2.0570	1.7179	0.1462
Treatment:Population	2	0.0023	289	2.0548	0.1738	0.8406
Treatment:Temperature	8	0.0824	281	1.9724	1.5905	0.1275
Population:Temperature	4	0.0112	277	1.9612	0.4322	0.7853
Treatment:Population:Temperature	8	0.0315	269	1.9297	0.6088	0.7702
Rate of endosperm rupture (d^{-1})	**Df**	**Deviance**	**Resid. Df**	**Resid. Dev**	***F***	***P* (>F)**
NULL			283	64.390		
Treatment	1	12.1639	282	52.226	1.047.452	< 2.2e-16 ***
Population	1	0.3706	281	51.855	3.1910	0.0751923
Temperature	4	20.3480	277	31.507	43.8047	< 2.2e-16 ***
Treatment:Population	1	0.9061	276	30.601	7.8028	0.0055985 **
Treatment:Temperature	4	2.4064	272	28.195	5.1804	0.0004941 ***
Population:Temperature	4	0.6684	268	27.527	1.4388	0.2214675
Treatment:Population:Temperature	4	0.4923	264	27.034	1.0599	0.3768206
Germination (%)	**Df**	**Deviance**	**Resid. Df**	**Resid. Dev**	***F***	***P* (>F)**
NULL			89	5098.6		
Treatment	2	3640.4	87	1458.2	445.2532	< 2.2e-16 ***
Population	1	55.4	86	1402.8	13.5411	0.0005014 ***
Temperature	4	206.9	82	1196.0	12.6505	1.584e-07 ***
Treatment:Population	2	149.2	80	1046.8	18.2441	6.461e-07 ***
Treatment:Temperature	8	457.8	72	589.0	13.9978	3.024e-11 ***
Population:Temperature	4	165.5	68	423.5	10.1215	2.501e-06 ***
Treatment:Population:Temperature	8	167.1	60	256.4	5.1091	7.146e-05 ***

The mean critical embryo lengths calculated on seeds incubated at different germination conditions after W+C pre-treatment with a split seed coat but without endosperm rupture (as well as no radicle protrusion), were 0.115 ± 0.020 and 0.117 ± 0.023 mm for RO and RC populations, respectively (Fig. 2A). Within all treatments, no statistical significant differences ($P > 0.05$) were highlighted on the different temperatures tested in each population (Fig. 2A). However, incubation temperatures had a statistically significant effect ($P < 0.001$) on final embryo length respect to the initial embryo length, or that calculated at the end of W+C pre-treatment (Fig. 2A). Temperatures in

the control (0) had no effect on the embryo growth, and the differences between initial embryo lengths were due to the elapsed period from the initial to final (120 days) measurements (Fig. 2A). In both populations, values obtained at the end of W+C and during GA_3 showed values similar to critical embryo length, while at the end of 0 they were similar to initial embryo length (Fig. 2A).

Seeds exhibited a two-step germination which followed embryo growth, with a delay detected between testa rupture, when the endosperm was exposed due to the embryo elongation, and endosperm rupture due to radicle emergence (Fig. 2B). Treatments and temperatures had a statistical significant effect ($P < 0.001$) on the rate of endosperm rupture in both populations (Table 1; Fig. 2B). After W+C treatment, the mean time course from testa to endosperm rupture (i.e. radicle protrusion) decreased with increasing temperature, ranging from 0.17 ± 0.05 $days^{-1}$ at 10°C to 0.77 ± 0.28 $days^{-1}$ at 20°C for RO, and from 0.16 ± 0.08 $days^{-1}$ at 10°C to 0.75 ± 0.30 $days^{-1}$ at 20°C for RC populations (Fig. 2B). At 25°C and at 25/10°C the mean time course increased with a rate of 0.41 ± 0.25 and 0.55 ± 0.21 $days^{-1}$ for RO, and 0.35 ± 0.23 and 0.53 ± 0.28 $days^{-1}$ for RC population (Fig. 2B). In the GA_3 treatment, the mean time course from testa to endosperm rupture was slower respect to after the W+C treatment (Fig. 2B) and, in particular, this difference was more evident in seeds of RO population, with a rate of 0.05 ± 0.01 $days^{-1}$ at 10°C and of 0.28 ± 0.22 $days^{-1}$ at 25°C (Fig. 2B).

While no seeds germinated during the control (0), they germinated with high percentages (> 50%) both after W+C and during GA_3 treatments in each population (Fig. 2C). Statistically significant differences ($P < 0.001$) among temperatures were detected within each treatment, except for seeds of RC treated with GA_3 ($P > 0.05$) where the germination range were from ca. 52% (at 10°C) to ca. 80% (at 25°C; Fig. 2C). GA_3 treated seeds of the RO population germinated with a range from 12% (at 10°C) to 62% (at 20°C; Fig. 2C). After W+C, high germination was detected at 25°C (88 ± 6%) for RO, and at 15°C (81 ± 12%) for RC (Fig. 2C).

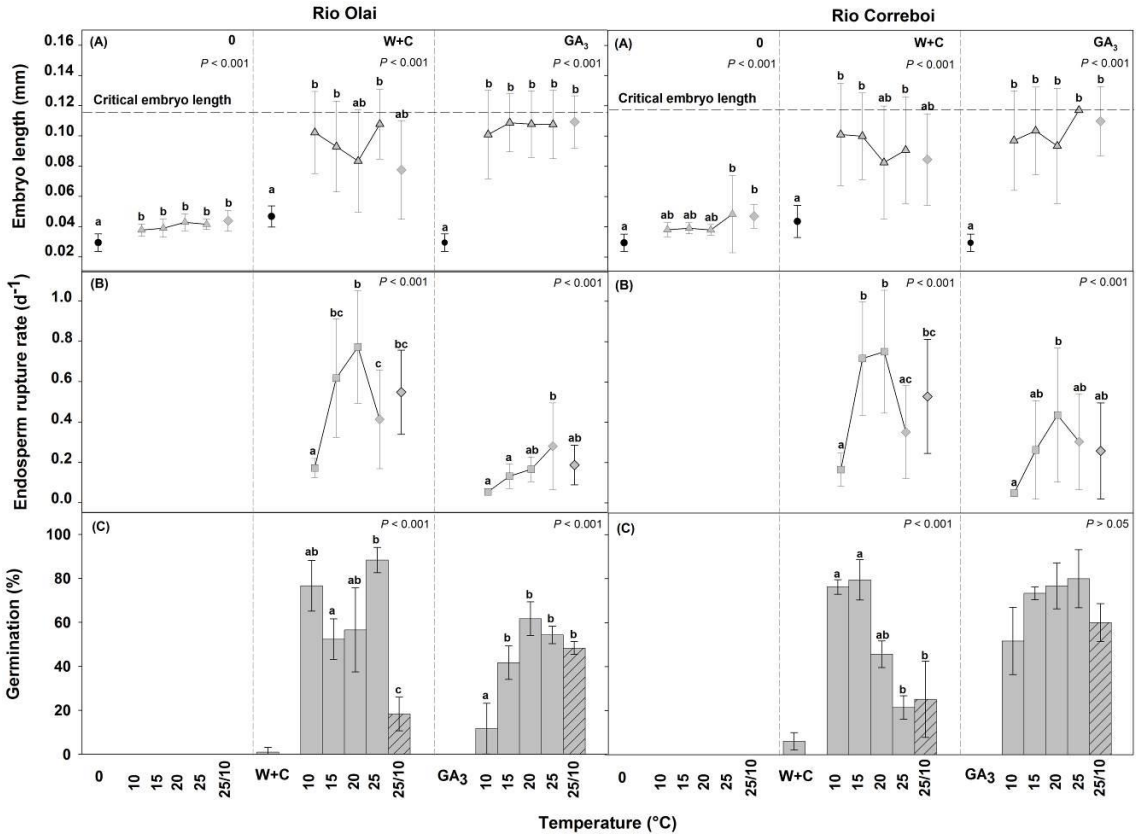

Figure 2 – Final embryo length values (A), time from seed coat splitting to endosperm rupture (B) and cumulative germination percentages (C) achieved at the end of germination tests (120 days), after each pre-treatment (0, control; W+C, 25°C for 3 months and then 5°C for another 3 months; GA_3, 250 mg l^{-1} in the germination substrate) for each population (Rio Olai and Rio Correboi). Embryo lengths measured at the start of germination tests (initial embryo length) are reported as a reference for the control and GA_3 while the value assessed at the end of pre-treatment is reported for W+C (black circles; A). The results in the alternating temperature regime (25/10°C) are here highlighted with a grey diamonds (A and B) and grey coarse bar (C). Data are the mean of 10 seeds (± SD) for embryo measurements, 20 (± SD) seeds (when available) for endosperm rupture rate and 3 replicates (± SD) of 20 seeds each for germination data. Dash lines (A) correspond to the Critical embryo length. General linear models (GLMs) were carried out within each treatment to test the effect of temperature on embryo growth, rate of endosperm rupture and germination. Values with the same letter are not different at $P > 0.05$ by *post hoc* pairwise *t*-test comparisons (with Bonferroni adjustment).

Thermal time approach on embryo growth

GLM analysis (Table 1) did not show statistically significant differences ($P > 0.05$) on embryo growth between populations, therefore a combined population response dataset was used to evaluate embryo thermal requirements, ascribing this characteristic to the species level. Seeds germinated after W+C and during GA_3 treatments showed differences on both critical embryo length rate ($1/t_e$) and cardinal temperatures (Fig. 3). Based on embryo length rate responses for each 10^{th} percentile (from 10% to 90%) of seeds that reached the critical embryo length, it was possible to estimate the mean base

temperature (T_{be}) in the sub-optimal temperature range for W+C and GA_3, and the mean ceiling temperature (T_{ce}) in the supra-optimal temperature range, and subsequently the optimal temperature for embryo growth (T_{oe}) for W+C (Fig. 3). Linear regressions for the different percentiles of sub-optimal temperature range for W+C were calculated passing through 5°C, which corresponds to an embryo growth rate equal to 0, value obtained at the end the W+C pre-treatment (see figure 1), and after were constrained to pass through the common value of T_{be}. For the supra-optimal temperature range, linear regressions were constrained to pass through the common value of T_{ce}. Linear regressions for the different percentiles for GA_3 were constrained to the common value of T_{be}. These models showed higher values of r^2 for all of the linear regression equations, than the model where T_{be} and T_{ce} varied for each percentile. Average T_{be} were 5.20 ± 0.60 and 5.30 ± 2.56°C for W+C and GA_3 treatments, respectively (Fig. 3), without statistically significant differences among treatments ($P > 0.05$). Average T_{ce} for W+C was 29.52 ± 2.37°C, and the average T_{oe} was 15.00 ± 1.02°C (Fig. 3) whereas in GA_3 treatment T_{oe} may be assumed as ≥ 25°C (Fig. 3).

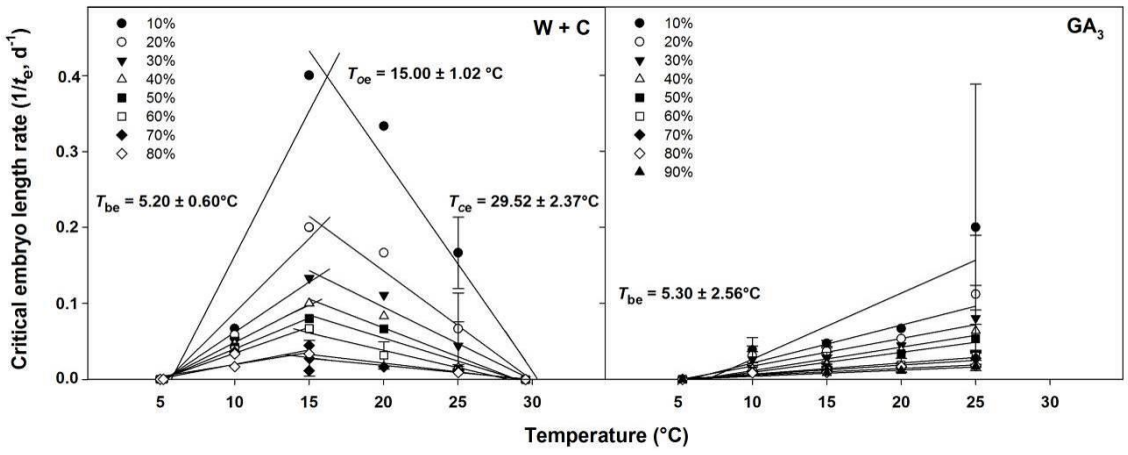

Figure 3 - Cardinal temperatures (T_{be}, base temperature, T_{oe}, optimal temperature and T_{ce}, ceiling temperature) to reach critical embryo length for seeds of *A. barbaricina*, calculated after W + C (25°C for 3 months and then 5°C for another 3 months) and incubated at a different range of germination temperatures (10, 15, 20 and 25°C), and T_{be} calculated after GA_3 (250 mg l^{-1} in the germination substrate) treatment and incubated at constant temperatures in the suboptimal range (≤ 25°C). Linear regressions for the different percentiles of sub-optimal temperature range for W+C were calculated passing through 5°C, which corresponds to an embryo growth rate equal to 0, value obtained at the end of the W+C pre-treatment (see figure 1), and after were constrained to pass through the common value of T_{bg}; for the supra-optimal temperature range, linear regressions were constrained to pass through the common value of T_{ce}. Linear regressions for the different percentiles for GA_3 were constrained to the common value of T_{bg}. Percentiles for which regression lines had a $P > 0.05$, T_{bg} and T_{ce} values were not calculated.

Figure 4 shows the relationship between log thermal time (θ_e) and percentages of seeds that reached the critical embryo length expressed in probits, calculated according to Eq. 5. The relationship between log θ_e and probit critical embryo length had better residual sums of square (0.1420 for W+C and 0.1228 for GA_3) and r^2 (0.95 and 0.97 for W+C and GA_3, respectively) than when expressed on a linear scale (data not shown). Thermal time required for 50% of seeds to reach the critical embryo length (θ_{e50}) was greater for the GA_3 with value of 2.64 log °Cd compared to the W+C treated seeds with value of 2.10 log °Cd. However, seed of W+C and GA_3 that reach the critical embryo length showed a very similar σ value (0.51 and 0.43°Cd, respectively; Fig. 4).

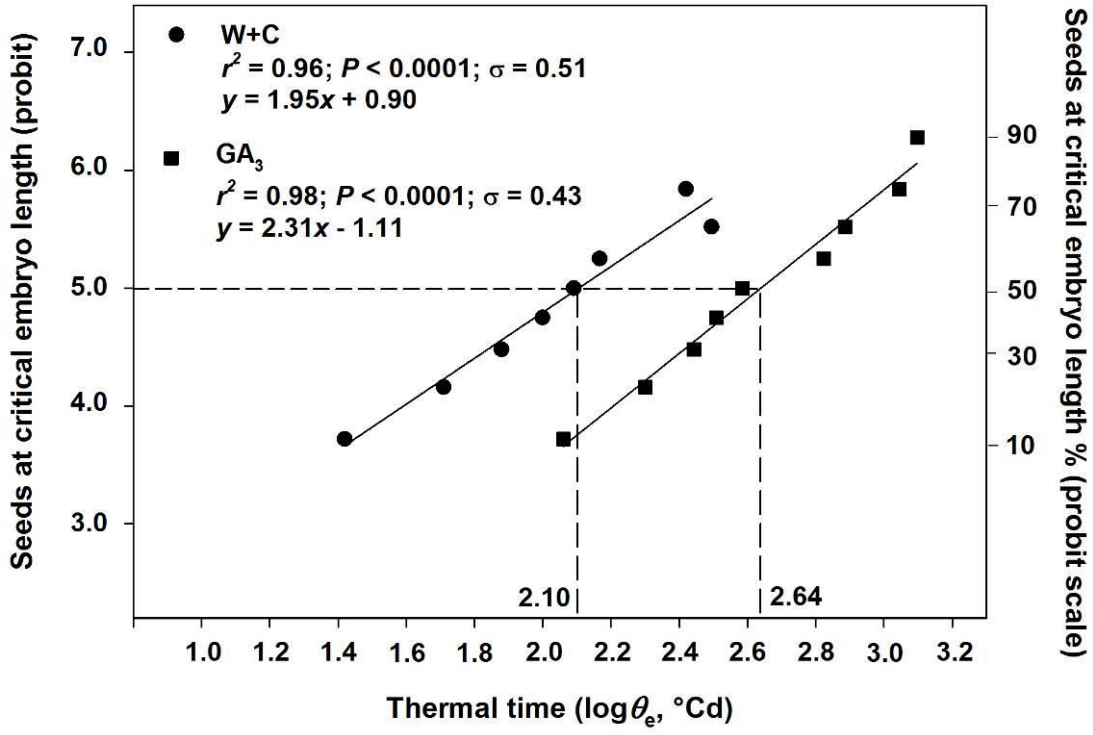

Figure 4 - Probit percentages of seeds of *A. barbaricina* that reached the critical embryo length after W + C (25°C for 3 months and then 5°C for another 3 months) and after GA_3 (250 mg l^{-1} in the germination substrate) treatments as a function of log thermal time requirement (log θ_e). Thermal times were calculated from critical embryo length time-courses assuming T_b of 5.20 and 5.30°C, for W + C and GA_3, respectively. Thermal times to reach 50% of seeds that reached the critical embryo length (θ_{e50}) are also reported.

Thermal time approach on seed germination

The T_{bg} for RO population were 6.85 ± 0.26°C for W+C and 8.43 ± 1.53°C for GA_3 treatment, while for RC population were 5.34 ± 1.38 and 5.42 ± 0.26°C for W+C and

GA_3 treatment, respectively (Fig. 5). These values were statistically different ($P < 0.01$) by GLM and a *post-hoc* pairwise comparisons *t*-test (with Bonferroni adjustment) highlighted that this difference was determined by the T_{bg} value of GA_3 treated seeds belonging to RO population (Fig. 5). For each treatment on both populations, the linear regressions were re-calculated for each percentile, constraining them to pass through the mean T_{bg} (Fig. 5). This model led to no differences in residual sum of squares compared with when T_{bg} was allowed to vary for each percentile, and showed highest values of r^2 for all of the linear regression equations ($r^2 > 0.91$ for RO W+C, $r^2 > 0.58$ for RC W+C, $r^2 > 0.88$ for RO GA_3 and $r^2 > 0.57$ for RC GA_3).

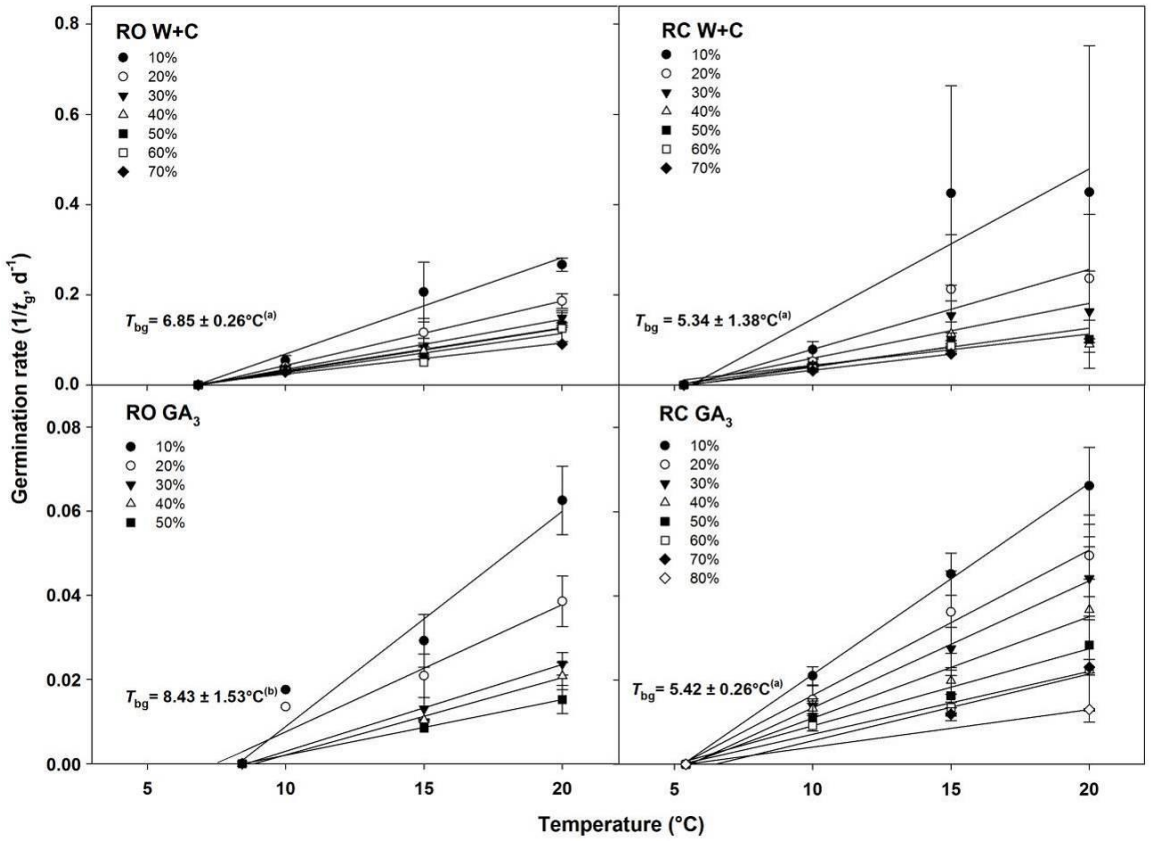

Figure 5 - Base temperatures for germination (T_{bg}) for the two populations (RO, Rio Olai; RC, Rio Correboi) of *A. barbaricina*, calculated after W+C (25°C for 3 months and then 5°C for another 3 months) and GA_3 (250 mg l^{-1} in the germination substrate) treatments, and incubated at constant temperatures (10–20°C). Within each population, the linear regressions for the different percentiles were constrained to the common value of T_{bg}. Percentiles for which regression lines had a $P > 0.05$, T_{bg} values were not calculated.

Figure 6 shows the relationship between log thermal time (θ_g) and germination expressed in probits, calculated according to Eq. 2. The relationship between log θ_g and probit germination had better residual sums of square both in W+C pre-treated seeds

(0.1349 and 0.1851 for RO and RC populations, respectively) and in the GA_3 treated seeds (0.0098 and 0.1477 for RO and RC populations, respectively) as well as for r^2 with values of 0.94 for RO and 0.92 for RC population in W+C pre-treated seeds, and 0.99 and 0.96 in GA_3 treated seed for RO and RC populations, respectively, than when expressed on a linear scale (data not shown). Thermal time required for 50% of germination (θ_{g50}) was greater for the GA_3 treated seeds (2.88 and 2.72 log °Cd for RO and RC, respectively), compared to the W+C pre-treated seeds (2.04 and 2.02 log °Cd for RC and RO, respectively; Fig. 6). In addition, GA_3 treated seeds of RO had a greater σ value (0.45 log °Cd) than the seeds belonging to RC population (0.33 log °Cd) and of those W+C pre-treated seeds (0.38 log °Cd and 0.26 log °Cd for RC and RO populations, respectively; Fig. 6).

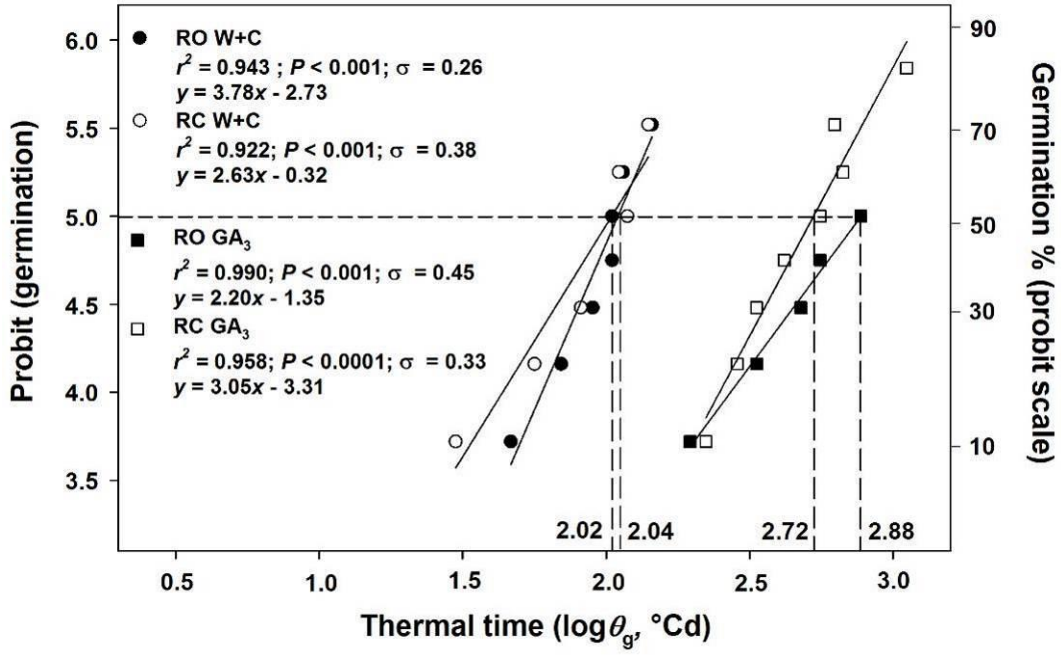

Figure 6 – Probit germination after W+C (25°C for 3 months and then 5°C for another 3 months) and after GA_3 (250 mg l^{-1} in the germination substrate) treatments for each populations (RO, Rio Olai; RC, Rio Correboi) as a function of log thermal time requirement (log θ_g). Thermal times were calculated from germination time-courses assuming T_b of 6.85°C and 5.34°C for W+C, and 8.43°C and 5.42°C for GA_3, for RO and RC, respectively. Thermal times to reach 50% of germination (θ_{g50}) are also reported. Linear regression of W+C for RC was calculated without the value obtained for g = 40. Thermal times to reach 50% of germination (log θ_{g50}) are also reported.

Discussion

Type of dormancy

The embryo in seeds of *Aquilegia barbaricina* is small at dispersal and must grow before radicle emergence. Therefore, following the dormancy classification system (Baskin and Baskin 1998, 2004), these seeds are morphologically dormant (MD). Generally, if embryos have only MD, growth is completed in a relatively short period, and seeds germinate in 30 days or less (Baskin and Baskin, 2004). *A. barbaricina* seeds of each population did not germinate without any treatment, even after 120 days. After warm and cold stratification or GA_3 treatment, seeds started to germinate (radicles emerged) at all tested temperature, due to increased embryo growth. Thus, seeds of this species also have a physiological component of dormancy (PD), and are morphophysiologically dormant (MPD), as previously reported by Mattana *et al.*, (2012). The request of both warm and cold stratifications, as well as the ability of exogenous GA_3 to overcome the physiological dormancy detected for this species, indicate an intermediate simple MPD (*sensu* Baskin and Baskin, 2004). A wide variety of dormancy types in Ranunculaceae species exists (Baskin and Baskin, 1998). For example, seeds of *Delphinium tricorne* and *Caltha leptosepala* of North America, and *Aconitum lycoctonum* of Western Europe have deep complex MPD (Baskin and Baskin, 1994; Forbis and Diggle, 2001; Vandelook *et al.*, 2009); non-deep complex MPD has been observed in seeds of the South European perennial *Eranthis hiemalis* (Frost-Christensen, 1974); epicotyl dormancy has been found in the North American *Hepatica acutiloba* and *Cimicifuga racemosa* (Baskin and Baskin, 1985), and deep simple epicotyl MPD has been observed in seeds of the European *Anemone nemorosa* (Mondoni *et al.*, 2008). The difference of dormancy type present in Ranunculaceae may result from an adaptation to specific habitat conditions and to the wide distribution of this family. Vandelook *et al.* (2009) reported that more distantly related species of the Ranunculaceae with similar habitat preferences have developed different dormancy breaking requirements, and this might be the result of independent evolutionary adaptations within the Ranunculaceae, resulting in differing dormancy breaking requirements.

Multiphasic seed germination

Testa and endosperm rupture has been identified as two sequential steps during seed germination in many species (i.e. Liu *et al.*, 2005; Manz *et al.*, 2005; Müller *et al.*, 2006; Petruzzelli *et al.*, 2003). This study confirmed the presence of two-step germination in Ranunculaceae member, as previously reported by Hepher and Roberts (1985) in *Trollius ledebouri* seeds. Data obtained from this study highlighted that seeds of *Aquilegia barbaricina* exhibited a multi-step seed germination. In particular, at least 3 phases may be identified after imbibition: (I) the embryo grows inside the seed, (II) seed coat splits and, (III) the endosperm weakens allowing the radicle protrusion. Is known that the inhibitory effect of ABA is counteracted by gibberellin and that endosperm rupture is under the control of an ABA – gibberellin antagonism (Koornneef *et al*., 2002; Leubner-Metzger, 2003; Kucera *et al*., 2005; Weitbrecht *et al.*, 2011). In seeds of *A. barbaricina,* the effect of GA_3 increased the meantime course rate from testa and endosperm rupture, compared to W+C stratified seeds. In addition, statistical difference was detected between temperatures in each treatment, leading to the hypothesis that, as occurs in the treatments, also the temperature may have effects on meantime course of this events. Therefore, the effects of gibberellins not only promoted embryo growth, but also endosperm rupture and radicle protrusion.

A an overall, without considering the incubation temperature, non-dormant seeds (i.e. after warm and cold stratification), reached their critical embryo length after less than 2 days of incubation, while the seed coat started to split and the radicle to protrude after ca. 6 days and then they continue with an overlap among all the phases (Fig. 7). This overlap suggests that the seeds coat may start to split when embryos are still growing, before they reach their "critical length" for germination and that radical protrusion follows immediately the split of the seed coat (Fig. 7).

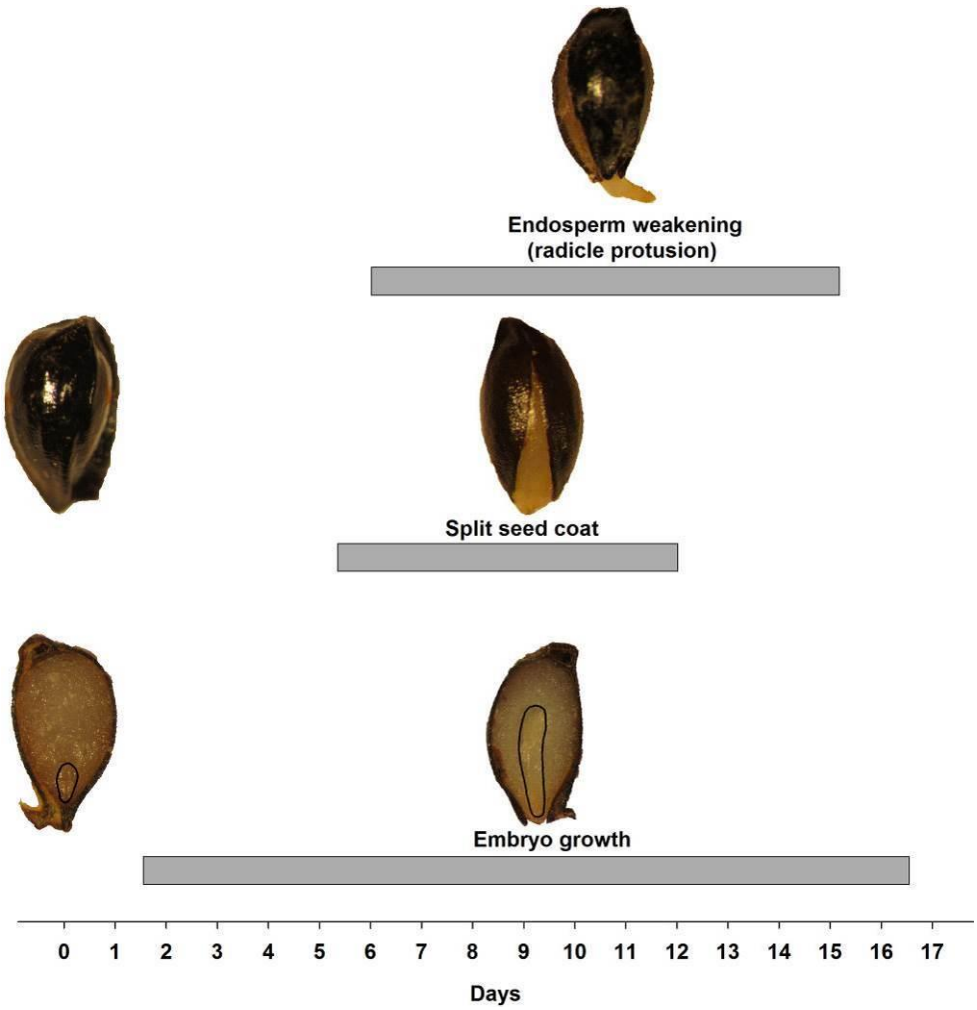

Figure 7 – Interval of time (in days) to complete the critical embryo growth, split seed coat and radicle protrusion events in non-dormant (i.e., during incubation after warm and cold stratification) seeds of *Aquilegia barbaricina*.

Thermal thresholds for embryo growth and seed germination

The base temperature for embryo growth rate (T_{be}) of non-dormant seeds of *A. barbaricina* was approximately 5°C both in W+C-stratified and GA_3 treated seeds. For W+C pre-treated seeds it was possible to calculate all cardinal temperatures, with optimal temperature for embryo growth to ca. 15°C and ceiling temperature to ca. 29°C. Base temperature for germination (T_{bg}) varied from ca. 5 to 7°C in W+C stratified seeds, and from 5 to 8°C for GA_3 treated seeds, depending on the provenance. To our knowledge, this is the first report of T_b calculated for embryo growth. Considering that no seeds of *A. barbaricina* germinated without treatment at the tested constant temperatures, a $T_b \geq 25$ °C (i.e. the highest temperature tested) may be supposed for dormant seeds of the two investigated populations. However, this remains to be confirmed by incubating seeds without pre-treatments at higher temperatures (i.e. up to

30 °C). Similar trend was detected in seeds of *Vitis vinifera* subsp. *sylvestris* (Orrù *et al.*, 2012). As constraining the linear regressions of each percentile for germination through the mean T_b improved the residual sum of squares and r^2 values, T_b for embryo growth and for germination can be used to describe the whole population response in *A. barbaricina* seeds, as previously reported for other species (e.g. Covell *et al.*, 1986; Ellis *et al.*, 1987; Pritchard and Manger, 1990; Orrù *et al.*, 2012; Porceddu *et al.*, 2013). The best model was obtained by fitting germination expressed in probit and log-normal (log °Cd) rather than normal distributed thermal times (°Cd), as previously reported for other herbaceous (Covell *et al.*, 1986; Ellis and Butcher, 1988) and tree species (Pritchard and Manger, 1990; Porceddu *et al.*, 2013). Also regarding the thermal times of embryo growth rate was obtained the best model by fitting the values in probit and log-normal (log °Cd) compared to when normal distributed, confirming that this methodology increases the goodness of the model.

Seeds of *A. barbaricina* varied in their thermal time estimates to reach θ_{50}, depending on treatment. Pritchard *et al.* (1999) reported that treatments for dormancy release can modify the T_b for seeds belonging to the same population. In this study, W+C pre-treatment increased the rate of accumulation of thermal units for embryo growth (°Cd), leading to a reduction in θ_{50} values from 2.64 log °Cd (ca. 440 °Cd) for GA_3 treated seeds to 2.10 log °Cd (128 °Cd) for W+C stratified seeds. Same trend was detected also for germination, with similar behaviour for Rio Correboi and Rio Olai populations, recording a reduction in θ_{50} values from ca. 2.80 log °Cd (ca. 650 °Cd) for GA_3 treated seeds to 2.03 log °Cd (110 °Cd) for W+C stratified seeds. Porceddu *et al.* (2013) detected, for *Rhamnus persicifolia* seed germination, a cold-induced decrease in θ_{50} from 2.59 log °Cd (385°Cd) to about 2.18 log °Cd (150°Cd), for untreated and cold stratified seeds, respectively. Similarly, a cold-induced decrease in θ_{50} have been reported also in *Polygonum aviculare* and in *V. vinifiera* subsp. *sylvestris* seeds (Batlla and Benech-Arnold, 2003; Orrù *et al.*, 2012). In *A. barbaricina* little differences in θ_{50} values for W+C treated seeds were detected between embryo growth (2.10 log °Cd) and seed germination (2.03 log °Cd); this allows to affirm that, to achieving of the units of thermal time for critical embryo length, the seeds reached also an amount of units of thermal time useful for germination. Therefore, the achievement of the critical embryo length and radicle protrusion can be described as two phases more or less overlapping in time, supporting the hypothesis showed in Figure 7. The analysis carried out in this study showed that in *A. barbaricina* the thermal requirements for embryo growth did

not vary among populations, while for seed germination these were different among populations. Embryo growth could be strictly related to the seeds biology of the species, while germination could be more related to the habitat of provenance of the species.

Conclusions

In conclusion, intermediate simple morphophysiological dormancy (MPD) was identified for *A. barbaricina* seeds. Thermal time model developed in this work allowed to identify the thermal thresholds (T_b and θ_{50}) requirements of embryo growth and seed germination of this species. In addition, results indicate that *A. barbaricina* showed a multi-step seed germination, with embryo growth representing the riskiest phase for the seed germination process of this species. This first attempt to model thermal requirement for embryo growth using a thermal time approach was confirmed by the morphological observations. This model has significant advantages over some previous models for estimation of germination, in particular for seeds that highlight a morphological component to dormancy, and may be useful to predict with a good accuracy the seedling emergence in the field (see Chapter 4).

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Chapter III - Sequential temperature control of multiphasic growth and germination of Paeonia corsica seeds

Abstract

- *Background and Aims*: Morphophysiological dormancy (MPD) is a class of seed dormancy in which the embryo is both underdeveloped and physiologically dormant. In this study, MPD was investigated in seeds of *Paeonia corsica* (Paeoniaceae).
- *Methods*: Seeds were incubated in the light at a range of temperatures (10 – 25 and 25/10 °C), without any pre-treatment, after W (3 months at 25 °C), C (3 months at 5 °C), W+C stratification (3 months at 25 °C followed by 3 months at 5 °C), and a GA_3 treatment (250 250 mg l^{-1} in the germination substrate). During germination tests, the time of seed coat and endosperm rupture were scored and embryo growth assessed. Epicotyl-plumule emergence were scored at 10, 15 and 20°C after control, C and W, and at 15°C during GA_3 treatment.
- *Key Results*: Embryos were small at seed dispersal, with an initial embryo:seed (E:S) ratio of ca. 0.3 (embryo length, ca. 1.4 mm), whereas the critical E:S ratio for germination was twice as long (ca. 0.6 with embryo length of ca. 3.9 mm). Testa and endosperm ruptures were identified as sequential events in seeds of this species. GA_3 and W followed by low temperature (≤ 15°C) promoted embryo growth (maximum growth rate of ca. 0.04 mmd^{-1}) and subsequent seed germination (i.e. radicle emergence; ca. 65%). Low germination occurred at warmer temperatures (> 20°C) only for GA_3 treated seeds, and cold stratification induced secondary dormancy, even when applied after warm stratification. After radicle emergence, epicotyl–plumule emergence was delayed for ca. 3 months. Mean time of epicotyl–plumule emergence was positively affected by cold stratification and GA_3.
- *Conclusions*: Seeds of this species showed non-deep simple (root) - non-deep simple (epicotyl) morphophysiological dormancy. *P. corsica* seeds exhibited differential temperature sensitivity for the sequential steps in the development process that resulted in the precise and optimal timing of seedling emergence.

Keywords: cold stratification; embryo growth; epicotyl dormancy; morphophysiological dormancy; Paeoniaceae; warm stratification.

Introduction

Seed dormancy is an important adaptation to prevent germination before or during unfavourable environmental conditions for seedling development (Baskin and Baskin, 1998). The process of seed germination starts when the dry seeds come into contact with water and ends when the radicle has emerged through all the coats enveloping the embryo (Finch-Savage and Leubner-Metzger, 2006; Weitbrecht *et al.*, 2011). Testa rupture and endosperm rupture are two sequential steps during germination (e.g. Karssen 1976; Hepher and Roberts, 1985; Krock *et al.*, 2002; Liu *et al.*, 2005; Müller *et al.*, 2006; Linkies *et al.*, 2009). In many plant species the seed-covering layers impose a physical constraint to radicle protrusion, which has to be overcome by the growth potential of the embryo (Kucera *et al.*, 2005; Muller *et al.*, 2006). Two-step germination is widespread over the entire phylogenetic tree and has been described for many plant families, e.g. for Ranunculaceae (Hepher and Roberts, 1985), Amaranthaceae (Karssen, 1976), Solanaceae, (Krock *et al*., 2002; Petruzzelli *et al*., 2003) and Brassicaceae (Liu *et al.*, 2005; Muller *et al.*, 2006).

In seeds with underdeveloped embryos, if embryo growth and radicle emergence are completed in about 30 days under suitable conditions, seeds have morphological dormancy (MD); if germination is delayed for more than about 30 days and seeds require a dormancy-breaking treatment such as exposure to moist cold (0–10°C) and/or to moist warm (≥ 15°C) stratification to germinate, they are described as having morphophysiological dormancy (MPD; Nikolaeva, 1977; Baskin and Baskin, 1990, 1998). Nine types of MPD have been defined, based on temperature requirements for embryo growth, the breaking of physiological dormancy (PD), and on the ability of gibberellic acid to overcome dormancy (Baskin and Baskin, 2004; Baskin *et al.*, 2008).

Abscisic acid (ABA) and gibberellic acid (GA) play an important role in a number of physiological processes of seed germination. ABA induces dormancy while GA plays a key role on dormancy release and germination (Finch-Savage and Leubner-Metzger, 2006). High ABA:GA ratio maintains dormancy, while dormancy release involves a net shift to increased GA biosynthesis and ABA degradation resulting in low ABA:GA ratio (Ali-Rachedi *et al.*, 2004; Cadman *et al.*, 2006; Liu *et al.*, 2010). These two hormones may act also in the promotion of testa and endosperm rupture (Finch-Savage and Leubner-Metzger, 2006). In *Lepidium sativum* L. and *Arabidopsis thaliana* (L.) Heynh. endosperm rupture is promoted by GA and inhibited by ABA and *Lepidium*

endosperm weakening is known to be promoted by GA and inhibited by ABA (Müller *et al.*, 2006).

According to Martin (1946) and Baskin and Baskin (1998), Paeoniaceae have seeds with rudimentary embryos, thus they need to grow before the seed germinates. Seed dormancy has been studied in different species of Paeoniaceae, and all of them have MPD (Barton, 1933; Nikolaeva *et al.*, 1985; Wang and van Staden, 2002). Saunders (1918) observed that epicotyl growth was delayed after radicles emerged from seeds of *Paeonia suffruticosa* Andrews, and Barton (1933) found that epicotyl dormancy of seeds of this species could be broken by exposing germinated seeds at temperature of ca. 5°C. Deep simple epicotyl MPD has been found in *P. officinalis* L. and *P. ostii* T.Hong & J.X.Zhang var. *lishizhenii* B.A.Shen (Nikolaeva *et al.*, 1985; Wang and van Staden, 2002), and non-deep simple morphophysiological dormancy in *P. californica* Nutt.(Schlising, 1976). More recently, Hao *et al.* (2013) correlated root length and epicotyl–plumule germination in *P. ludlowii* (Stern & G.Taylor) D.Y.Hong seeds, highlighting an essential root lengths ≥ 6 cm for epicotyl dormancy release by cold stratification. Germination of Paeoniaceae is hypogeal. During hypogeal germination, cotyledons remain inside the seed coat and stay below the surface of the soil as there is no substantial elongation of hypocotyl, while the epicotyl is released from the seed through extension of the cotyledonary petioles (Sadhu, 1989). Schlising (1976) reported that elongation of cotyledon bases during hypogeal germination of *P. californica* seems to permit optimal germination at soil depths of only 2-3 cm.

The taxonomy of the genus *Paeonia* in central Mediterranean islands was extremely controversial and unclear, especially in Sardinia (see Moris, 1837; Cullen and Heywood, 1964; Pignatti, 1982; Akeroyd, 1993; Cesca *et al.*, 2001). Currently, as reported by Hong (2005), De-Yuan and Xiao-Quan (2006) and Bacchetta *et al.* (2012), only *Paeonia corsica* Sieber *ex* Tausch grows in Sardinia, and there is no information on seed dormancy and germination strategies for this species.

Therefore, the main aim of this study was to investigate the seed germination ecology of *P. corsica*, in order to: (i) identify the class of dormancy *sensu* Baskin & Baskin (2004), and if MPD is present, at what level; (ii) evaluate the sequential steps during seed germination.

Materials and Methods

Study species and seedlot details

Paeonia corsica is a geophyte entirely glabrous, very occasionally pubescent on the lower surface of leaves. This species is characterized by mostly 9 leaflets often rather densely holosericeous beneath and mostly short-tomentose carpels with the widest part above the middle, which distinguish it from the related species, *P. mascula* (L.) Mill., *P. coriacea* Boiss. and *P. cambessedesii* (Willk.) Willk. (De-Yuan and Xiao-Quan, 2006). *P. corsica* is confined only to Corsica and Sardinia (Bacchetta *et al.*, 2012). In Sardinia, the species grows from 400 to 1700 m a.s.l. in different geological substrates (sedimentary, volcanic and metamorphic rocks), and it prefers deep, rich and wet soils. Flowering occurs from late March to early May, and the fruiting period usually occurs from early September to October.

Seeds of *P. corsica* were collected directly from plants near and under riparian woods of *Alnus glutinosa* (L.) Gaertn. at the time of natural dispersal in September 2011, at ca. 1,200 m a.s.l. along the Rio Correboi (Villagrande Strisaili, OG) in CE Sardinia (Italy).

Experimental trials

3 replicates of 20 seeds each per condition (Table 1), were sown in September 2011, on the surface of 1% agar water in 90-mm diameter plastic Petri dishes and incubated in the light (12 h light / 12 h dark) at a range of germination temperatures (10, 15, 20, 25 and 25/10°C). In the alternating temperature regime, the 12-h light period coincided with the elevated temperature period. Further replicates were given a warm (W = 25°C for 3 months) and a cold stratification (C = 5°C for 3 months) and a combination of them (W+C), before being incubated at the range of germination temperatures (Table 1). 3 extra replicates of 20 seeds each were sown on the surface of 1% agar water with 250 $mg \cdot l^{-1}$ GA_3 and incubated at the range of germination temperatures (Table 1).

As different developmental steps on the seed germination of this species were identified during this study, embryo growth, time from testa to endosperm rupture, radicle and epicotyl-plumule emergence were measured as separate phases.

Table 1 - Experimental design.

Condition		Embryo growth measurements	
Code	**Description**	**Number of measurements**	**Timing**
0	Control	5	After 15, 30, 60, 90 and 120 days at 10-25°C and 25⁄10°C.
W	3 months, 25°C	8	After 30, 60 and 90 days during warm stratification (W), 15, 30, 60, 90 and 120 days after sowing for germination at 10-25°C and 25⁄10°C.
C	3 months, 5°C	8	After 30, 60 and 90 days during cold stratification (C), 15, 30, 60, 90 and 120 days after sowing for germination at 10 - 25 and 25⁄10°C..
W + C	3 months, 25°C (W) → 3 months, 5°C (C)	12	After 30, 60 and 90 days during warm (W), 15, 30, 60 and 90 days during cold (C), 15, 30, 60, 90 and 120 days after sowing for germination at 10-25°C and 25⁄10°C.
GA_3	GA_3 (250 $mg \cdot l^{-1}$) in germination medium	5	After 15, 30, 60, 90 and 120 days at 10-25°C and 25⁄10°C.

Embryo measurements

Embryo growth, during the above described conditions and germination temperatures, was assessed at different times (Table 1) by measuring 10 seeds for each sample interval. Seeds were cut in half under a dissecting microscope and images of embryos acquired using a Zeiss SteREO Discovery.V8, with an objective Achromat S 0.63x, FWD 107mm (Carl Zeiss MicroImaging GmbH) at 5.0x magnification, coupled to a Canon (Power shot G11) digital camera. Embryo (E) and seed (S) lengths were measured using the image analysis software ImageJ 1.41o (National Institutes of Health, Bethesda, MA, USA). Seed length was measured excluding the thickness of the seed coat and the embryo to seed length (E:S) ratio calculated for each seed. The initial E:S ratio was calculated by measuring 20 randomly selected seeds before the start of the experiments. The critical E:S ratio of seeds with a split seed coat but no radicle protrusion (i.e. when the endosperm was exposed) was determined for 20 randomly selected seeds and used for seeds that had germinated before measurements commenced (Vandelook *et al.*, 2007).

Endosperm rupture and radicle emergence

During tests, seeds with split seed coat were scored, and the time from seed coat splitting to endosperm rupture (i.e. when the radicle emerges) was monitored in 15 seeds for each condition. Germination was defined as visible radicle emergence. Germinated seeds were scored 3 times a week. Germination tests lasted for a minimum of 1 month and a maximum of 4 months. When no additional germination had occurred for 2 weeks, a cut-test was carried out to estimate the viability of the remaining seeds. The final germination percentage was calculated as the mean of 3 replicates (± 1 SD), on the basis of the total number of firm seeds.

Epicotyl dormancy release

To evaluate the delay of the epicotyl–plumule germination (i.e. when the epicotyl or the first true leaf was emerged) after radicle protrusion in seeds of *P. corsica*, a warm pre-treatment (i.e. W = 3 months at 25°C on the surface of 1% agar water, see Table 1), was applied in March 2012 to 200 seeds before incubation for germination at 15°C. Germinated seeds were then: (A) kept at 15°C on agar water for an additional 2-weeks period in order to allow root growth, before transplanting to a sterilised soil substrate of sand ⁄ soil / peat (1:1:1) at 10, 15 and 20°C; (B) moved to 5°C for 2 months on agar water, before transplanting to the soil substrate at 10, 15 and 20°C; (C) moved to 25°C for 2 months on agar water, before transplanting to the soil substrate at 10, 15 and 20°C; and (D) kept at 15°C for 2 months on the surface of 1% agar water with GA_3 (250 mg l^{-1} in the germination substrate). For each condition 15 seeds were used. Epicotyl–plumule germination were scored twice per week. The mean time to epicotyl–plumule emergence for each condition was calculated on the basis of the total number of seedlings with the epicotyl–plumule emerged. When no additional radicle or epicotyl–plumule germination occurred for 2 weeks, after a minimum of 4 months, both experiments were stopped.

Statistical analysis

Generalized Linear Models (GLMs) were used to evaluate the effect of pre-treatments (i.e. 0, W, C, W+C and GA_3) on embryo growth rate, E:S ratio and rate of endosperm rupture event. The GLM for final seed germination percentages was unbalanced due to

many 0 values and was not carried out. The effect of incubation temperature within each pre-treatment was also assessed by GLM for embryo growth rate, E:S ratio, rate of endosperm rupture event and final germination percentages. The effect of each condition on the percentages of epicotyl–plumule emergence were analyzed by GLM, based on the number of seeds with epicotyl–plumule emerged on the total of 15 germinated seeds for each condition, while GLM, based on the total of seeds with epicotyl–plumule emerged, was used to evaluate the effects of each condition on the time between radicle emergence and epicotyl–plumule emergence. Significant differences highlighted by GLM on embryo growth rate, E:S ratio, rate of endosperm rupture event and epicotyl–plumule, were then analysed by a *post-hoc* pairwise comparisons *t*-test (with Bonferroni adjustment). A log link function and quasipoisson error structure was used for analysing embryo growth rate, E:S ratio, rate of endosperm rupture event and epicotyl–plumule emergence. A logit link function and quasibinomial error structure was used for analysing seed germination percentages while a logit link function and binomial error structure was used for analysing epicotyl–plumule germination percentages. Quasipoisson and quasibinomial error structures and *F* tests with an empirical scale parameter instead of chi-squared on the subsequent ANOVA were used in order to overcome residual overdispersion (Crawley, 2007). All statistical analyses used R v. 2.14.0 (R Development Core Team, 2011).

Results

Embryo growth and root emergence

GLM highlighted a highly statistically significant effect ($P < 0.001$) of W and GA_3 treatments on embryo growth rate (Table 2). At 10 and 15°C, embryos of seeds from the W treatment grew with a mean rate of 0.027 ± 0.014 and 0.044 ± 0.004 mm d^{-1}, respectively, significantly faster ($P < 0.001$) than at 20 and 25°C and 25/10°C (ca. 0.01 mm d^{-1}; Fig. 1A). Seeds on GA_3 had embryos extending at ca. 0.03 mm d^{-1} at warm temperatures (≥ 15°C), significantly faster ($P < 0.01$) than at 10°C (0.016 ± 0.007 mm d^{-1}; Fig. 1A). Seeds of the control (0) and after C and W+C treatments embryos grew very slowly (≤ 0.01 mm d^{-1}) at all germination conditions, with no statistical differences among temperatures ($P > 0.05$; Fig. 1A).

The mean initial E:S ratio for *P. corsica* seeds was 0.27 ± 0.04, with a mean embryo length of 1.41 ± 0.21 mm and mean seed length of 5.19 ± 0.48 mm. The critical

E:S ratio for germination was 0.58 ± 0.09, with a mean embryo length of 3.95 ± 0.71 mm and mean seed length of 6.82 ± 0.56 mm. All treatments, except C, had a moderate statistically significant effect ($P < 0.05$) on E:S ratio (Table 2). At the last measurement (after 120 days from sowing or from moving after pre-treatments; see Table 1), seeds reached their critical E:S ratio for germination at 15°C for the W pre-treatment, while the mean E:S ratios were ca. 0.5 at 20°C, ca. 0.4 at 25°C and < 0.4 at 10 and 25/10°C, with these differences being statistically significant (Fig. 1B; $P < 0.001$). For control (0) and after C and W+C pre-treatments, E:S ratios were low (< 0.5) at all the tested temperatures. Highly statistically significant differences were detected among temperatures for the control (0) and W+C treatment ($P < 0.001$), while no statistical differences were detected after C ($P > 0.05$; Fig. 1B). GA_3 treated seeds reached their critical E:S ratio at warm temperatures (≥ 15°C) with high values (from ca. 0.6 to ca. 0.8), while at 10°C the mean E:S ratio was ca. 0.5, with these values being significantly different ($P < 0.001$; Fig. 1B).

Table 2 - GLMs results of embryo growth rate and E:S ratio of the following factors: 0, control; C, pre-chilling; W, warming; W+C; GA_3, 250 mg l^{-1}.

Embryo growth rate (mmd^{-1})	**Estimate**	**Std. Error**	**z value**	**Pr(>|z|)**
(Intercept)	0.001852	0.164247	0.011	0.991002
C	-0.366370	0.238691	-1.535	0.124804
W	0.704285	0.195433	3.604	0.000314 ***
W+C	0.084849	0.217609	0.390	0.696599
GA_3	1.008470	0.186343	5.412	6.24e-08 ***
E:S ratio	**Estimate**	**Std. Error**	**t value**	**Pr(>|t|)**
(Intercept)	-1.02452	0.04103	-24.972	< 2e-16 ***
C	0.01602	0.06068	0.264	0.79190
W	0.18858	0.05777	3.264	0.00122 **
W+C	0.13565	0.05829	2.327	0.02060 *
GA_3	0.46214	0.05238	8.823	< 2e-16 ***

*** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$

Germination followed the same trend as that detected for embryo growth, with high germination at 15°C, with 63 ± 10% and 63 ± 3% for W and GA_3 treatments, and low germination (7 ± 3%) at the end of the Control (0; Fig. 1C). Seeds also germinated at 10°C in W and GA_3 treatments (10 ± 9% and 15 ± 0%, respectively; Fig. 1C), but no germination was detected in the Control (Fig. 1C). Germination of 37 ± 21% was

obtained at 20°C in GA_3 treatment, but no germination was detected after W treatment and in the Control (Fig. 1C). Low germination (< 10%) was detected at 25 and 25/10°C in GA_3 treatment (Fig. 1C), while no germination occurred at warm (> 20°C) temperatures after W treatment and at temperatures > 15°C in the Control (Fig. 1C). No germination was detected at all temperature tested after C and W+C treatments (Fig. 1C).

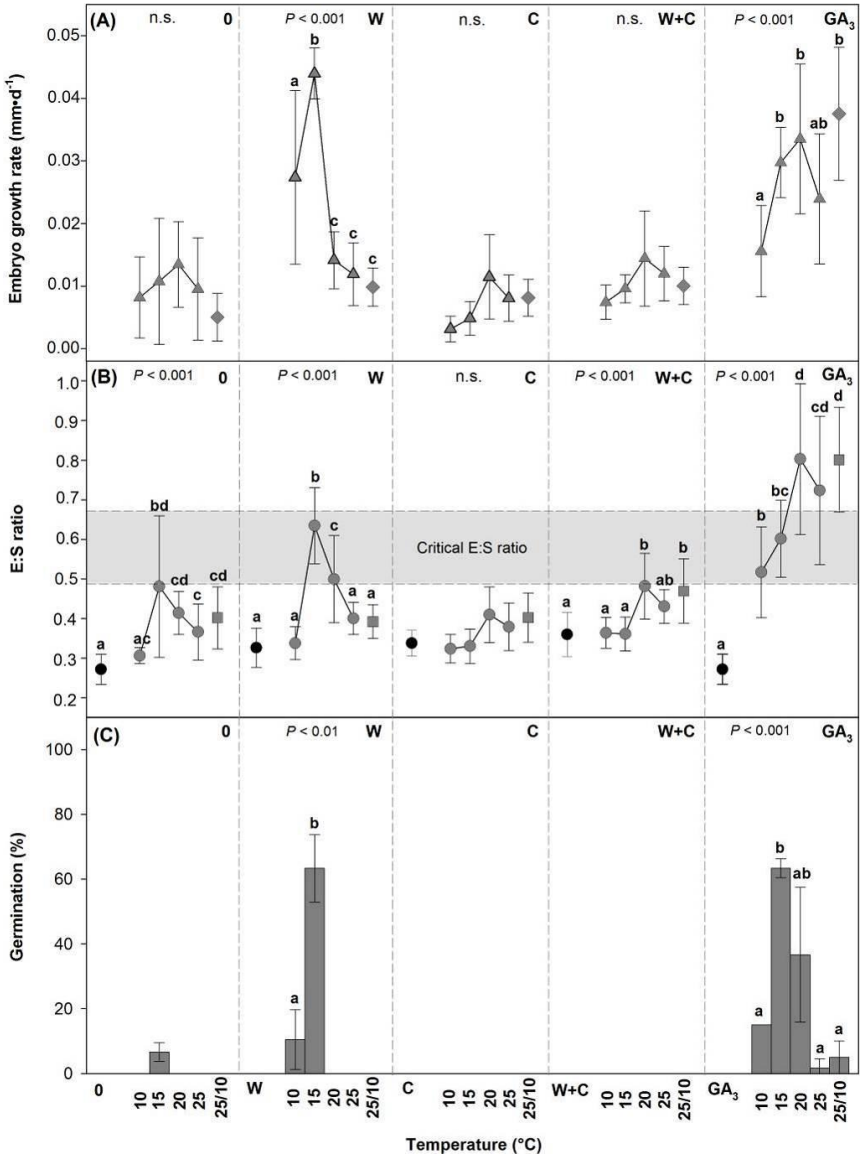

Figure 1 - Rate of embryo growth (A), final values of E:S ratio (B), and cumulative germination percentages (C) achieved at the end of germination tests (120 days), after each pre-treatment (0, control; W, 25°C for 3 months; C, 5°C for 3 months; W+C, 25°C for 3 months and then 5°C for another 3 months; GA_3, 250 mg l^{-1} of GA_3 in the germination substrate). E:S ratio measured at the start of germination tests and at the end of pre-treatments for W, C and W+C are reported here as a reference, with black circles (B). The results in the alternating temperature regime (25/10°C) are highlighted with a different symbol (diamonds and squares for embryo growth rate and E:S ratio, respectively) compared to constant temperature values. Data are the mean of 10 seeds (± SD) for embryo growth rate and E:S ratio, and of 3 replicates (± SD) for germination data. The gray band (B) corresponds to the range of values of the

critical E:S ratio calculated on 20 seeds. GLM was carried out within each pre-treatment to test differences in values of either embryo growth rate, E:S ratio, and germination data. Values with the same letter are not different at $P > 0.05$ by *post-hoc* pairwise comparisons *t*-test (with Bonferroni adjustment).

Testa and endosperm rupture events during germination

Paeonia corsica seeds exhibit different steps of germination, with a delay detected between testa rupture (i.e. when the endosperm was exposed by a split seed coat), and endosperm rupture (i.e. with radicle emergence). At 15°C after W and during GA_3 treatments, the mean time course from testa to endosperm rupture were 0.09 ± 0.07 $days^{-1}$ and 0.07 ± 0.07 $days^{-1}$, respectively, with these differences being not statistically significant at $P > 0.05$ (Fig. 2).

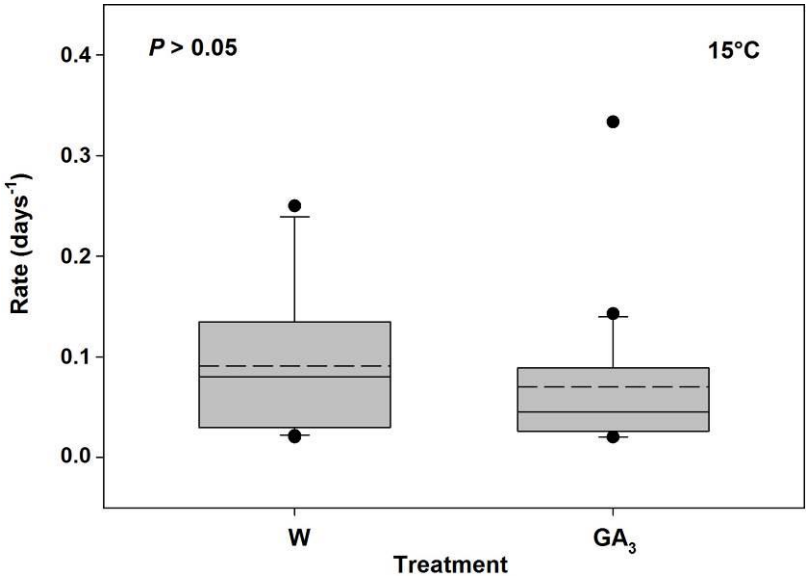

Figure 2 - Time course from testa to endosperm rupture at 15°C after W (3 months at 25°C) and with GA_3 (250 mg l^{-1} in the germination substrate) treatments. Data are the mean of 20 seeds. Dashed lines corresponds to the means.

Epicotyl–plumule germination

The epicotyl–plumule of seeds having radicle emerged, incubated without any treatment, emerged only at 10°C, with a value of 93% (Fig. 3A). After 2 months of warm stratification, epicotyl–plumule emerged only at 10°C with a value of 42% (Fig. 3A). After 2 months of cold stratification, 92% and 58% of epicotyl–plumule emerged at 10 and 15°C, respectively, and only 1 seed at 20°C (Fig. 3A). During 2 months of GA_3 treatment, epicotyl–plumule emerged from all germinated seeds (Fig. 3A). These differences in epicotyl–plumule emergence were statistically significant at $P < 0.001$,

but no statistical differences ($P > 0.05$) were detected at 10°C among treatments (Fig. 3A).

The different applied conditions of treatments and temperatures had a significant effect on the mean time for epicotyl–plumule germination ($P < 0.001$; Fig. 3B). After C, the highest mean rate was detected at 15°C (0.039 ± 0.022 $days^{-1}$) and then at 10°C after C (0.031 ± 0.013 $days^{-1}$) and GA_3 treatment at 15°C (0.023 ± 0.003 $days^{-1}$; Fig. 3B). Lower mean values were detected at 10°C after 0 and W (0.011 ± 0.002 $days^{-1}$ and 0.008 ± 0.000 $days^{-1}$, respectively; Fig. 3B), without statistical differences ($P > 0.05$) between these two conditions (Fig. 3B).

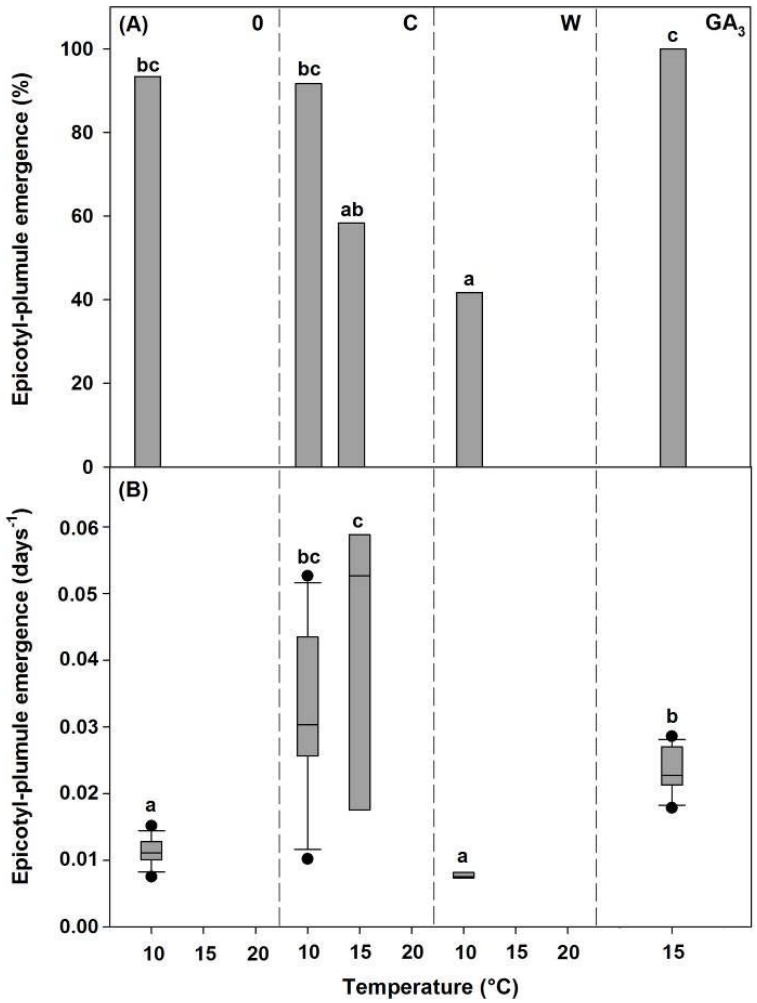

Figure 3 – Percentages (A) and days (B) of epicotyl–plumule emergence at 10, 15 and 20°C after each pre-treatment (0, control; C, 5°C for 2 months; W, 25°C for 2 months) and at 15°C during GA_3 treatment (GA_3, 250 mg l^{-1} in the substrate). N = 15 germinated seeds (when available) for each condition. GLM was carried out to test differences in values. Bars or box plot with the same letter are not different at $P > 0.05$ by *post-hoc* pairwise comparisons *t*-test (with Bonferroni adjustment).

Discussion

Morphophysiological dormancy

The embryo in seeds of *Paeonia corsica* is small at dispersal time and must grow before radicle emergence. Therefore, following the dormancy classification system (Baskin and Baskin 1998, 2004), these seeds are morphologically dormant (MD). Generally, if embryos have only MD, growth is completed in a relatively short period, and seeds germinate in 30 days or less (Baskin and Baskin, 2004). *P. corsica* seeds germinated to very low levels (ca. 7%) at 15°C without any treatment and more than 100 days were required to reach this percentage of germination. After warm stratification or GA_3 treatment, seeds start to germinate (radicles emerged) in ca. 30 days, due to increased embryo growth and seed germination rates. GA_3 treatment also widened the temperature range for germination in *P. corsica* seeds. Thus, seeds of this species also have a physiological component to dormancy (PD), and are morphophysiologically dormant (MPD). In addition, cold stratification (C) failed to break PD, and imposed secondary dormancy, also when preceded by warm stratification (W+C), delaying embryo growth and preventing seed germination even at 10 and 15°C. More recently, secondary dormancy induced by cold stratification has been reported also in seeds of *Poa laxa* subsp. *laxa* (Mondoni *et al.*, 2012) and *Ribes multiflorum* subsp. *sandalioticum* (Mattana *et al.*, 2012). Probert *et al.* (1989) highlighted that prolonged chilling of high-dormancy batches of *Ranunculus sceleratus* led to a reduction in both the rate and capacity of germination, indicating that chilling induced a secondary dormancy.

The MPD identified in *P. corsica* confirms the presence of this class of dormancy for Paeoniaceae, as already reported by other authors (Barton, 1933; Nikolaeva *et al.*, 1985; Wang and van Staden, 2002). In addition, the delay of about 1 month after pre-chilling and of 3 months after warming treatment, detected between onset of seed germination (root emergence) and epicotyl–plumule emergence in this species, suggested the presence of epicotyl dormancy, and can be described as a kind of simple epicotyl MPD. Epicotyl MPD have been found also in *P. suffruticosa*, *P. officinalis* and *P. ostii* var. *lishizhenii* seeds (Barton, 1933; Nikolaeva *et al.*, 1985; Wang and van Staden, 2002). Roots and shoots can have different levels of PD (Baskin and Baskin, 1983, 1986); therefore, to describe dormancy in seeds with simple epicotyl MPD, the level of PD (deep, intermediate and non-deep; Baskin and Baskin, 2004) in both the root and shoot must be described (Baskin *et al.*, 2009). Warm stratification and

GA_3 treatment enhanced embryo growth and subsequent seed germination at low temperatures; therefore, *P. corsica* roots have non-deep PD. Furthermore, epicotyl–plumule emergence was affected positively by chilling pre-treatment and GA_3 treatment, and this suggested that epicotyls of *P. corsica* have non-deep PD. Therefore, it is possible affirm that seeds of *P. corsica* have non-deep simple (root) – non-deep simple (epicotyl) MPD. This level of epicotyl MPD have been found by other authors in *Viburnum odoratissimum* (Baskin *et al.*, 2008), *Daphniphyllum glaucescens* subsp. *oldhamii* var. *oldhamii* (Baskin *et al.*, 2009) and in *Ribes multiflorum* subsp. *sandalioticum* (hereafter *R. sandalioticum*) seeds (Mattana *et al.*, 2012).

Morphophysiological dormancy is common among basal angiosperms (Baskin and Baskin, 1998), and embryo size increases from basal families having small embryos to the most derived families having larger embryos (Martin 1946; Forbis *et al*., 2002). However, a few families within the Saxifragales (i.e. Paeoniaceae and Grossulariaceae) have relatively small embryos, as does Parnassiaceae within the Celastrales (Forbis *et al*., 2002). *P. corsica* (Paeoniaceae) and *R. sandalioticum* (Grossulariaceae), two species that growth in the same ecosystem and ecological conditions in Sardinia, show the same class of dormancy, and the presence of MPD may be evidence to adaptation to common environmental conditions as suggested by Forbis *et al.* (2002).

Embryo growth and germination under GA_3 treatment

In non-dormant seeds of *P. corsica*, 10 and 15°C were the temperatures that stimulate embryo growth, with higher E:S ratio values than other temperatures (see Fig. 1). GA_3 treatment had a strong effect on E:S ratio value at all temperature tested, influencing also the embryo growth rate. Several authors have suggested a minimum embryo length for germination in species where embryos must elongate before radicle emergence (see Kondo *et al.*, 2004; Copete *et al.*, 2011; Mattana *et al.*, 2012). In *P. corsica* seeds, the final mean critical E:S ratio occurred in seeds incubated at 15°C in the control and at 10°C after W pre-treatment was lower for germination, while in GA_3 treated seeds the value was higher than the final mean critical E:S ratio calculated for germination. Therefore, germination can occur also when the critical value was not reached. In addition, GA_3 treated seeds incubated at high temperatures (> 20°C) showed that embryo can grow further the critical E:S ratio. Therefore, although GA_3 promoted embryo growth, it had no effect on germination at high temperatures (i.e. > 20°C). In

this work, the value of the mean critical E:S ratio for germination in *P. corsica* was reported as value comprised from minimum to maximum critical E:S ratio, according to Newton *et al.* (2013) that showed that, in seeds of *Narcissus pseudonarcissus* and *Galanthus nivalis*, the mean germination embryo length was attained or exceeded, while, germination at cooler temperatures, in some seeds, commenced before the germination embryo length was reached.

GA_3 treatment, moreover, besides acting on the embryo growth, also widened the temperature range for germination in *P. corsica* seeds, allowing low percentages of seed germination also at temperatures > 15°C. Similar behaviour was found by Mattana *et al.* (2012) in GA_3 treated seeds of *R. sandalioticum*.

Testa and endosperm rupture events during germination

The endosperm is known to act as a barrier for radicle protrusion and thereby the completion of germination in seeds from several angiosperm clades (see Hepher and Roberts, 1985; Karssen, 1976; Bewley, 1997; Leubner-Metzger, 2003; Muller *et al.*, 2006). Seeds of Paeoniaceae are anatropous, with testa constituted of many cells thick and the inner epidermis of the outer integument (endotesta) is unspecialized (Corner, 1976). Data from this study highlighted that *P. corsica* seeds exhibited a two-step germination, with a lapse of time from testa to endosperm rupture. However, statistical analysis showed no difference in the meantime course from testa to endosperm rupture in warm stratified and GA_3 treated seeds. It is known that the inhibitory effect of ABA is counteracted by gibberellin and that endosperm rupture is under the control of an ABA– gibberellin antagonism (Koornneef *et al*., 2002; Leubner-Metzger, 2003; Kucera *et al*., 2005; Weitbrecht *et al.*, 2011); in *P. corsica* it would appear that the effects of gibberellins promote and / or facilitate to endosperm rupture and radicle protrusion, however, specific tests on this topic need to be conducted (see Muller *et al.*, 2006).

Ecological correlates of seed germination

Seeds of *P. corsica* ripen in late summer and dispersal takes place in autumn, mainly by barochory. Following dispersal in early autumn, the seeds are exposed to a mean soil temperature < 20°C without having experienced a warm stratification, thus they stay dormant in the ground until the next summer when the seeds are exposed at a cycle of warm temperature, i.e. when the mean soil temperature is > 20°C. Once imbibed,

embryos may start to grow inside the seeds. However, it is only when mean soil temperatures drop below 15°C in October - November and following the periods of annual maximum precipitation that embryo growth reaches the critical E:S ratio (mean embryo length of ca. 4.0 mm), thereby allowing seeds to germinate. Germinated seeds go through the winter with an emerged radicle, and epicotyl–plumule emerge only after ca. 3 months (March - April), when mean soil temperatures again reach 10 – 15°C. Seedling establishment is completed before the end of the second wet season (May - June). Seedling growth can take place for 2 months, until the start of summer, so that the seedlings enter the dry summer period (June – August) with well-developed root and shoot systems.

Secondary dormancy of non-germinated seeds imposed by cold stratification in the first winter prevents radicle emergence in late spring and exposure of recently emerged seedlings to the dry summer conditions that would most likely kill them. However, the request of low temperatures (10 - 15°C) for the embryo growth and germination of non-dormant seeds, could suggest that these phases are the most sensitive to temperature, and could impact on germination phenology and/or could reduce the level of natural emergence in the field, highlighting an increasing threat from global warming.

Conclusions

Paeonia corsica showed non-deep simple (root) - non-deep simple (epicotyl) morphophysiological dormancy and a multi-step of seed germination from dispersal to seedling establishment was observed in this species. Similar pattern on seeds germination was detected for *R. sandalioticum*, where the species showed high specialisation with the Mediterranean seasonality (Mattana *et al.*, 2012), suggesting for this species and *P. corsica* that their embryo morphology and seed germination characteristics are closely tied to ecology, as a convergent response to similar environmental conditions due by the same ecosystem (Forbis *et al.*, 2002). This was confirmed in a related study (see Chapter 4) where the seeds were sown in their natural population and results highlight similar requirements for dormancy breaking, germination and epicotyl dormancy release for this two species.

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Chapter IV - Thermal time model predicts long-term in situ germination of endospermic seeds of three endemic Mediterranean mountain species

Abstract

- *Background and Aims*: The effect of environmental temperature conditions on embryo growth, seed germination and epicotyl emergence was assessed for three endemic Mediterranean mountain species and a soil heat sum model was used to predict their seed germination in the field.
- *Methods*: Seeds of each species were buried in the soil in their natural populations, both underneath and outside the tree canopy, and exhumed at regular intervals. Embryo growth measurements, seed germination and epicotyl emergence were assessed at different exhumation times. Soil temperatures were recorded using data loggers and soil heat sum (°Cd) was calculated and predicted on the basis of the estimated T_b, soil temperatures, and field germination. A soil heat sum approach was then applied to predict germination timing under two simulated IPCC scenarios (B1: +1.8 °C; A2: +3.4 °C).
- *Key Results*: θ_{50} for embryo growth (2.10 log °Cd) and germination (2.04 log °Cd) for *A. barbaricina* were reached in April. The highest field germination of *P. corsica* was recorded from September to November and estimated θ_{50} values falls within the range of 339.26 - 1367.68 °Cd, while highest germination of *R. sandalioticum* was recorded in December allowed to estimate the θ_{50} values falls within the range of 0 °Cd - 715.92 °Cd. Soil heat sum under the two different IPCC scenarios could lead the completion of germination forward by about 1 month for all three species.
- *Conclusions*: Field observations allowed to validate the thermal niche requirements for seed germination obtained in controlled condition, and the developed model based on soil heat sum approach allowed to estimate the thermal accumulation requirements and to predict with good accuracy the seed germination in the field. The model developed may have applicability to predictions of *in situ* regeneration under different scenarios of increasing temperatures.

Keywords: global warming, IPCC scenarios, thermal time, embryo growth, phenology, endemic vascular flora, Sardinia

Introduction

The Mediterranean climate is characterized by a high seasonality in temperature and precipitation, which leads to a hot drought in summer and a cool, wet, winter (Joffre *et al.*, 1999). This peculiarity has important implications for plant germination physiology, since dry summer conditions limit water availability and thus germination and growth, while cool winter temperatures can limit germination during the season with high water availability (Rundel, 1996). In seasonal climates, temperature is usually the main environmental factor influencing seed germination in moist soils (Fenner and Thompson, 2005).

Seed dormancy is an important adaptation to prevent germination before or during unfavourable environmental conditions for germination and subsequent seedling development (Baskin and Baskin, 1998). Dormancy breaking and germination requirements are specific for each species and depend on phylogeny, geographical distribution, habitat preference and life cycle (Vandelook *et al.*, 2008). Morphological, physical and physiological are three fundamentally different types of seed dormancy, and they may be found combined among them (e.g. morphophysiological dormancy, MPD; Fenner and Thompson, 2005). MPD is frequent in parts of the world with moist seasonal climate (Fenner and Thompson, 2005). Breaking MPD requires embryo growth and a treatment to overcome their physiological component (Baskin and Baskin, 2004). Embryo morphology has important implications for dormancy and germination. Generally, embryo size increases within the angiosperms from basal families to the most derived families: a small embryo surrounded by abundant endosperm is considered to be a plesiomorphic condition, whereas more derived species often have a more developed embryo (Martin, 1946; Forbis *et al.*, 2002). However, different eudicot *taxa*, such as Santalales, Paeoniaceae, Grossulariaceae and Parnassiaceae, present an underdeveloped embryo, thus suggesting that embryo morphology is closely tied to ecology, as a convergent response to similar environmental conditions (Forbis *et al.*, 2002). Vandelook *et al.* (2012) reported that the relative embryo length in Apiaceae may have evolved as an adaptation to habitat and life cycle, and Mattana *et al.* (2013) indicated that embryo morphology and type of dormancy in Grossulariaceae are closely tied to plant ecology.

In non-dormant seeds, the germination response to accumulated temperature has been modelled using a thermal time (θ) approach (Garcia-Huidobro *et al.*, 1982; Covell

et al., 1986; Ellis *et al.*, 1986, 1987; Pritchard and Manger, 1990; Trudgill *et al.*, 2000; Hardegree, 2006). In this model, seeds accumulate units of thermal time (°Cd) to germinate for a percentile *g* of the population and, as reported in the Chapter 2 for *Aquilegia barbaricina* Arrigoni *et* E.Nardi, this approach may be applied to identify the thermal thresholds (T_b and θ_{50}) requirements for embryo growth. Seed germination may be predicted in relation to thermal time accumulation (heat sum, °Cd) above a gradually reducing T_b (Steadman and Pritchard, 2004). This approach has been used to predict seed germination in the field (i.e. Hardegree and Van Vactor, 2000; Steadman *et al.*, 2003; Chantre *et al.*, 2009) and to assess the impact of different simulated climate change scenarios on seed dormancy release and germination (Orrù *et al.*, 2012). Recently, Porceddu *et al.* (2013) used a soil heat sum model to predict *in situ* seed germination of *Rhamnus persicifolia* Moris. Soil heat sum approach may be used to predict seed germination phenology in the field under the current environmental conditions (i.e. temperature), but could also be useful to predict the impact of climate warming on seed germination.

The Intergovernmental Panel on Climate Change (IPCC) has predicted temperature increases of approx. 2–4 °C by 2090 – 2099. In particular, large increases in temperature have been predicted and reported for the Mediterranean mountain ranges (Peñuelas and Boada, 2003; Bravo *et al.*, 2008). Mediterranean mountains represent one of the most important centre of biodiversity and differentiation of the world (Médail and Quézel, 1997), and Supramontes and Gennargentu massif have been recognised as two of micro-hotspots within Sardinian region (Cañadas *et al.*, 2014). Mountains of Central Northern Sardinia (Italy) are characterized by riparian vegetation constituted by *Alnus glutinosa* (L.) Gaertn. with other associated taxa such as *Taxus baccata* L., *Ilex aquifolium* L. and *R. persicifolia*. The canopies of woody plants modify the microclimate beneath and around them through interception of precipitation and by shading, which influence maximum soil temperature (Breshears *et al.*, 1998). Rare and threatened Sardinian endemic species such as *Ribes multiflorum* Kit *ex* Roem *et* Schult. subsp. *sandalioticum* Arrigoni (Grossulariaceae), *A. barbaricina* (Ranunculaceae), and *Paeonia corsica* Sieber *ex* Tausch (Paeoniacea) grow in the same ecosystem and ecological conditions under and close to the canopy of such riparian woods.

Embryo in seeds of *R. multiflorum* subsp. *sandalioticum* (hereafter *R. sandalioticum*), *A. barbaricina* and *P. corsica* are linear underdeveloped *sensu* Baskin and Baskin (2007), and all these species show a morphophysiological dormancy

(MPD); in particular *A. barbaricina* shows an intermediate simple MPD (Chapter 2), while *R. sandalioticum* and *P. corsica* show non-deep simple (root) - non-deep simple (epicotyl) MPD (Mattana *et al.*, 2012b; Chapter 3).

In this study, embryo morphology, seed germination and thermal requirements of *R. multiflorum* subsp. *sandalioticum*, *A. barbaricina* and *P. corsica* were correlated with the environmental temperature conditions, in order to: (1) investigate the field embryo growth and seed germination of these Sardinian endemic mountain species with endospermic seeds; (2) develop a model based on soil heat sum approach and predict their seed germination phenology in the field, under present climatic conditions and two different IPCC scenarios of increasing temperatures.

Materials and Methods

Study species

Aquilegia barbaricina (Ranunculaceae), *Paeonia corsica* (Paeoniaceae) and *Ribes sandalioticum* (Grossulariaceae) are endemic species of Sardinia and grow in the same localities from ca. 1,000 m a.s.l. to the higher elevation of CE-Sardinia mountains, in wet woodlands, meadows and stream margins under and near riparian woods (see Tables 1 and 2). Information of embryo and seed germination obtained in controlled conditions are reported in Chapters 2 and 3 for *A. barbaricina* and *P. corsica*, respectively, and those for *R. sandalioticum* from Mattana *et al.* (2012b). Warm stratification release dormancy in *R. sandalioticum* (Mattana *et al.*, 2012b) and *P. corsica* (Chapter 3), while warm plus cold stratification is needed to break dormancy in *A. barbaricina* (Mattana *et al.*, 2012a; Chapter 2).

Seed lot details

Seeds of *A. barbaricina*, *P. corsica* and ripe berries of *R. sandalioticum* were collected directly from plants near and under riparian woods of *A. glutinosa* at the time of natural dispersal in 2011. Seeds of *R. sandalioticum* were immediately separated from the pulp by rubbing fruits through sieves under running water. The cleaned seeds were then spread out and left to dry at room temperature.

Table 1 – Seed lot details, embryo and germination characteristics of each study species. T_{be}, $T_{oe,}$ and T_{ce} correspond to the base, optimal and ceiling temperature for embryo growth, respectively, while T_b correspond to the base temperature for seed germination.

Species	**Population**	**Date of collecting**	**Seed mass (mg)**	**T_{be} (°C)**	**T_{oe} (°C)**	**T_{ce} (°C)**	**T_b (°C) dormant seeds**	**T_b (°C) non-dormant seeds**	**Initial embryo length**	**Critical embryo length**	**Max germination in laboratory (%)**	**Viability (%)**	**Source**
A. barbaricina	Rio Correboi (Villagrande Strisaili, OG)	29/06/2011	1.26 ± 0.06	5.20 ± 0.60	15.00 ± 1.02	29.52 ± 2.37	> 25	5.34 ± 1.38	0.03 ± 0.01	0.12 ± 0.02	81± 12	98 ± 2	Chapter 2
P. corsica	Rio Correboi (Villagrande Strisaili, OG)	26/08/2011	89.11 ± 14.78	ND	ND	ND	15*	10*	0.14 ± 0.02	0.39 ± 0.07	63± 10	85 ± 7	Chapter 3
R. sandalioticum	Monte Novo San Giovanni (Orgosolo, NU)	26/08/2011	4.71 ± 0.48	ND	ND	ND	10*	5*	0.05 ± 0.01	0.18 ± 0.02	95 ± 5	95 ± 5	This work

* T_b values estimated as the lowest tested temperature at which germination occurred (see Trudgil *et al.*, 2000)

Germination and embryo growth in controlled conditions

Whilst information on embryo and seed germination under controlled conditions of the seed lots of *A. barbaricina* and *P. corsica* are reported in Chapters 2 and 3, to obtain this data for the seed lot of *R. sandalioticum* 3 replicates of 20 seeds of this *taxon* were sown on the surface of 1% agar water in 90 mm diameter plastic Petri dishes, stratified for 3 months at 25°C and then incubated at 5, 10 and 15°C in the light (12 h light / 12 h darkness; Mattana *et al.*, 2012b). Germination was defined as visible radicle emergence (> 1 mm). Germinated seeds were scored 3 times a week. At the end of the germination tests, when no additional germination had occurred for 2 weeks, a cut test was carried out to determine the firmness of remaining seeds and the number of empty seeds. Firm seeds were considered to be viable. Germination results are reported in Table 2.

Seed germination and embryo growth in natural conditions

According to the methodology in Porceddu *et al.* (2013; see Chapter 1) seeds of each species were placed in fine-mesh polyester envelopes (3 replicates of 25 seeds) and buried in the soil at a depth of 2-3 cm, within ca. 20 days after seed collection (Table 2). Envelopes were buried both underneath (IN) and outside (OUT) the tree canopy, with a distance between them of ca. 6 m, in each natural population (Table 2). Envelopes buried in the experimental sites were exhumed at about 3-months intervals from September 2011 to June 2012 (with an intermediate exhumation also in April 2012) for *A. barbaricina*, from September 2011 to March 2012 for *R. sandalioticum*, and from September 2011 to December 2012 for *P. corsica*. A further exhumation for *P. corsica* was performed also in March 2013 to evaluate the number of seed with epicotyl-plumule (hereafter "epicotyl"; see Chapter 3) emerged. Retrieved envelopes were analysed in the laboratory, where they were washed under running water and opened. The number of germinated and epicotyl emerged seeds was recorded. In addition, embryo growth in the field was assessed during each exhumation time (Table 2), by measuring 20 randomly chosen seeds. Seeds were cut in half under a dissecting microscope and images of embryos were acquired using a Zeiss SteREO Discovery.V8, with an objective Achromat S 0.63x, FWD 107mm (Carl Zeiss MicroImaging GmbH) at a 6.3x magnification for *A. barbaricina* and *R. sandalioticum* and at a 4.0x magnification for *P. corsica*, coupled to a Canon (Power shot G11) digital camera. Embryo and seed lengths were measured using the image analysis software ImageJ

1.41o (National Institutes of Health, Bethesda, MA, USA). Seed length was measured ignoring the seed coat. The initial embryo length was calculated by measuring 20 randomly selected seeds before starting the experiments. The embryo length of seeds with a split seed coat but no radicle protrusion (i.e. critical embryo length) was determined for 20 randomly selected seeds and used for seeds that had germinated before measurements (Vandelook *et al.*, 2007).

Table 2 - Locations, habitat characteristics and dates of experimental trials carried out in each sites (Rio Correboi: RC IN and RC OUT; Monte Novo San Giovanni: MSG IN and MSG OUT) of the natural populations of each species. For each experimental site, IN and OUT differentiate between underneath and outside the tree canopy, respectively.

Species	Population	Experimental sites	Habitat	Altitude (m a.s.l.)	Aspect	Date of field sowing	Dates of exhumation and days after sowing
A. barbaricina	Rio Correboi (Villagrande Strisaili, OG)	RC IN	Riparian wood of black alder (*Glechomo-Alnetum glutinosae*) – Mantle shrubs of elm-leaf blackberry (*Pruno-Rubion ulmifolii*)	1267	0	18/07/2011	16/09/2011 (60 days) 09/12/2011 (144 days) 29/03/2012 (255 days) 26/04/2012 (283 days) 25/06/2012 (343 days)
		RC OUT	Open grassland of *Carici-Genistetea lobelioidis*		NE		
P. corsica	Rio Correboi (Villagrande Strisaili, OG)	RC IN	Riparian wood of black alder (*Glechomo-Alnetum glutinosae*) – Mantle shrubs of elm-leaf blackberry (*Pruno-Rubion ulmifolii*)	1267	0	16/09/2011	09/12/2011 (84 days) 29/03/2012 (195 days) 25/06/2012 (283 days) 19/09/2012 (369 days) 28/12/2012 (469 days) 10/04/2013 (572 days)
		RC OUT	Open grassland of *Carici-Genistetea lobelioidis*		NE		
R. sandalioticum	Monte Novo San Giovanni (Orgosolo, NU)	MSG IN	Mantle shrubs of elm-leaf blackberry (*Pruno-Rubion ulmifolii*)	1225	0	16/09/2011	09/12/2011 (84 days) 29/03/2012 (195 days)
		MSG OUT	Open grassland of *Carici-Genistetea lobelioidis*		0		

Soil heat sum approach

A soil heat sum approach was used to predict field germination phenology for all the investigated species according to Porceddu *et al.* (2013). Soil temperatures at the level of the envelopes were recorded both underneath (IN) and outside (OUT) the tree canopy of the natural population sites at 90-minutes intervals, using data loggers (TidbiT® v2 Temp logger, Onset Computer Corporation, Cape Cod, Massachusetts, U.S.). Soil temperatures above T_b of each species were used to assess the temperature accumulation till the achievement of θ_{50}. Soil heat sum was calculated, starting from the date of sowing, according to the following equation:

$$\textit{Soil heat sum}\ (°\text{Cd}) = \{\sum [(T_S - T_b) \times t]\}/16, \text{ (Eq. 1)}$$

where T_S is the temperature at each logging interval recorded by data loggers, T_b is the base temperature for seed germination of each species (see Table 1), t is the length of the logging interval expressed in hours and 16 is the number of logging records per day (Porceddu *et al.*, 2013). In addition, for *A. barbaricina* it was also possible to build a predictive model for embryo growth according to the following equations:

$$\textit{Soil heat sum}\ (°\text{Cd}) = \{\sum [(T_S - T_{be}) \times t]\}/16, \text{ (Eq. 2)}$$

for the sub-optimal temperature range (i.e. soil temperatures < 15°C; see Chapter 2) and:

$$\textit{Soil heat sum}\ (°\text{Cd}) = \{\sum [(T_{ce} - T_s) \times t]\}/16, \text{ (Eq. 3)}$$

for the supra-optimal temperature range (i.e. soil temperatures > 15°C; see Chapter 2).

Pluviometric data for Rio Correboi (monthly averages of rainfall from 1922 to 2009 from the nearby climatic station of Fonni, NU) and Monte Novo San Giovanni (monthly averages of rainfall from 1936 to 2009 from the nearby climatic station of Montes, Orgosolo, NU), were acquired from Regione Autonoma della Sardegna (http://www.regione.sardegna.it/j/v/25?s=131338&v=2&c=5650&t=1). The presence / absence of the tree canopy of riparian wood was observed at each field excursion during

this study, and the different periods described by Porceddu *et al.* (2013) identified (see Chapter 1).

Statistical analysis

Generalized Linear Models (GLMs) were used to compare the field embryo length, seed germination and epicotyl emergence percentages of each species at different exhumation dates, both IN and OUT the tree canopy (see Table 2). GLM with a log link function and quasipoisson error structure was used for analysing embryo length values, while GLMs with a logit link function and quasibinomial error structure were used when analysing germination and epicotyl percentages. Quasibinomial and quasipoisson error structures and *F* tests with an empirical scale parameter instead of chi-squared on the subsequent ANOVA were used in order to overcome residual overdispersion (Crawley, 2007). All statistical analyses were carried out with R v. 2.14.0 (R Development Core Team, 2011).

Results

Embryo growth and germination tests in natural conditions

Soil temperatures recorded by data loggers were very similar for the 2 localities (RC and MSG; Fig. 1A), with an annual mean temperature of ca. 9.5°C for IN in both populations, and of ca. 10.2°C and ca.11.8°C for RC OUT and MSG OUT, respectively, ranging from a minimum of -0.6°C (OUT; January 12, 2012) to a maximum of 29.6°C (OUT; July 12, 2012) in RC, and a minimum of 0.2°C (OUT; February 23, 2012) to a maximum of 27.7°C (OUT; July 13, 2012; Fig. 1A) in MSG. The lowest mean temperatures (ca. 1°C) were detected in the period III in all experimental sites, whereas the highest mean temperatures were reached in the period VI with ca. 18°C for RC IN and MSG IN, and ca. 22° for RC OUT and MSG OUT (Fig. 1A). The length of the effective cold stratification periods (i.e. mean daily temperatures < 5°C) was 92 days for RC IN and MSG IN (both with 41 days of snow cover), and 98 days for RC OUT (with 47 days of snow cover) and 93 days for MSG OUT (with 44 days of snow cover), and occurred from December to March (Fig. 1A). The length of the warm stratification periods (i.e. mean daily temperatures > 20°C) was 64 and 44 days for RC IN and MSG IN, respectively, and 80 days for RC OUT and 96 days for MSG OUT, and occurred from June to August-early September (Fig. 1A).

Embryos of *A. barbaricina* seeds did not grow from July 2011 (date of field sowing) to December 2011 (period II; Fig. 1B). In March 2012 (period IV) embryos started to grow and reached an embryo length of ca. 0.11 mm both in RC IN and OUT; at the same time a few seeds (ca. 30%) had started to germinate in RC IN (Fig. 1B-C). In April 2012, between period IV and V, the seeds reached their critical embryo length (ca. 0.12 mm) and the majority of the seeds had germinated, reaching values of approx. 80% both in RC IN and OUT (Fig. 1B-C). In June 2012 (period VI), the percentage of germinated seeds of *A. barbaricina* was ca. 95% both IN and OUT RC experimental sites (Fig. 1B-C). More specifically, in *A. barbaricina* the month where both the critical embryo length was reached and maximum germination achieved was April (Fig. 1).

Embryos of *P. corsica* did not grow respect to the initial embryo length (ca. 0.15 mm) from the date of sowing (September 2011) to June 2012 (period VI; Fig. 1B). In September 2012 (period I), the embryo started to grow and reached length values of 0.26 and 0.34 mm for RC IN and OUT, respectively (Fig. 1B). Seeds also started to germinate, with approx. 56 and 10% of germinated seeds for RC OUT and IN,

respectively (Fig. 1C). Critical embryo length (ca. 0.40 mm) was reached at the end of December 2012, between the end of period II and the start of period III (i.e. start of cold stratification period), and germination was approx. 78% in RC IN and approx. 50% in RC OUT (Fig. 1B-C). In this exhumation time, ca. 30% of seeds in RC OUT had emerged epicotyls, while in RC IN all seeds were without emerged epicotyl (Fig. 1D). At the last exhumation, in April 2013 (period IV), the percentage of seeds with emerged epicotyl was approx. 45 and 70% for RC IN and OUT, respectively (Fig. 1D). Each phase of seed germination in *P. corsica* occurred in the second year after sowing; critical embryo length was reached from September to December (depending on the position), seed germinated in September in RC OUT and in December in RC IN, but epicotyl emerged in April in both experimental sites (Fig. 1).

Germination of *R. sandalioticum* seeds was faster with respect to the other two species. From the date of field sowing (September 2011) to December 2011 (period II) the embryo grew from the initial embryo length (ca. 0.05 mm) to a value near the one of critical embryo length (ca. 0.18 mm), and the seeds germinated with a percentage of ca. 58% in RC IN and ca. 84 % in RC OUT, but no seeds had emerged epicotyls (Fig. 1B-D). At the last exhumation, in March 2012 (period IV), the germinated seeds with emerged epicotyls reached values of approx. 55% in RC IN and ca. 82 % in RC OUT, while ca. 12 and 15 % of the seeds had a protruded radical but no emerged epicotyl in RC IN and RC OUT, respectively (Fig. 1C-D). To summarize, the critical embryo length in *R. sandalioticum* seeds was reached in December, and at the same time the seeds germinated, while epicotyl emergence occurred in March (Fig. 1).

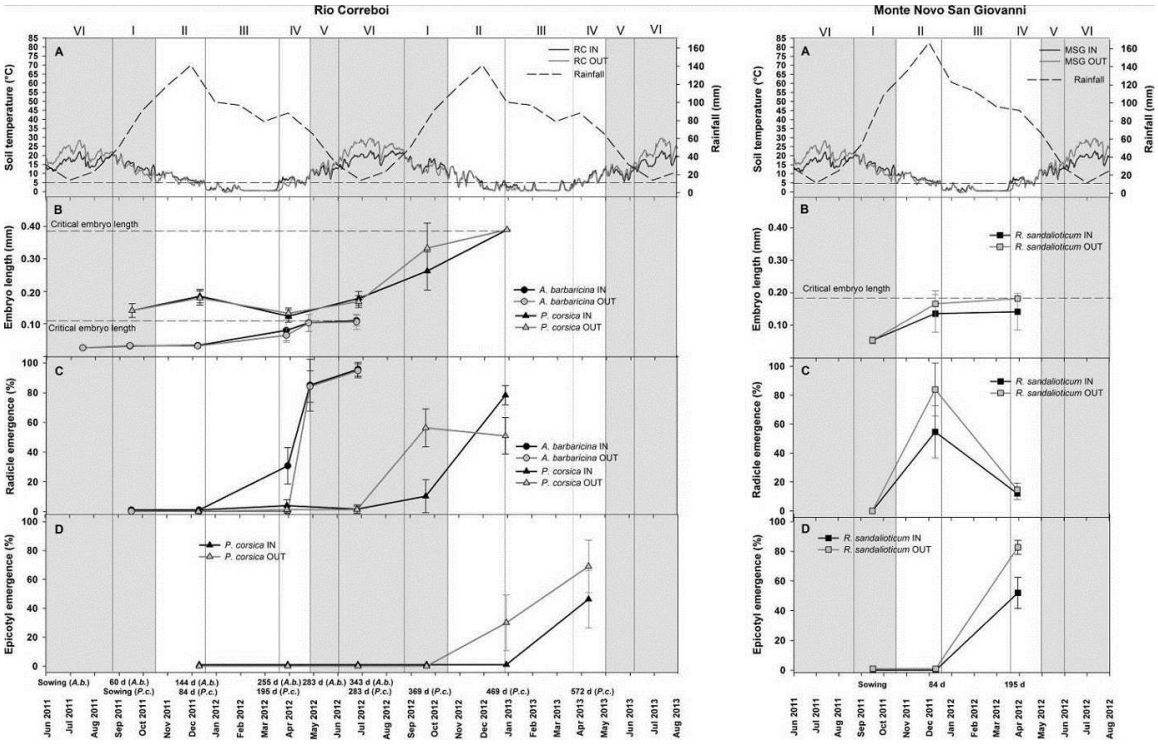

Figure **1** - (A) Annual trends of mean daily temperatures recorded in the soil both underneath (IN) and outside (OUT) the tree canopy for Rio Correboi (RC) and Monte Novo San Giovanni (MSG), and mean monthly rainfall obtained from the nearby weather stations of Fonni for RC and of Montes for MSG; (B) Embryo length in mm (data are the mean of 20 seeds at each exhumation time); (C) field germination (data are the mean 3 replicates of 25 seeds each) IN and OUT at each time of exhumation; and (D) field epicotyl emergence (data are the mean of 3 replicates of 25 seeds each) IN and OUT at each exhumation time of *P. corsica* and *R. sandalioticum*. The background grey squares correspond to the presence of the tree canopy. I, II, III, IV, V and VI correspond to different periods identified by Porceddu *et al.* (2013; see Chapter 1).

Generalized linear models (GLM) identified a high statistically significant ($P < 0.001$) effect for all three factors ("Date of exhumation", D; "Position", P; "Species", S; Table 3) for embryo length. For seed germination and epicotyl emergence, GLMs highlighted a high statistically significant difference ($P < 0.001$) for the "D" and "S" factors and a statistically significant ($P < 0.05$) effect for the "P" factor (Table 3). A highly statistically significant difference ($P < 0.001$) was found for all the two-way interactions (D × P, D × S and P × S) on embryo length, seed germination and epicotyl emergence (Table 3). No statistically significant differences ($P > 0.05$) were detected for the three-way interaction (D × P × S) for embryo length, seed germination and epicotyl emergence (Table 3).

Table 3 - GLMs results for the effect on (I) embryo length, (II) seed germination and (III) epicotyl emergence in the field of the following factors: "Date of exhumation" (D: see table 2), "Position" (P: IN and OUT) and "Species" (S: *A. barbaricina*, *R. sandalioticum* and *P. corsica*).

(I) Embryo	**Df**	**Deviance**	**Resid. df**	**Resid. dev**	***F***	***P* (>F)**
NULL			557	354.55		
Date (D)	6	222.432	551	132.12	662.8910	< 0.001
Position (P)	1	0.637	550	131.48	11.3866	< 0.001
Species (S)	2	66.182	548	65.30	591.7089	< 0.001
D × P	6	1.569	542	63.73	4.6769	< 0.001
D × S	5	29.825	537	33.91	106.6599	< 0.001
P × S	2	1.033	535	32.87	9.2371	< 0.001
D × P × S	5	0.527	530	32.35	1.8855	> 0.05
(II) Germination	**Df**	**Deviance**	**Resid. df**	**Resid. dev**	***F***	***P* (>F)**
NULL			71	6210.7		
D	6	1802.1	65	4408.7	47.4112	< 0.001
P	1	37.4	64	4371.2	5.9072	< 0.05
S	2	3454.5	62	916.8	272.6571	< 0.001
D × P	6	129.5	56	787.3	3.4066	< 0.001
D × S	3	193.8	53	593.5	10.1971	< 0.001
P × S	2	240.8	51	352.7	19.0097	< 0.001
D × P × S	3	14.0	48	338.7	0.7360	> 0.05
(III) Epicotyl emergence	**Df**	**Deviance**	**Resid. df**	**Resid. dev**	***F***	***P* (>F)**
NULL			71	5644.2		
D	6	2883.90	65	2760.3	105.5932	< 0.001
P	1	23.25	64	2737.1	5.1079	< 0.05
S	2	2018.71	62	718.3	221.7438	< 0.001
D × P	6	134.13	56	584.2	4.9113	< 0.001
D × S	3	136.81	53	447.4	10.0186	< 0.001
P × S	2	214.37	51	233.0	23.5471	< 0.001
D × P × S	3	0.00	48	233.0	0.0000	> 0.05

Soil heat sum for embryo growth and seed germination of Aquilegia barbaricina

Figure 2 reports the soil heat sum accumulation till the achievement of θ_{50} threshold value for embryo growth (Fig. 2A) and germination (Fig. 2B) in the field for *A. barbaricina* seeds, both IN and OUT the tree canopy, according to field germination and temperature recorded by each data logger. θ_{50} values were expressed in log °Cd, according to the best model obtained under controlled conditions (see Chapter 2). Immediately after sowing (period VI), and during periods I, II and III, T_b of dormant seed of *A. barbaricina* was higher than the mean soil temperatures, and this prevented the soil heat sum accumulation both for embryo growth and germination. However, after cold stratification (period IV), when the seed dormancy was broken, the lower T_b values and the increasing soil temperatures allowed the threshold of 2.10 log °Cd (for embryo growth) and 2.04 log °Cd (for germination) to be reached from late April to early May (period V; Fig. 2A-B). More specifically, θ_{50} for embryo growth was reached in April 29 for IN and in the May 03 for OUT (287 and 291 days after sowing for IN and OUT, respectively; Fig. 2C), while θ_{50} for germination was reached in April 28 for IN and in May 02 for OUT (286 and 289 days after sowing for IN and OUT, respectively; Fig. 2D). This estimated time was confirmed by the embryo measurements and germination recorded in the field (see Fig. 1).

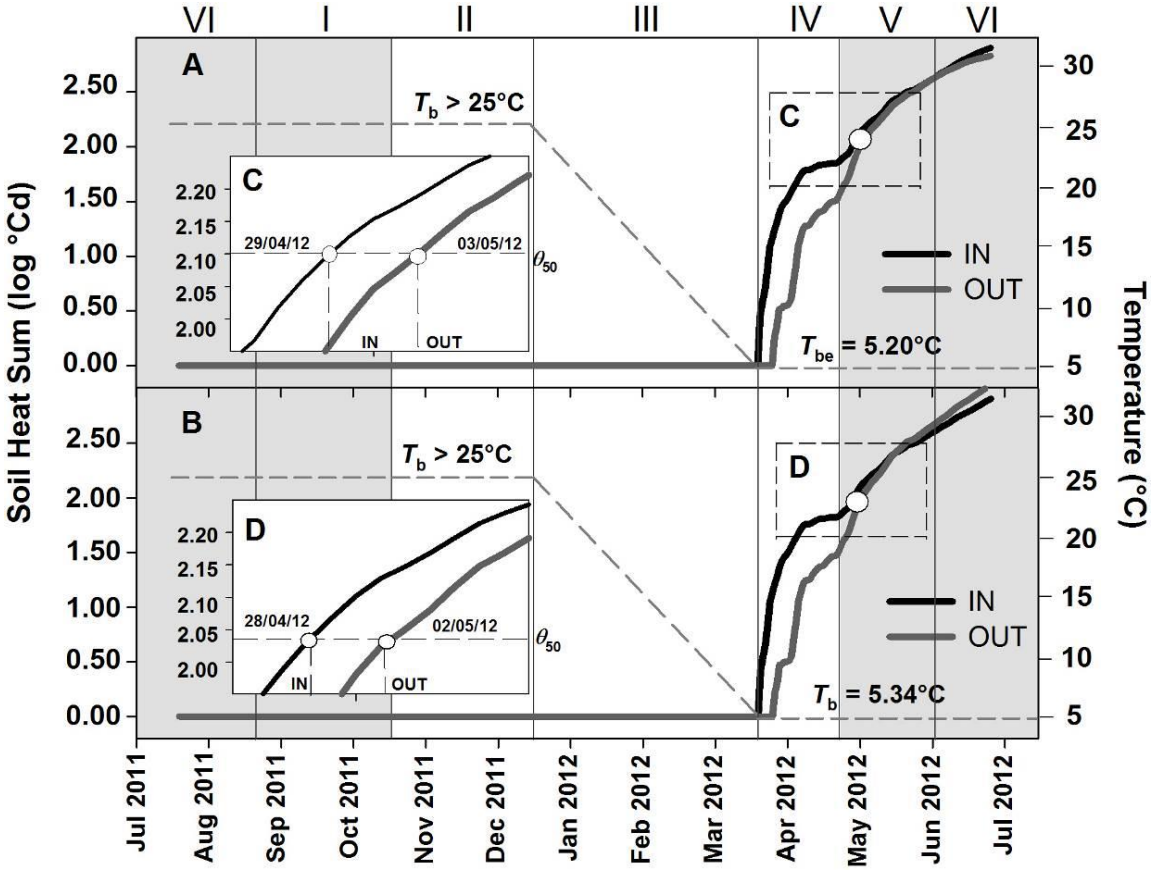

Figure **2** – Soil Heat Sum (expressed in log °Cd) to achievement the θ_{50} threshold value for (A) embryo (50% of seeds that reached the critical embryo length) and (B) germination (50% of seed germination in the field) of *A. barbaricina*, both underneath (IN) and outside (OUT) the tree canopy. The inset plots (C and D) show the details of the achievement of the θ_{50} threshold value (2.10 and 2.04 log °Cd, for embryo and germination respectively). Dark grey short dashes represent the base temperature before (T_b > 25°C) and after (5.20 and 5.34°C for embryo growth and seed germination, respectively) cold stratification. The background grey squares correspond to the presence of the tree canopy. I, II, III, IV and V correspond to different periods identified by Porceddu *et al.*, (2013) and described in the Chapter 1.

The linear regression analysis carried out to detect relationships between estimated and calculated values of soil heat sum highlighted a significant correlation for embryo growth ($P < 0.05$; $r^2 = 0.84$; $y = 0.67x + 1.09$), while showed not statistically significant relationships for seed germination ($P > 0.05$; $r^2 = 0.72$; $y = 0.39x + 1.53$).

Soil heat sum estimates for seed germination of Paeonia corsica and Ribes sandalioticum

In controlled conditions, non-dormant seeds of *P. corsica* germinated only at two of the range of tested temperatures (see Chapter 3). The same trend was identified for *R.*

sandalioticum (see Table 1) which germination of non-dormant seeds occurred at 5°C (57 ± 15%) and 10°C (95 ± 5%), and only few seeds (< 5%) germinated at 15°C.
Therefore the dataset was not large enough to correlate germination rate and temperature for germination for both species and, consequently, it was not possible to build a thermal time model to calculate their T_b, as for *A. barbaricina* seeds. Therefore for these species T_b was estimated using the lowest tested temperature at which germination was recorded. These values were 15 and 10°C, for dormant and non-dormant seed of *P. corsica*, respectively, and 10 and 5°C for dormant and non-dormant seed of *R. sandalioticum*, respectively (see Table 1).

The soil heat sum accumulation range to achievement the θ_{50} threshold value, both IN and OUT (Fig. 3), was estimated by using these values and according to field seed germination percentages obtained during different exhumation times (Fig. 1C) and to temperatures recorded by each data logger (Fig. 1A). From the date of sowing (September; period I) to the end of period V (June), seeds of *P. corsica* did not experience the warm stratification period (i.e. period VI) and T_b estimated for dormant seeds was higher than the mean soil temperatures, and this prevented the soil heat sum accumulation (Fig. 3A). The increasing soil temperatures during period VI allowed the beginning of soil heat sum accumulation. During this period, seeds of *P. corsica* released PD dormancy and T_b estimate decreased to a value of 10°C; this increased the rate of soil heat sum accumulation. The absence of germination (0%) observed in June and the germination obtained in September in RC OUT (ca. 56 %) allowed to estimate that the θ_{50} values for seed germination falls within the range of 339.26 - 1367.68 °Cd in this experimental site, while the germination of ca. 10% and ca. 76 % recorded in RC IN in September and in December, respectively (see Fig. 1C), indicated that the θ_{50} estimated falls within the range 670.04 - 901.95 °Cd (Fig. 3A). However, the value of 901.95 °Cd was reached in November 16th, and after this date the seeds did not accumulate heat sum.

As regards of *R. sandalioticum* (Fig. 3B), the T_b estimated for dormant and non-dormant seeds of this species was lower than the mean soil temperatures, and this promoted the soil heat sum accumulation immediately after sowing (in September; period I). During the first exhumation carried out in December (period II), seeds germinated were > 50% in both experimental sites of MSG (see Fig. 1C); by this time seeds had accumulated 659.27 °Cd in MSG IN and 715.92 °Cd in MSG OUT (Fig. 3B).

This allowed to estimate that θ_{50} falls within the range 0 °Cd - 659.27 °Cd in MSG IN, and 0 °Cd - 715.92 °Cd in MSG OUT (Fig. 3B).

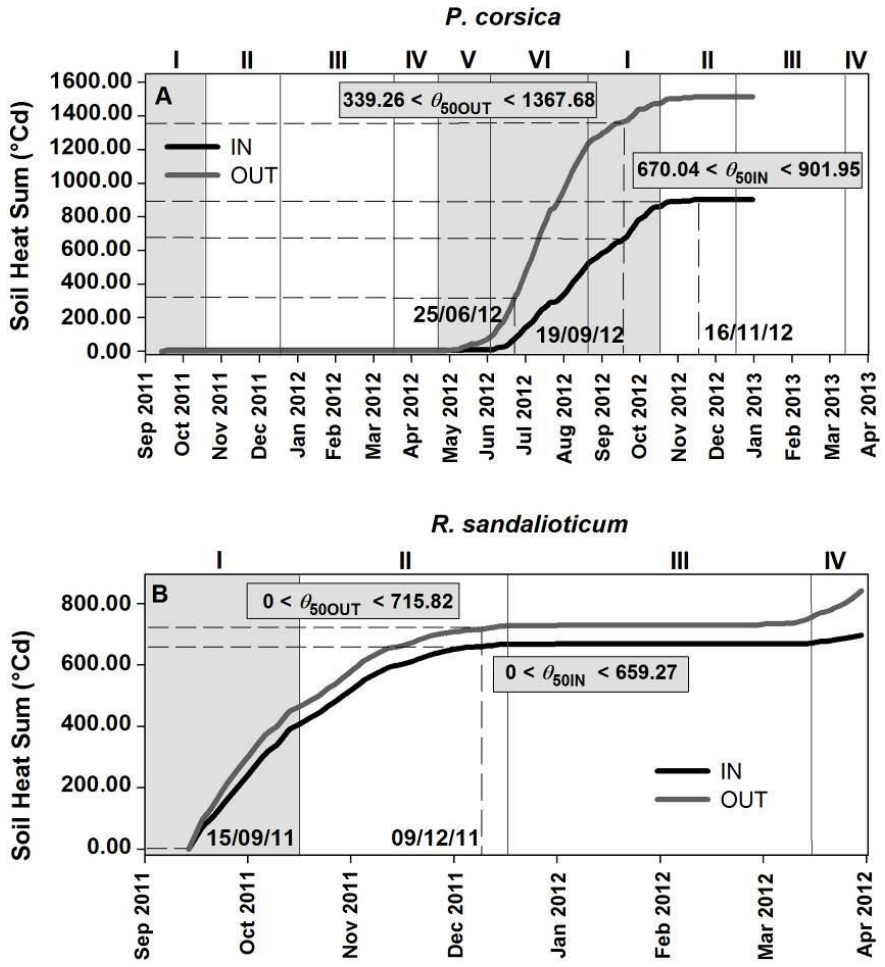

Figure **3** - Soil Heat Sum (°Cd) for the achievement of the predicted minimum and maximum values for germination near to θ_{50} threshold value (50% of seed germination in the field) of *P. corsica* and *R. sandalioticum*, both underneath (IN) and outside (OUT) the tree canopy, calculated according to their exhumation times. The background grey squares correspond to the presence of the tree canopy. I, II, III, IV and V correspond to different periods identified by Porceddu *et al.*, (2013) and described in Chapter 1.

Seed germination phenology under different climate scenarios

Figures 4, 5 and 6 show the soil heat sum accumulation and the achievement of the θ_{50} of each species in the field, both IN and OUT, for the present climate data and under two different IPCC scenarios (B1, +1.8 °C and A2, +3.4 °C; IPCC, 2007). Figure 4 shows the soil heat sum accumulation and the achievement of the θ_{50} threshold value for *A. barbaricina*. The increase in temperature of +1.8 °C (B1 scenario) and +3.4°C (A2 scenario) in RC should lead to a reduction of the period III (i.e. cold stratification) from ca. 90 days in B1 scenario to ca. 45 days in A2 scenario, with an increase of the mean soil temperature of ca. 3°C in the latter scenario with respect to the present mean soil

temperature (ca. 1°C; Fig. 4A). The increase in the mean temperature during period III would not compromise seed dormancy release in *A. barbaricina*. However, after cold stratification period, the increased temperature would accelerate germination of non-dormant seeds, bringing it forward from late-April to middle-April in RC IN and from early-May to late-April in RC OUT for the B1 scenario, and to early-April and middle-April in RC IN and RC OUT, respectively, for the A2 scenario (Fig. 4B).

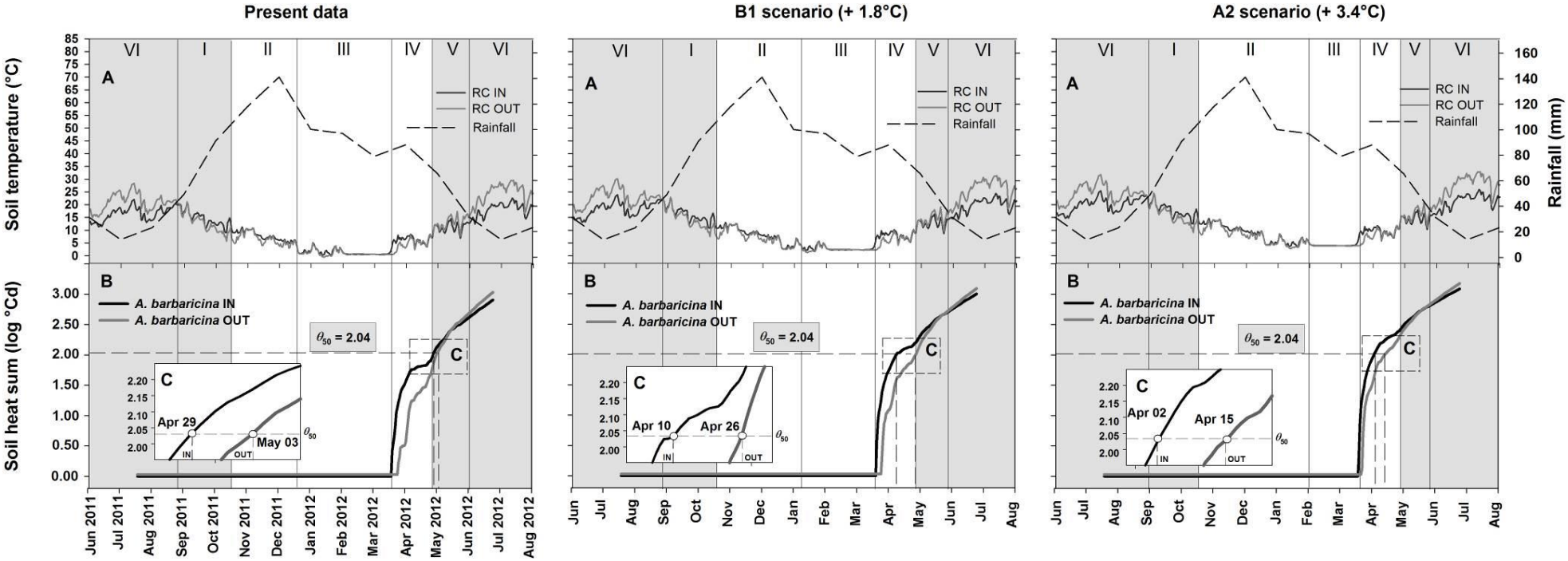

Figure **4** - (A) Annual trends of mean daily temperatures recorded in the soil for Rio Correboi (RC) with mean monthly rainfall obtained from the nearby weather stations of Fonni, and (B) Soil heat sum (expressed in log °Cd) to achievement the θ_{50} threshold value (2.04 log °Cd) for *A. barbaricina* seed germination, both underneath (IN) and outside (OUT) the tree canopy, for the present data and under two different IPCC scenarios (B1, +1.8 °C and A2, +3.4 °C). The inset plots (C) show the details of the achievement of the θ_{50} threshold value. The background grey squares correspond to the presence of the tree canopy. I, II, III, IV, V and VI correspond to different periods identified by Porceddu *et al.*, (2013). Results of the present data was already reported in figure 1A and 2, and it is presented again to better understand the differences with the other two scenarios.

In addition, the increase in temperature predicted in both scenarios, should not affect the length of period VI (i.e. the summer drought period) in RC, but should increase its mean soil temperature. The mean soil temperature in RC IN should increase from approx. 19°C to approx. 21 and 23°C in B1 and A2 scenarios, respectively; while in RC OUT it should increase from approx. 24°C to 26 and 28°C in B1 and A2 scenario, respectively (Fig. 5). In particular, an increased soil heat sum would accelerate the achievement of the θ_{50} threshold value in *P. corsica* seed germination both in RC IN and RC OUT (Fig. 5). The increase in temperature predicted in B1 scenario should anticipate seed germination of this species in June-August for RC OUT and in August-September for RC IN (Fig. 5). The increase in temperature predicted in A2 scenario should bring forward seed germination in June-early August for RC OUT and in July-August for RC IN (Fig. 5).

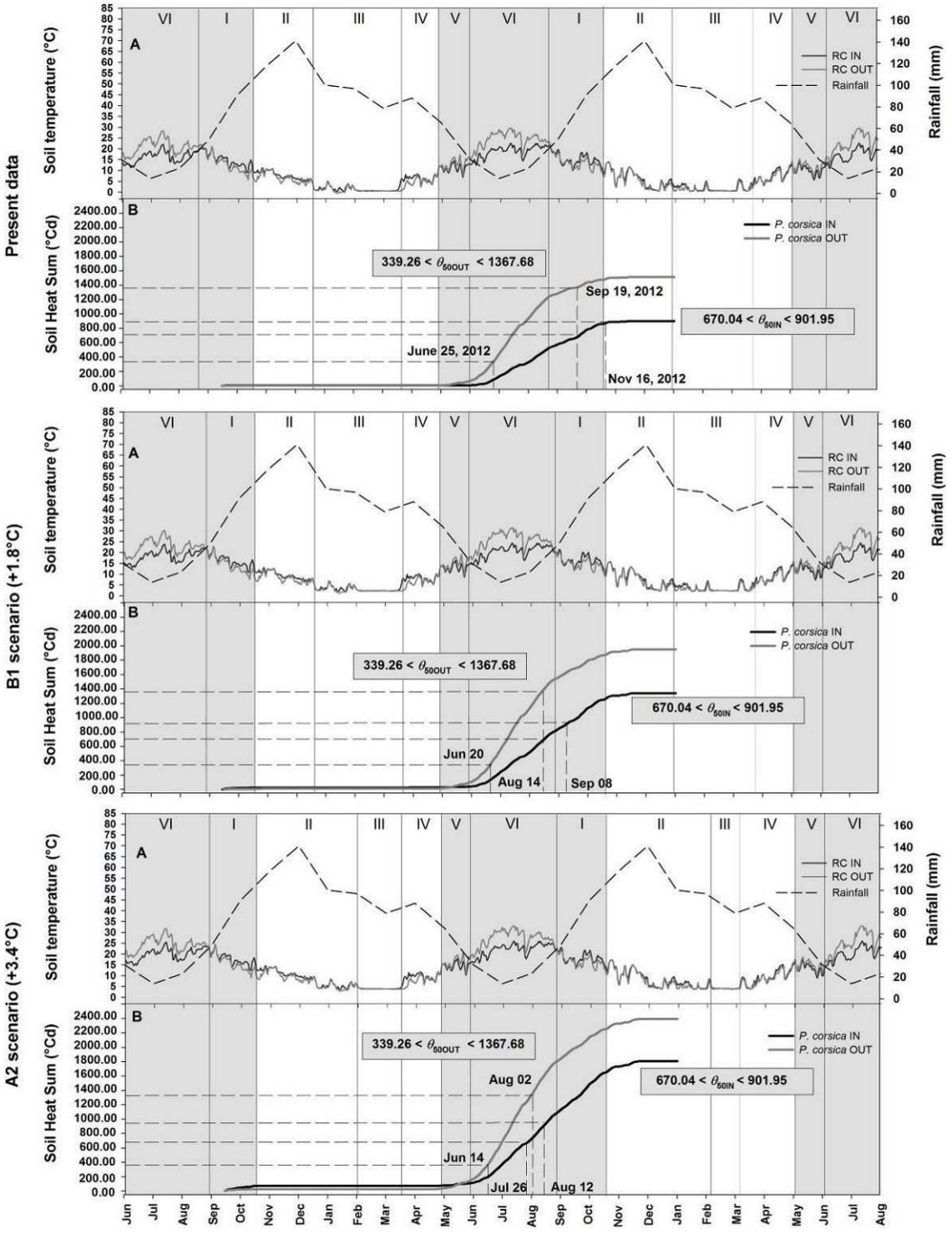

Figure **5** – (A) Annual trends of mean daily soil temperatures for Rio Correboi (RC) with mean monthly rainfall obtained from the nearby weather stations of Fonni. (B) Soil Heat Sum (°Cd) to achievement of the predicted minimum and maximum values for germination near to the θ_{50} threshold value, both underneath (IN) and outside (OUT) the tree canopy, for the present data and under two different IPCC scenarios (B1, +1.8 °C and A2, +3.4 °C). The background grey squares correspond to the presence of the tree canopy. I, II, III, IV, V and VI correspond to different periods identified by Porceddu *et al.*, (2013) and described in Chapter 1.

An increased soil heat sum would accelerate the achievement of the θ_{50} threshold value in *R. sandalioticum* seeds in MSG. In B1 scenario, it should bring forward its seed germination to November, while in A2 scenario it could bring it forward to October (Fig. 6). More specifically, seed germination of this species would occur ca. 38 and 45 days earlier in B1 and A2 scenarios, respectively (Fig. 6).

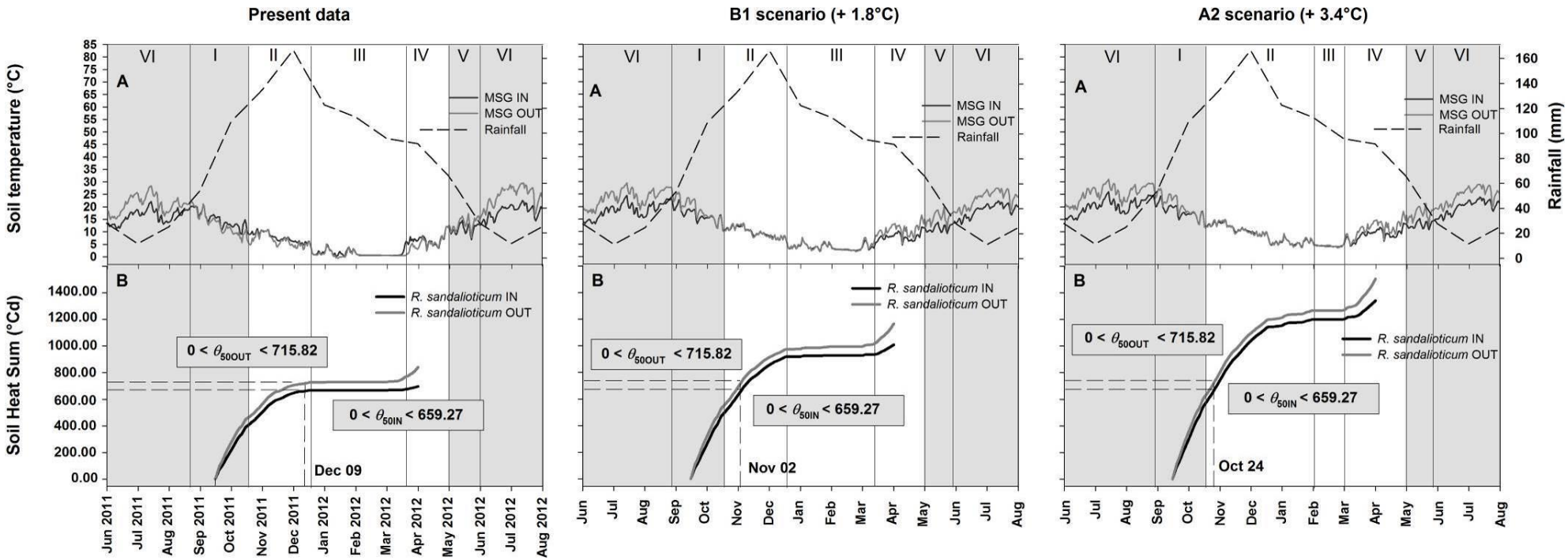

Figure 6 - (A) Annual trends of mean daily temperatures recorded in the soil for Monte Novo San Giovanni (MSG) with mean monthly rainfall obtained from the nearby weather stations of Montes, and (B) Soil Heat Sum (°Cd) to achievement of the predicted minimum and maximum values for germination near to the θ_{50} threshold value, both underneath (IN) and outside (OUT) the tree canopy, for the present data and under two different IPCC scenarios (B1, +1.8 °C and A2, +3.4 °C). The background grey squares correspond to the presence of the tree canopy. I, II, III, IV, V and VI correspond to different periods identified by Porceddu *et al.*, (2013). Results of the present data was already reported in figure 1A and 3, and it is presented again to better understand the differences with the other two scenarios.

Discussion

Seed dormancy

Baskin and Baskin (1998) discussed the phylogenetic relationships of dormancy at the family level, and reported that MD and MPD are basal in the evolutionary trend for the angiosperms, while PD, PY and (PY+PD) are derived. Martin (1946) placed several plant families, including Aquifoliaceae, Araliaceae, Magnoliaceae, Papaveraceae, and Ranunculaceae, at the base of his phylogenetic tree. Forbis *et al.* (2002) reconstructed the E:S (the ratio of embryo to seed) value of the angiosperm ancestor with standard phylogenetic methods, and showed that the low embryo size to seed size ratio (E:S) increased between ancestral and derived angiosperms. Forbis *et al.* (2002) confirmed that the Ranunculaceae are an ancestral family and placed the Paeoniaceae and the Grossulariaceae as derived families. *A. barbaricina* (Ranunculaceae) showed an initial embryo length of ca. 0.03 mm and a seed length of ca. 0.19 mm (i.e. E:S = 0.16; Chapter 2), while *R. sandalioticum* and *P. corsica* (Saxifragales) showed an initial E:S ratio of ca. 0.3 and of ca. 0.2, respectively (Mattana *et al.*, 2012b; Chapter 3). The similar E:S values detected between these three species confirm that there was a convergent evolution in embryo size at dispersal time in this *taxa* although belonging to different families, as a response to similar environmental conditions due to the same habitat and ecosystem, as suggested by Forbis *et al.* (2002).

Ecological correlates of embryo growth, seed germination, epicotyl emergence and seedling establishment in natural conditions

The phenology of embryo growth and of the radicle and cotyledon emergence in the seeds of *A. barbaricina*, *P. corsica* and *R. sandalioticum* were studied in this work. All species disperse between period VI and I, in presence of a tree canopy. All three species developed specific mechanisms for achieving seed germination, which are useful to avoid unfavourable environmental conditions for plant establishment.

Seeds of *A. barbaricina* are dispersed in summer and germinate the following spring/early summer, therefore the seeds experience warm stratification during the summer and cold stratification during autumn/winter (Mattana *et al.*, 2012a). Embryos start to grow inside the seeds after cold stratification, and when the critical embryo

length is reached, seeds germinate during the early spring. In this way, seedlings can grow before the dry summer period.

Seeds of *P. corsica* are dispersed in autumn and stay dormant until the next summer when seeds are exposed to warm temperature. During the warm stratification period, the embryos can grow inside the seeds, however, the critical embryo length can be reached before the cold stratification period, allowing seeds to germinate. Germinated seeds go through the winter with an emerged radicle, and the cold stratification period allows epicotyls to emerge in April. Seedlings establishment is completed before the end of the second wet season so that they could grow with well-developed roots and shoots before the dry summer period.

Berries of *R. sandalioticum* are dispersed in late summer and the seeds are exposed to warm temperatures. Once imbibed, embryos can grow inside the seeds, reaching the critical embryo length before the cold stratification period, thereby allowing seeds to germinate. Germinated seeds go through the winter with an emerged radicle, epicotyls emerge and seedlings establishment is complete before the end of the wet season and they could grow until the start of the summer. This allows the seedlings to enter the dry summer period with a well-developed root and shoot systems (Mattana *et al.*, 2012b).

Seed germination of *A. barbaricina* and *R. sandalioticum* was obtained when the tree canopy was absent, therefore its seems to have no influence on seed germination *sensu stricto* for these two species; similar behavior was found in *R. persicifolia*, species that growth in the same ecosystem and ecological conditions in Sardinia (Porceddu *et al.*, 2013; see Chapter 1). In *P. corsica*, on the contrary, the tree canopy seems to have negatively influence on seed germination; maximum germination for OUT (i.e. outside the tree canopy) was obtained in September and only few germinated seeds was found for IN in this period, while maximum germination for IN was obtained in December when the canopy was absent. In all species, however, closure of the tree canopy could influence survival of newly established seedlings due to microclimate amelioration (moister and cooler) during the dry and hot Mediterranean summers (Valiente-Banuet *et al.*, 1991; Greenlee and Callaway, 1996; Gómez-Aparicio *et al.*, 2005). The seeds of the all the investigated species showed a high synchronisation with the Mediterranean seasonality with respect to embryo growth, seed germination and seedling establishment, and thus demonstrating to have developed peculiar adaptations to the

harsh Mediterranean climatic conditions, as previously reported for *R. sandalioticum* by Mattana *et al.* (2012b).

Soil heat sum for in situ seed germination

The quantification of thermal time for germination has been used in different studies to characterize changes in seed dormancy and in the subsequent germination in the field (i.e. Forcella *et al.*, 2000; Hardegree and Van Vactor, 2000; Steadman *et al.*, 2003; Chantre *et al.*, 2009; Porceddu *et al.*, 2013). In this work we used the soil heat sum model applied by Porceddu *et al.* (2013; see Chapter 1), and the thermal threshold requirements (θ_{50}) of *A. barbaricina* calculated previously in controlled conditions (Chapter 2) to predict embryo growth and seed germination phenology in the field under the current environmental conditions. The obtained results showed a high correlation between soil heat sum accumulation to reach θ_{50} for the critical embryo length and seed germination. The model applied in this work demonstrated that, in the original population, these values were reached between April and May, thus confirming that the θ_{50} for embryo growth and the θ_{50} for seed germination are reached approximately at the same time. Results were validated through field observations of embryo growth measurements and seed germination. The model can also be used to estimate the range of θ_{50} for seed germination in species where the thermal time value (θ_{50}) in controlled conditions is unknown. The model allowed to estimate the soil heat sum accumulation for seed germination of *P. corsica* and *R. sandalioticum* seeds and to know approximately their thermal requirements.

Phenology of seed germination under a changing climate

Knowledge of thermal requirements for each species may be used to predict the seed germination phenology under increasing temperatures due to global warming. Orrù *et al.* (2012) used an environmental heat sum approach to predict germination timing under two simulated IPCC scenarios (+1.8 °C for B1 and + 3.4 °C for A2; IPCC, 2007) for *Vitis vinifera* subsp. *sylvestris* seeds, highlighting an altitude-related risk from climate warming, in particular under A2 scenario where the higher winter temperature would not allow seed dormancy loss in the lowest populations. Porceddu *et al*. (2013; see Chapter 1) reported that in *R. persicifolia* seeds, the warmer temperatures predicted by two simulated IPCC scenarios may reduce the cold stratification period useful for

dormancy release, and may anticipate the field germination, however, the increasing temperatures and the consequent reduction of the stratification period would not be detrimental *per se* for seed germination of this species. Results of this present study highlighted that the B1 and A2 scenarios may affect the rate of heat sum accumulation of all the studied species. In particular, soil heat sum under these two different IPCC scenarios bring completion of germination forward by about 1 month for all three species. The increasing temperature predicted in both scenarios should not compromise the dormancy release of *A. barbaricina*; however, the phenological shift of seed germination increases the temporal distance from the period of summer drought and could therefore enhance the seedlings growth of this species before the harsh period. On the contrary, this shifting, in particular in A2 scenario, could increase the risk of late frosts in spring which could damage young seedlings and increase the potential mortality of plants. The increasing temperature might be a disadvantage for *P. corsica*; in fact, the shifting could concentrate the process of seed germination during the summer drought period, increasing the risk of mortality for young seedlings. In addition, it could inhibit the establishment of seedlings at lower elevations, as previously reported for *V. vinifera* subsp. *sylvestris* (Orru *et al.*, 2012), with a consequent shift of the species at higher altitudes. However, these latter are areas lack of riparian woods, which represent the typical habitat where these species grow. This elevation range shift would thus lead to a reduction of their distribution and of their ecological range. The bringing forward of seed germination in *R. sandalioticum* would not cause particular problems for the seedlings growth because it would coincide with the period of maximum rainfall, when the seedlings could benefit of water availability and mild temperatures. However, the sensitivity of *R. sandalioticum* to low temperatures for seed germination highlighted the presence of an increasing threat due to global warming, which could reduce the level of natural emergence in the field (Mattana *et al.*, 2012b).

Conclusion

In conclusion, the seed germination phenology of three endemic Mediterranean mountain species with endospermic seeds growing in the same ecosystem was characterized. The thermal niche requirements for seed germination obtained in controlled condition in previous works (Chapters 2 and 3) were validated through field observations, and the developed model based on soil heat sum approach proposed by

Porceddu *et al.* (2013) allowed to predict with good accuracy the seed germination in the field and to estimate the thermal accumulation requirements for seed germination of each species. In addition, the model developed may have applicability to predictions of *in situ* regeneration under different IPCC scenarios of increasing temperatures. This work could confirmed that species, belonging to different families placed in different phylogenetic clades (Forbis *et al.*, 2002), could have experienced a convergent evolution on their seed morphology and type of seed dormancy, as a response to similar environmental and climatic conditions due by the same habitat and ecosystem (Forbis *et al.*, 2002).

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General conclusions

The main conclusions achieved in the present thesis are summarized in the following points:

1- Type 2 non deep physiological dormancy (PD) was identified for *R. persicifolia* seeds, the thermal niche requirements for dormancy release and germination were quantified and predictions for germination validated through field observations of emergence. The soil heat sum model developed in chapter 1 for seed germination in *R. persicifolia* may have applicability to predictions of *in situ* regeneration of other species growing on Mediterranean mountain waterways and of physiologically dormant species of temperate and alpine regions, where spring germination prevails due to a requirement for cold stratification over winter.

2- Intermediate simple morphophysiological dormancy (MPD) was identified for *A. barbaricina* seeds. Thermal time model developed in chapter 1 allowed to identify the thermal thresholds (T_b and θ_{50}) requirements of seed germination of this species. In addition, a similar model developed to embryo measurements allowed also to identify T_b and θ_{50} requirements of embryo growth for *A. barbaricina*. The modelling of the thermal time approach applied on embryo growth is an important first study that correlates the thermal threshold with seed morphology. In addition, results obtained in chapter 2 indicate that *A. barbaricina* showed a multi-step seed germination. The empirical knowledge of how the different phases occur during seed germination were confirmed by their thermal requirements. This model has significant advantages over some previous models for estimation of germination, in particular for seeds that highlight a morphological component to dormancy.

3- *Paeonia corsica* showed non-deep simple (root) - non-deep simple (epicotyl) morphophysiological dormancy and a multi-step of seed germination from dispersal to seedling establishment was observed also in this species, as individuated in *A. barbaricina*. Similar pattern on seeds germination was detected for *R. sandalioticum*, showing a high specialisation with the Mediterranean seasonality.

4- In chapter 4, the seed germination phenology of three endemic Mediterranean mountain species with endospermic seeds growing in the same ecosystem (i.e. *A. barbaricina*, *P. corsica* and *R. sandalioticum*) was characterized. The thermal niche requirements for seed germination obtained in controlled condition, reported in chapter 2 and 3, were validated through field observations, and the developed model based on

soil heat sum approach proposed in chapter 1 allowed to predict with good accuracy the seed germination in the field and to estimate the thermal accumulation requirements for seed germination of each species. In addition, the model developed may have applicability to predictions of *in situ* regeneration under different IPCC scenarios of increasing temperatures. In addition, chapter 4 could suggest that the studied species with endospermic seeds, belonging to different families placed in different phylogenetic clades, could have experienced a convergent evolution on their seed morphology and type of seed dormancy, as a response to similar environmental and climatic conditions due by the same habitat and ecosystem.

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Annexe I

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Thermal niche for *in situ* seed germination by Mediterranean mountain streams: model prediction and validation for *Rhamnus persicifolia* seeds

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• *Background and Aims* Mediterranean mountain species face exacting ecological conditions of rainy, cold winters and arid, hot summers, which affect seed germination phenology. In this study, a soil heat sum model was used to predict field emergence of *Rhamnus persicifolia*, an endemic tree species living at the edge of mountain streams of central eastern Sardinia.
• *Methods* Seeds were incubated in the light at a range of temperatures (10–25 and 25/10 °C) after different periods (up to 3 months) of cold stratification at 5 °C. Base temperatures (T_b), and thermal times for 50 % germination (θ_{50}) were calculated. Seeds were also buried in the soil in two natural populations (Rio Correboi and Rio Olai), both underneath and outside the tree canopy, and exhumed at regular intervals. Soil temperatures were recorded using data loggers and soil heat sum (°Cd) was calculated on the basis of the estimated T_b and soil temperatures.
• *Key Results* Cold stratification released physiological dormancy (PD), increasing final germination and widening the range of germination temperatures, indicative of a Type 2 non-deep PD. T_b was reduced from 10·5 °C for non-stratified seeds to 2·7 °C for seeds cold stratified for 3 months. The best thermal time model was obtained by fitting probit germination against log °Cd. θ_{50} was 2·6 log °Cd for untreated seeds and 2·17–2·19 log °Cd for stratified seeds. When θ_{50} values were integrated with soil heat sum estimates, field emergence was predicted from March to April and confirmed through field observations.
• *Conclusions* T_b and θ_{50} values facilitated model development of the thermal niche for *in situ* germination of *R. persicifolia*. These experimental approaches may be applied to model the natural regeneration patterns of other species growing on Mediterranean mountain waterways and of physiologically dormant species, with overwintering cold stratification requirement and spring germination.

Key words: Base temperature, climate change, cold stratification, physiological dormancy, Rhamnaceae, *Rhamnus persicifolia*, seed germination model, soil heat sum, thermal time.

INTRODUCTION

Seed dormancy prevents germination in a specified period of time, under any combination of environmental factors that otherwise favour germination (Baskin and Baskin, 2004). Thus, dormancy is an adaptive trait that optimizes the distribution of germination over time in a population of seeds (Copete *et al.*, 2011). In seasonal climates, temperature is usually the main environmental factor governing seed germination in moist soil (Fenner and Thompson, 2005). Seeds of many temperate plant species are dormant at the time of dispersal, and specific temperature requirements must be met before dormancy is lost and germination is possible (Baskin and Baskin, 1998). Depending on the species and timing of dispersal, seeds may experience a warm period before autumn and winter begin, or be subjected to cold stratification during winter immediately after autumn shedding (Baskin and Baskin, 1989; Noronha *et al.*, 1997). The requirement for chilling, widespread amongst temperate species, represents a natural mechanism which ensures that germination occurs in the spring (Probert, 2000). During exposure to low temperatures, the range of temperatures over which seeds will germinate, as well as germination percentages, increases (Baskin and Baskin, 1988).

The Mediterranean climate is characterized by its seasonality in temperature and precipitation, which leads to a hot drought in summer and a cool, wet, winter (Joffre *et al.*, 1999). This peculiarity has important implications for plant germination physiology, since dry summer conditions limit water availability and thus germination and growth, while cool winter temperatures can limit germination during the season with high water availability (Rundel, 1996).

The canopies of woody plants modify the microclimate beneath and around them through interception of precipitation and by shading, which influence maximum soil temperature and the amount of soil moisture available to plants (Breshears *et al.*, 1998). As the course of action and relative importance of factors regulating germination in the laboratory may be quite different from those occurring under field conditions (Thompson, 1973), linkage between field, garden and laboratory studies is crucial (Brändel and Schütz, 2005).

As reproduction niche and reproductive success are related to temperature, all aspects of the plant reproductive cycle are

potentially sensitive to climate change (Bykova *et al.*, 2012). The Intergovernmental Panel on Climate Change (IPCC) has predicted temperature increases of approx. 2–4 °C by 2090–2099. Furthermore, in the Mediterranean region, a declining trend of precipitation was observed from 1900 to 2005 (IPCC, 2007). In response to climate change, plants can adapt to the new environmental conditions or, when possible, migrate to track their climatic niches (Meineri *et al.*, 2013).

In non-dormant seeds, the germination response to accumulated temperature has been modelled by a thermal time (θ) approach (Garcia-Huidobro *et al.*, 1982; Covell *et al.*, 1986; Ellis *et al.*, 1986, 1987; Pritchard and Manger, 1990; Hardegree, 2006). In this model, seeds accumulate units of thermal time (°Cd) to germinate for a percentile g of the population. When seeds are subjected to temperatures (T) above a base temperature for germination (T_b), the germination rate increases linearly with temperature to an optimum temperature (T_o), above which germination rate starts to decrease (Garcia-Huidobro *et al.*, 1982). Thus, in this sub-optimal range (T_o–T_b), germination occurs in the time t_g, when the thermal time accumulated has reached the critical value (θ_g) for a percentile g of the population, and can be described as $\theta_g = (T - T_b)t_g$.

Intraspecific variation in T_b among seed populations may be due to different environmental conditions during seed development (Daws *et al.*, 2004). However, T_b has also been found to change with dormancy status. In particular, Pritchard *et al.* (1999) found that T_b decreased by 1 °C every 6 d of pre-chilling at 6 °C in *Aesculus hippocastanum* seeds. Thus seed dormancy release in this species could be described simply in terms of T_b reduction, gradually allowing germination to occur at progressively lower temperatures (Pritchard *et al.*, 1999). In addition, subsequent seed germination may be predicted in relation to thermal time accumulation (heat sum, °Cd) above a gradually reducing T_b (Steadman and Pritchard, 2004). This approach has been used to predict seed germination in the field (i.e. Hardegree and Van Vactor, 2000; Steadman *et al.*, 2003; Chantre *et al.*, 2009) and, more recently, to assess the impact of different simulated climate change scenarios on seed dormancy release and germination timing in *Vitis vinifera* subsp. *sylvestris* (Orrù *et al.*, 2012).

Sardinian massifs represent a southern European refugium for some temperate tree species *sensu* Tzedakis *et al.* (2002). In this region, vegetation among mountain waterways is mainly constituted by *Alnus glutinosa* woods, where the rare Sardinian endemic *Rhamnus persicifolia* may also be found. Seeds of the Rhamnaceae have an investing embryo (Martin, 1946) and can be non-dormant or, following the dormancy classification system (Baskin and Baskin, 1998, 2004), show physiological (PD), physical dormancy (PY) or combined (physical and physiological; PY + PD) dormancy. Physical dormancy is the most represented class in this family (61 % of the investigated species), followed by PY + PD (22 %), PD (12 %) and non-dormancy (ND) (6 %; Walck *et al.*, 2012). Mattana *et al.* (2009) reported that germination of *R. persicifolia* seeds could be achieved, without any scarification, at warm temperatures (≥20 °C), excluding the presence of PY. Whilst there was no obligate requirement for alternating temperature or light, pre-chilling had a positive effect on the germination rate, reducing T_{50} by >50 % and indicating the presence of PD in seeds of this species. However, the effect of pre-chilling on seed germination over a wide range of temperatures, and the identification of the type of PD according to the seed dormancy classification system (Baskin and Baskin, 2004), remain to be investigated.

The aims of this work were to (1) investigate the thermal requirements for seed dormancy release and germination of the rare *R. persicifolia* and (2) develop a thermal-time model, based on a soil heat sum approach, in order to characterize the thermal niche for seed germination and predict the seed germination phenology in the field.

MATERIALS AND METHODS

Study species

Rhamnus persicifolia is a small dioecious tree or shrub. It is closely related to *R. cathartica*, but with elliptic–lanceolate leaves and reddish ripe drupes. It is endemic to Central–Eastern Sardinia (Italy), occurring at 600–1500 m a.s.l. on both limestone and siliceous substrata. This species grows in scattered groups or as single trees, in riparian woods or hygrophilous scrubs along mountainous waterways (Mattana *et al.*, 2009). *Rhamnus persicifolia* is included in the Italian Red Book as vulnerable (Conti *et al.*, 1992, 1997), because of its narrow distribution and population decline, induced by human activities and by climate change (Arrigoni, 1977). To date, only six populations are known; half of these are threatened by low plant numbers or an unbalanced sex ratio (Bacchetta *et al.*, 2011).

Seed lot details

Fruits of *R. persicifolia* were collected directly from 15 plants on 16 September 2011 along the Rio Correboi (RC; Villagrande Strisaili, Ogliastra) and from five plants on 30 September 2011 along the Rio Olai (RO; Orgosolo, Nuoro) streams in Central–Eastern Sardinia (see Table 1). The low number of sampled plants was due to the few female individuals found in these two populations (see Bacchetta *et al.*, 2011). Seeds were immediately separated from the pulp by rubbing the fruits through sieves under running water. The cleaned seeds were then spread out and left to dry at room temperature, until the experiments started, as specified below.

Germination tests under controlled conditions

For the RC provenance collection, three replicates of 20 seeds were sown on the surface of 1 % agar water in 90 mm diameter plastic Petri dishes and incubated in the light (12 h light/12 h darkness) for 1–4 months under a range of constant temperatures (10, 15, 20 and 25 °C) and under an alternating temperature regime (25/10 °C). In the alternating temperature regime, the 12 h light period coincided with the elevated temperature period. At the same time, three different cold stratification periods were started (5 °C in 1 % agar water in 90 mm diameter plastic Petri dishes for 1, 2 and 3 months: C1, C2 and C3 treatments, respectively) and, at the end of each pre-treatment, seeds were incubated, as detailed above.

Due to the low availability of seeds collected in the RO (see Table 1), these seeds were only stratified for 3 months at 5 °C

TABLE 1. *Locations, habitat characteristics and dates of experimental trials carried out in each site (Rio Correboi, RC; Rio Olai, RO) of the two natural populations of* R. persicifolia

Population	Soil substrate type	Experimental sites	Habitat	Altitude (m a.s.l.)	Aspect	Date of field sowing	Dates of exhumation and days after sowing
Rio Correboi (Villagrande Strisaili, Ogliastra), RC	Metamorphytes	RC1 IN	Riparian wood with *Alnus glutinosa* – Mantle shrubs with *Rubus ulmifolius*.	1209	0	30/09/2011	26/04/2012 (209 d), 25/06/2012 (269 d)
		RC1 OUT	Open grassland of *Carici-Genistetea lobelioidis*.		NE		
		RC2 IN	Riparian wood with *A. glutinosa* – Mantle shrubs with *R. ulmifolius*.	1267	0	30/09/2011	09/12/2011 (70 d), 29/03/2012 (181 d), 26/04/2012 (209 d), 25/06/2012 (269 d)
		RC2 OUT	Open grassland of *Carici-Genistetea lobelioidis*.		NE		
		RC3 IN	Shady rocky outcrop with *Ribes multiflorum*. subsp. *sandalioticum* and *Rubus ulmifolius*.	1347	NE	30/09/2011	26/04/2012 (209 d), 25/06/2012 (269 d)
		RC3 OUT	Open grassland of *Carici-Genistetea lobelioidis*.		NE		
Rio Olai (Orgosolo, Nuoro), RO	Metamorphytes	RO IN	Riparian wood with *A. glutinosa* – Mantle shrubs with *R. ulmifolius*.	970	0	05/10/2011	09/12/2011 (65 d), 29/03/2012 (176 d), 26/04/2012 (204 d), 25/06/2012 (264 d)
		RO OUT	Open grassland of *Carici-Genistetea lobelioidis*.		0		

For each experimental site, IN and OUT differentiate between underneath and outside the canopy, respectively.

and then incubated at 25 °C (12 h light/12 h darkness). These conditions were chosen on the basis of earlier findings (Mattana *et al.*, 2009).

Germination was defined as visible radicle emergence (>1 mm). Germinated seeds were scored three times a week. At the end of the germination tests, when no additional germination had occurred for 2 weeks, a cut test was carried out to determine the firmness of the remaining seeds and the number of empty seeds. Firm seeds were considered to be viable. This methodology was chosen on the basis of previous findings on seeds of this species, which highlighted a very high seed viability, with 100 % of non-empty seeds staining uniformly red in 1 % solution of 2,3,5-triphenyl-tetrazolium chloride (Mattana *et al.*, 2009).

Field experiments

Within 15–20 d of collection, seeds were placed in fine-mesh polyester envelopes (three replicates of 25 seeds) and buried in soil at a depth of 2–3 cm. Envelopes were buried both underneath (IN) and outside (OUT) the canopy, with a distance between them of approx. 6 m, at each experimental site of the two original populations, for a total of six experimental sites for RC, in order to cover the whole altitudinal range of this population, and two for RO (Table 1). Envelopes buried in experimental sites RC2 and RO were exhumed at intervals of about 3 months from September 2011 to June 2012 (with an intermediate exhumation also in April 2012; Table 1). Alternatively, those buried in experimental sites RC1 and RC3 were exhumed only in April and June 2012. Retrieved envelopes were analysed in the laboratory, where they were washed under running water and opened. The number of germinated seeds was recorded, and a cut test was carried out to check the viability of any remaining non-germinated seeds, as described above.

Soil temperatures at the level of the envelopes were recorded IN and OUT of the canopy at 90 min intervals, using data loggers (TidbiT® v2 Temp logger, Onset Computer Corporation, Cape Cod, MA, USA).

Data analysis

The final germination percentage was calculated as the mean of the three replicates ± standard deviation (s.d.), on the basis of the total number of filled seeds. Generalized linear models (GLMs) were used to compare: (1) final germination percentages and T_b achieved under controlled conditions for seed collected in RC, followed by a *post hoc* pairwise comparisons *t*-test (with Bonferroni adjustment); and (2) the field germination percentages at each experimental site (RC1, RC2, RC3 and RO) on different exhumation dates (December 2011, March 2012, April 2012 and June 2012), both IN and OUT of the canopy (see Table 1). Generalized linear models, with a logit link function and quasi-binomial error structure, were used when analysing germination percentages, whereas a GLM with a log link function and quasi-poisson error structure was used for analysing T_b values. Quasi-binomial and quasi-poisson error structures and *F*-tests with an empirical scale parameter instead of χ^2 on the subsequent analysis of variance (ANOVA) were used in order to overcome residual overdispersion (Crawley, 2007).

Thermal time analyses were carried out for RC seeds germinating at constant temperatures for untreated seeds (0, control) and after each cold pre-treatment (C1, C2 and C3). Estimates of time

(t_g, d) taken for cumulative germination to reach different percentiles (g) for successive increments of 10 % germination were interpolated from the germination progress curves (Covell *et al.*, 1986). The germination rate ($1/t_g$) was regressed, using a linear model, as a function of temperature according to the following equation (Garcia-Huidobro *et al.*, 1982):

$$1/t_g(d^{-1}) = (T_g - T_b)/\theta \quad (1)$$

An average ($\pm$ s.d.) of the x-intercept among percentiles was calculated for the sub-optimal temperature range (10–20 °C) to establish the T_b for each treatment (Ellis *et al.*, 1986; Pritchard and Manger, 1990). Linear regression equations were then recalculated for each percentile, but constrained to pass through T_b (Hardegree, 2006). A comparison of regressions was then made between this model and one in which the T_b were allowed to vary for all the percentiles, and the best estimate was considered to be that which resulted in the smallest residual variance (Covell *et al.*, 1986). Thermal time (θ, °Cd) estimates for each treatment were then calculated separately as the inverse of the sub-optimal regression equations [Covell *et al.*, 1986; see eqn (1)].

The T_b values were fitted as a function of the length of the stratification period using a linear model. Generally, θ did not accumulate during pre-treatments because the stratification temperature (5 °C) was lower than the T_b. However, in seeds stratified at 5 °C for 120 d (C3), T_b reduced during stratification to below the stratification temperature itself. Using the relationship between rate of decline of T_b and temperature, and assuming that the rate of reduction of T_b continued unchanged, according to Steadman and Pritchard (2004), θ accumulated during the C3 stratification phase (θ_s) was calculated.

Germination percentages were transformed to probits using tabular data from Finney (1971). Linear regression was used to express probit (g) as a function of thermal time (θ_g) and the form of cumulative germination response of seeds described by the equation (Covell *et al.*, 1986):

$$\text{probit}(g) = K + \theta_g/\sigma \quad (2)$$

where K is an intercept constant when θ_g is zero, θ_g may be normal or log-normal distributed (and the best model evaluated on the basis of the r^2 values; Hardegree, 2006), and σ is the standard deviation of the response to θ_g (i.e. the reciprocal of the slope), and represents the sensitivity of the population to θ_g (Covell *et al.*, 1986). Thus the flatter the slope of the fitted line, the greater the variation in response to thermal time between individual seeds (Daws *et al.*, 2004).

A heat sum approach was used to predict seed germination in the field, according to Orrù *et al.* (2012). These authors used environmental temperatures of the original populations above T_b to assess the temperature accumulation until the achievement of θ_{50} (Orrù *et al.*, 2012). In this study, soil heat sum was calculated, starting from the date of sowing, according to the following equation, modified from Daws and Jensen (2011):

$$\text{Soil heat sum (°Cd)} = \{\sum[(T_S - T_b) \times t]\}/18 \quad (3)$$

where T_S is the temperature at each logging interval recorded by data loggers, T_b is the base temperature for germination calculated day by day, according to the length of stratification experienced in the field, t is the length of the logging interval expressed in hours and 18 is the number of logging records per day. All statistical analyses were carried out using R v. 2·14·0 (R Development Core Team, 2011).

Pluviometric data for RC (monthly rainfall averages from 1922 to 2009 from the nearby climatic station of Fonni, Nuoro) and RO (monthly rainfall averages from 1936 to 2009 from the nearby climatic station of Montes, Orgosolo, Nuoro) were acquired from Regione Autonoma della Sardegna (http://www.regione.sardegna.it/j/v/25?s=131338&v=2&c=5650&t=1). The presence/absence of the tree canopy of riparian wood with *A. glutinosa* was observed at each field excursion realized during this study.

RESULTS

Seed germination under controlled conditions

The fitted GLM highlighted a statistically significant effect ($P <$ 0·001) on germination of temperature (T) and treatment (S) factors and of their interaction ($T \times S$; Fig. 1) for seeds collected in RC (see Table 1). Untreated seeds (0) germinated at percentages ranging from approx. 50 % to approx. 87 % at all the tested temperatures, except at 10 °C where germination was <15 % (Fig. 1). The applied cold stratification treatments increased seed germination percentages and widened the range of germination temperatures (Fig. 1). In particular, the effect of cold stratification was positive and statistically significant ($P <$ 0·001) at 10 °C, with germination increasing with the length of stratification from 12 $\pm$ 8 % (0) to 92 $\pm$ 8 % (C3), and at 15 °C, with percentages increasing from 61 $\pm$ 5 % (0) to 87 $\pm$ 3 % (C3). Untreated and cold-stratified seeds reached high germination when incubated under the alternating temperature regime (25/10 °C), with percentages >80 % for 0, C1 and C2

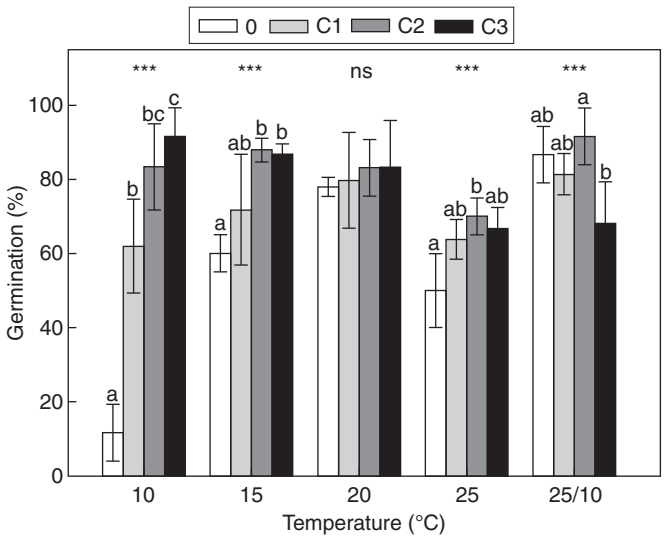

FIG. 1. Effects of temperatures and cold treatments (0, control; C1, C2 and C3 cold stratification at 5 °C for 1, 2 and 3 months, respectively) on final germination for *Rhamnus persicifolia* seeds collected in Rio Correboi. Data are the mean of three replicates (1 $\pm$ s.d.). Temperatures, treatments and their interaction are statistically significant ($P <$ 0·001 by GLM). *Post hoc* pairwise t-test comparisons (with Bonferroni adjustment) were carried out for each germination temperature, and bars with different letters indicate significant ($P <$ 0·05) variation.

treatments, without statistically significant differences ($P > 0{\cdot}05$); whereas after C3, germination significantly ($P < 0{\cdot}05$) decreased to 68 ± 11 % (Fig. 1).

Final germination for seeds collected in RO incubated at 25 °C after 3 months at 5 °C was 60 ± 7 %.

Thermal requirement for germination

Goodness of fit (r^2) for the linear regressions of $1/t$ against temperature for RC collections showed that the best sub-optimal model included data only up to 20 °C (i.e. excluding 25 °C; Fig. 2A). Based on germination rate responses for each 10th percentile from 10 to 80 % germination, it was possible to estimate the mean base temperature for germination (T_b) in the sub-optimal temperature range for each treatment (Fig. 2A). Average T_b values were 10·5 ± 0·6, 8·5 ± 0·9, 6·1 ± 1·4 and 2·7 ± 0·8 °C, for 0, C1, C2 and C3 treatments, respectively. For the different treatments, linear regressions were re-calculated for each percentile, constraining them to pass through the mean T_b. This model led to no differences in residual sum of squares and showed higher values of r^2 for all of the linear regression equations ($r^2 > 0{\cdot}75$ for 0, $r^2 > 0{\cdot}93$ for C1, $r^2 > 0{\cdot}81$ for C2

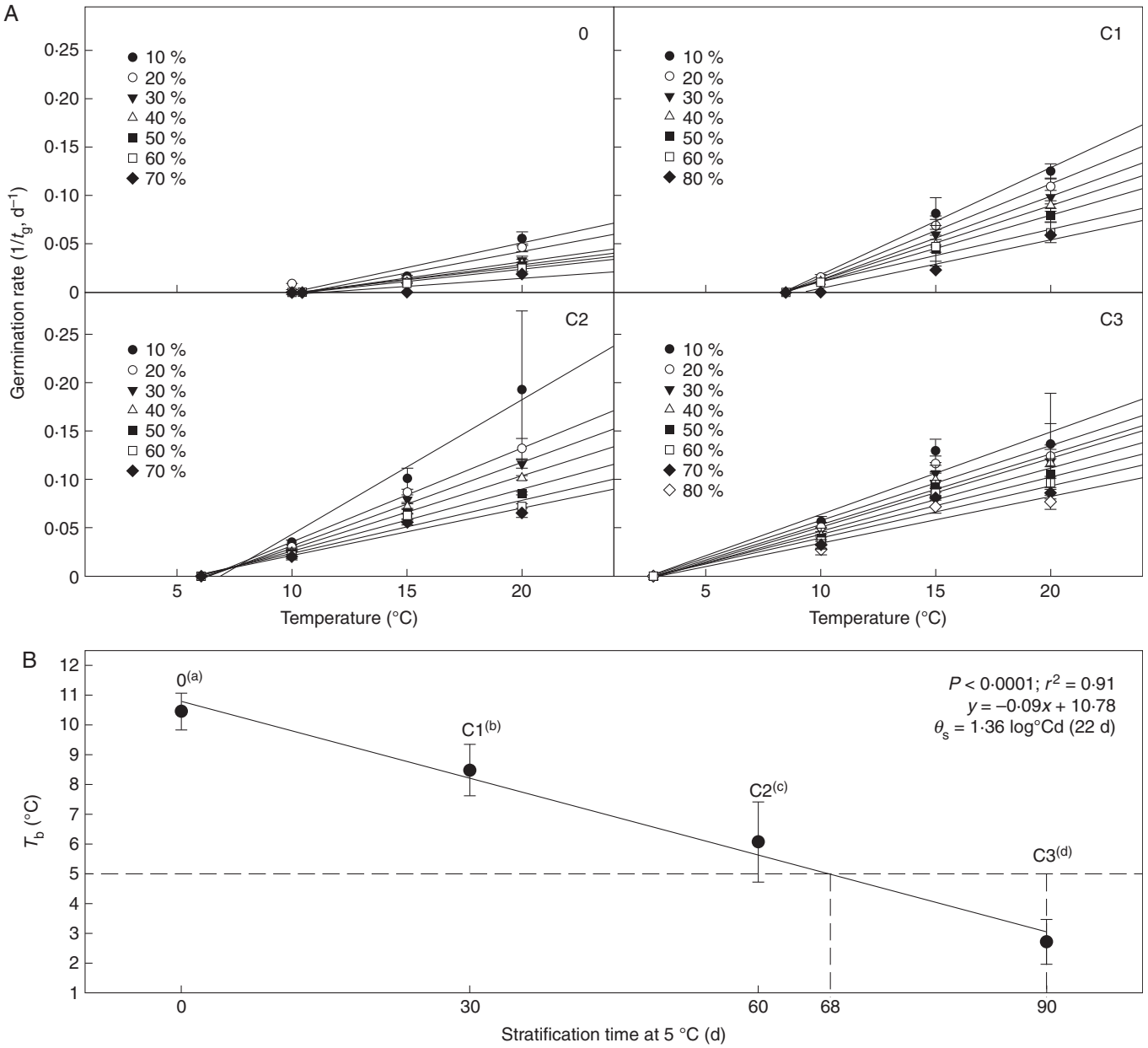

FIG. 2. (A) Base temperatures (T_b), calculated for different germination percentiles of *Rhamnus persicifolia* seeds, after each pre-treatment (0, control; C1, C2 and C3 cold stratifications at 5 °C for 1, 2 and 3 months, respectively) and incubation at the sub-optimal temperatures (10–20 °C). Within each pre-treatment, the linear regressions for the different percentiles were constrained to the common value of T_b. Linear regressions of percentiles with $P > 0{\cdot}05$ were not included. (B) Relationship between T_b and stratification time at 5 °C. Data are the mean ± s.d. of T_b of each percentile. Statistical differences among pre-treatments were analysed by GLM followed by *post hoc* pairwise *t*-test comparisons (with Bonferroni adjustment). Mean T_b values with different letters are significantly different at $P < 0{\cdot}05$.

and $r^2 > 0{\cdot}81$ for C3) than the model where T_b varied for each percentile ($r^2 > 0{\cdot}73$ for 0, $r^2 > 0{\cdot}87$ for C1, $r^2 > 0{\cdot}73$ for C2 and $r^2 > 0{\cdot}54$ for C3). The T_b values were statistically different ($P < 0{\cdot}001$) by GLM, and the *post hoc* pairwise *t*-test comparison (with Bonferroni adjustment) highlighted significant differences among all treatments (Fig. 2B). The relationship between T_b and the length of the stratification period at 5 °C is shown in Fig. 2B. The linear regression highlighted that this negative relationship was statistically significant ($r^2 = 0{\cdot}91$, $P < 0{\cdot}0001$; Fig. 2B), with T_b decreasing by 0·09 °C d^{-1} of stratification or by 1 °C for every 11 d of chilling. After 68 d of stratification, T_b decreased below 5 °C, and seeds accumulated 1·36 log °Cd (θ_s) in the next 22 d until the end of the C3 treatment at 90 d.

Figure 3 shows the relationship between log thermal time (θ) and germination expressed in probits, calculated according to eqn (2). The relationship between log θ and probit germination had better residual sums of square (0·1091, 0·0961, 0·0228 and 0·1366 for 0, C1, C2 and C3, respectively) and r^2 (0·95, 0·97, 0·99 and 0·96 for 0, C1, C2 and C3, respectively) than when expressed on a linear scale (data not shown). Thermal time for 50 % of germination (θ_{50}) was greater for the control (2·59 log °Cd) compared with the cold-treated seeds (from 2·17 to 2·19 log °Cd; Fig. 3). Seeds of 0 and C2 had a greater σ value (0·26 and 0·25 log °Cd, respectively) compared with C1 and C3 (0·18 and 0·12 log °Cd, respectively; Fig. 3).

Seed germination in the field

In December 2011, the great majority of seeds (>85 %) were dormant (Table 2), although a few seeds (<3 %) had started to germinate in RO. In March 2012, seeds also started germinating in RC, while the majority of the remaining seeds were still dormant, and the level of dead seeds was always <7 % (Table 2). In RO, the majority of the seeds germinated, reaching values of approx. 70 % both IN and OUT, and the remaining seeds were mainly dead (Table 2). By April 2012, germination in RC1 was approx. 60 %, with approx. 25 % of seeds remaining dormant and 15 % dead, for both IN and OUT. In RC2 IN and OUT, approx. 75 and 35 % of the seeds, respectively, had germinated; approx. 14 and 45 % of seeds were dormant and approx. 11 and 20 % of seeds were dead. For RC3 OUT, germination reached approx. 43 %, with approx. 10 and 47 % being dormant or dead, respectively. No germination data were available for RC3 IN due to predation by animals (Table 2).

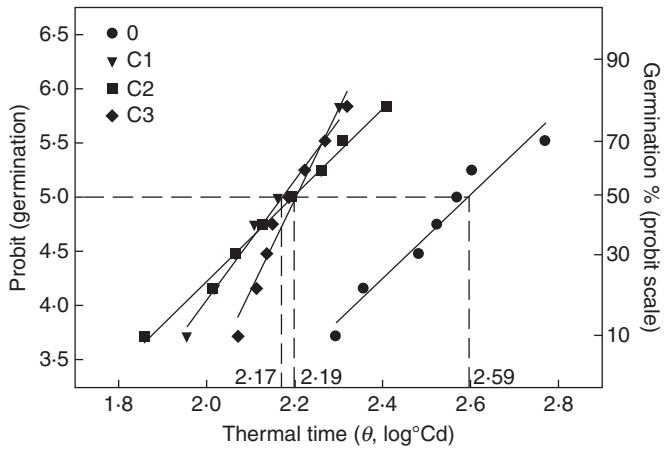

FIG. 3. Probit germination after each pre-treatment (0, control; C1, C2 and C3 cold stratification at 5 °C for 1, 2 and 3 months, respectively) as a function of log-thermal time requirement. Thermal times were calculated from germination time-courses from estimated T_b of 10·5, 8·5, 6·1 and 2·7 °C for 0, C1, C2 and C3, respectively. Thermal times to reach θ_{50} are also shown (dashed lines).

At the last exhumation, in June 2012, the percentage of dead seeds was high for all the experimental sites in both populations, ranging from 24 ± 9 % for RC1 OUT to 91 ± 4 % for RC2 OUT, and all the remaining seeds germinated (Table 2). The bag in RC1 IN could not be retrieved, as it was probably washed away, while seeds in that of RC3 IN were predated by animals (Table 2).

Generalized linear models highlighted a statistically significant ($P < 0{\cdot}001$) effect for all the factors (date, D; position, P; site S) as well as for their interactions, except for the two-way interaction D × P and the three-way interaction D × P × S for which $P > 0{\cdot}05$ (Table 3).

Soil heat sum and thermal niche for in situ *seed germination*

The establishment of the tree canopy of *A. glutinosa* woods was very similar in the two streams (RC and RO), starting at the end of April and disappearing in mid-October (Fig. 4). In detail, the annual trend of soil temperatures could be divided into six periods, according to the presence/absence of the canopy and to the seasons, for RC1, RC2 and RO experimental sites: (I) from the sowing at the end of September/early October to the disappearance of the tree canopy in mid-October; (II) from the disappearance of the canopy in mid-October to the start of the stratification period, when mean daily temperatures fell to 5 °C in December; (III) the main stratification period, from December to March, when mean daily temperatures are close to 5 °C; (IV) from the end of the stratification period in March to the appearance of the canopy in April; (V) from the appearance of the canopy in April to the start of the summer droughts in June/July; and (VI) the summer drought period when rainfall drastically reduces (Fig. 4, Table 2). The absence of a riparian wood in RC3 (see Table 1) led to only four environmental periods: (I) from sowing to the start of the stratification period in December; (II) the stratification period until March; (III) from the end of the stratification period to the start of the summer droughts in June/July; and (IV) the summer drought period.

By combining eqn (3) and the equation in Fig. 2B, where T_b was calculated day by day, for RC seeds, according to the length of stratification experienced in the field, it was possible to calculate the soil heat sum reached by the seeds at the different exhumation times for each experimental site of both populations (Table 2). The values calculated for RC2 and RO (for which there was a complete temporal sequence) were compared with those estimated using the thermal time (θ) model, expressed as probit germination and log °Cd (for germination values from 10 to 80 %; see Fig. 3). The linear regression highlighted a statistically significant relationship between calculated and estimated data ($n = 5$; $P = 0{\cdot}0018$; $r^2 = 0{\cdot}97$; $y = 1{\cdot}0992x - 0{\cdot}1739$).

In RC2 (Fig. 4A), the length of the effective stratification periods was 92 d for IN and 98 d for OUT (with 41 and 47 d of snow cover, respectively), leading to T_b values at the end of the stratification period of 2·9 and 2·5 °C for IN and OUT, respectively. Before (periods I and II) and during stratification (period III), mean soil temperatures were similar or lower than T_b (10·2 °C), preventing the soil heat sum accumulation for germination. However, after

TABLE 2. *Evaluation categories of the exhumed seeds (%), recorded soil temperatures (°C), calculated soil heat sum (log °Cd) and field germination percentages (mean ± s.d.) for each experimental site (Rio Correboi, RC; Rio Olai, RO) underneath (IN) and outside (OUT) the canopy at the different exhumation dates*

		Evaluation categories of the exhumed seeds (%, mean ± 1 s.d.)											Recorded mean soil temperature (°C)		Calculated soil heat sum (log °Cd)		Predicted soil heat sum (log °Cd)	
		IN					OUT											
Date of exhumation	Experimental site	G	V	D	NT	P	G	V	D	NT	P	Period	IN	OUT	IN	OUT	IN	OUT
09/12/2011	RC2	0 ± 0	95 ± 2	5 ± 2	–	–	0 ± 0	98 ± 2	1 ± 2	–	–	II	4·9	3·0	1·54	0·95	–	–
	RO	1 ± 2	83 ± 8	16 ± 11	–	–	3 ± 2	87 ± 6	11 ± 4	–	–	II	7·5	6·6	1·68	1·88	–	–
29/03/2012	RC2	32 ± 18	61 ± 19	7 ± 2	–	–	2 ± 3	95 ± 5	5 ± 5	–	–	IV	6·3	3·5	1·96	1·43	2·15	–
	RO	73 ± 12	6 ± 7	21 ± 16	–	–	75 ± 3	8 ± 7	17 ± 7	–	–	IV	8·2	11·0	2·16	2·43	2·29	2·28
26/04/2012	RC1	57 ± 24	28 ± 13	15 ± 15	–	–	61 ± 12	25 ± 13	13 ± 2	–	–	IV	8·9	10·4	2·44	2·37	2·22	2·23
	RC2	74 ± 5	14 ± 5	11 ± 2	–	–	35 ± 12	45 ± 8	20 ± 7	–	–	IV	9·5	9·7	2·34	2·28	2·28	2·16
	RC3	–	–	–	–	100	43 ± 25	10 ± 9	47 ± 33	–	–	III	6·7	16·2	1·94	2·69	–	2·18
	RO	55 ± 11	7 ± 4	38 ± 11	–	–	73 ± 3	14 ± 11	13 ± 8	–	–	IV	11·7	13·7	2·49	2·68	2·21	2·27
25/06/2012	RC1	–	–	–	100	–	76 ± 9	0 ± 0	24 ± 9	–	–	V	17·4	27·5	3·01	3·15	–	2·29
	RC2	71 ± 21	0 ± 0	29 ± 21	–	–	9 ± 4	0 ± 0	91 ± 4	–	–	V	19·3	27·0	3·02	3·11	2·26	–
	RC3	–	–	–	–	100	4 ± 4	0 ± 0	96 ± 4	–	–	III	16·6	27·6	2·90	3·22	–	–
	RO	45 ± 24	0 ± 0	55 ± 24	–	–	57 ± 11	0 ± 0	43 ± 11	–	–	V	17·1	27·1	3·02	3·19	2·18	2·22

The soil heat sum values, predicted on the basis of the thermal time (θ) model (expressed as probit germination and log °Cd; see Fig. 3), are also reported for the different germination percentages for values from 10 to 80 % (see Fig. 3).

G, germinated seeds; V, viable dormant seeds; D, dead seeds; P, predated seeds; NT, envelopes not retrieved.

Periods, identified according to the presence/absence of the canopy and to the seasons for all the experimental sites for RC1, RC2 and RO, correspond to: (I) from sowing to the disappearance of the tree canopy; (II) from the disappearance of the canopy to the start of the stratification period; (III) the stratification period; (IV) from the end of the stratification period to the appearance of the canopy; (V) from the appearance of the canopy to the start of the summer droughts; and (VI) the summer drought period. For RC3 they correspond to: (I) from sowing to the start of the stratification period; (II) the stratification period; (III) from the end of the stratification period to the start of the summer droughts; and (IV) the summer drought period.

TABLE 3. *GLM results for the effect on seed germination in the field of the following factors: 'Date' (D: December 2011, March 2012, April 2012 and June 2012), 'Position' (P: IN and OUT) and 'Experimental site' (S: RC1, RC2, RC3 and RO)*

	d.f.	Deviance	Residual d.f.	Residual deviance	*F*	*P*
Null			62	3105·85		
Date (D)	3	1371·24	59	1734·61	59·3520	<0·001
Position (P)	1	98·21	58	1636·40	12·7530	<0·001
Site (S)	3	456·67	55	1179·73	19·7661	<0·001
D × P	3	34·36	52	1145·37	1·4872	>0·05
D × S	5	408·83	47	736·54	10·6173	<0·001
P × S	2	385·86	45	350·68	25·0519	<0·001
D × P × S	3	10·34	42	340·34	0·4474	>0·05

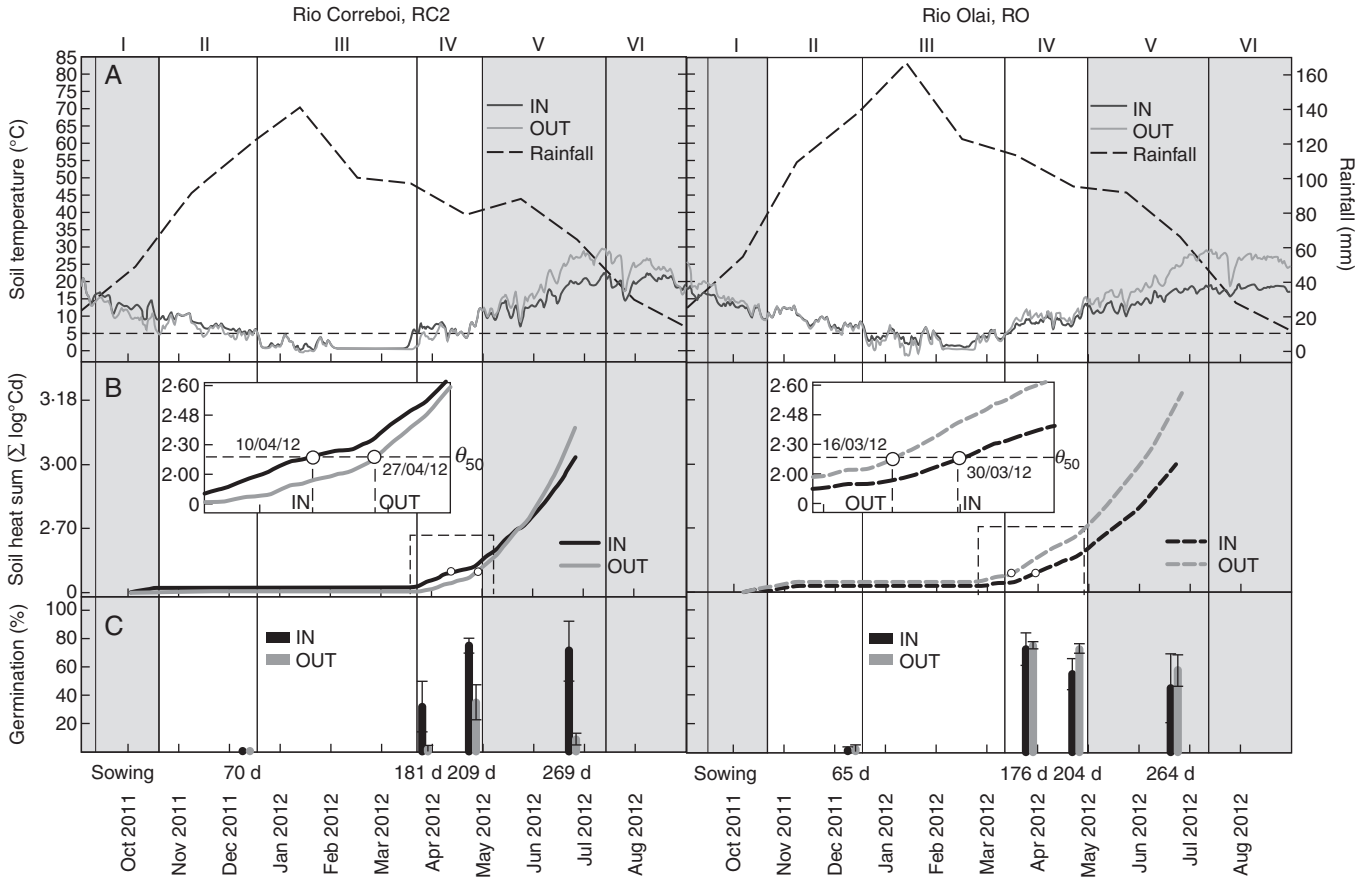

FIG. 4. Soil temperatures, soil heat sum and field germination for Rio Correboi (RC2) and Rio Olai (RO). (A) Annual trends of mean daily temperatures recorded in the soil both underneath (IN) and outside (OUT) the tree canopy and mean monthly rainfall (data from the nearby weather stations of Fonni and Montes for RC2 and RO, respectively); (B) soil heat sum (expressed in log °Cd); and (C) field germination (three replicates of 25 seeds each) IN and OUT at each time of exhumation, expressed in days after sowing. The inset plots in (B) show the detail of the achievement of the θ_{50} threshold value (2·19 log °Cd). The background grey squares correspond to the presence of the tree canopy. The details of periods I, II, III, IV, V and VI are as given for Table 2

stratification (period IV), the lower T_b values and the increasing soil temperatures allowed the threshold of 2·19 log °Cd (which corresponds to the value to achieve 50 % of germination in the laboratory, θ_{50}) to be reached 194 (IN) and 211 (OUT) d from sowing (Fig. 4B). This estimated time was confirmed by the germination recorded in the field (Table 2, Fig. 4C).

In RC1, the length of the stratification period was 90 d for IN and 104 d for OUT environmental conditions, leading to T_b values at the end of the stratification period of 2·9 and 1·9 °C for IN and OUT, respectively. After stratification, the threshold for θ_{50} was reached 186 and 200 d after sowing for IN and OUT, respectively, consistent with the field values presented in Table 2. In RC3, the effective stratification period was 116 d for IN and 93 d for OUT, leading to T_b values at the end of the stratification period of 2·5 and 2·9 °C for IN and OUT, respectively. Therefore, the threshold for θ_{50} was reached in period III, 171 (OUT) and 219 (IN) d after sowing. Although few field germination data were available for this experimental site, the

highest germination (43·0 ± 25·2 % for OUT) was recorded in April (Table 2).

In RO, the length of the stratification period was 75 d for both IN and OUT (with 15 and 20 d of snow cover, respectively), leading to T_b values at the end of the stratification period of 4·4 °C for each site (Fig. 4). Before (periods I and II) and during stratification (period III), mean soil temperatures were similar or lower than T_b (10·2 °C), leading to a slow accumulation of heat sum (1·73 log °Cd for IN and 1·97 log °Cd for OUT) by the end of period III (Fig. 4B). After stratification (period IV), the lower T_b values and the increasing soil temperatures enabled θ_{50} for RC seeds to be reached 164 (OUT) and 178 (IN) d after sowing (Fig. 4B). Although these times were estimated using data from seeds belonging to a different population (RC), the estimated dates were confirmed by the high germination percentages recorded in the field from March to April (Fig. 4C).

DISCUSSION

Type of dormancy

Final germination of *R. persicifolia* seeds was significantly improved by cold stratification (5 °C) at intermediate and low temperatures, confirming the presence of PD and supporting earlier observations (Mattana *et al.*, 2009). Physical dormancy is also known in seeds of *R. cathartica*, *R. caroliniana*, *R. frangula* and *R. purshiana* (Baskin and Baskin, 1998), *R. alaternus* and *R. cathartica* (Dupont *et al.*, 1997; García-Fayos *et al.*, 2001), and *R. alnifolia* and *R. lanceolata* (Sharma and Graves, 2005). As just 1 month of cold stratification is sufficient to break *R. persicifolia* seed dormancy, the seeds appear to have non-deep PD (Baskin and Baskin, 2004). Further, as the temperature range at which the *R. persicifolia* seeds could germinate widened from higher to lower, the seeds have Type 2 non-deep PD (Baskin and Baskin, 2004).

Thermal requirements for germination

The optimal temperature for germination of non-dormant seeds of *R. persicifolia* is presumed to be around 20 °C, as the best fit of the germination rate data in the sub-optimal temperature range excluded 25 °C, which fell in the supra-optimal temperature range. The T_b in seeds of *R. persicifolia* varied from approx. 10 °C for non-treated seeds to approx. 3 °C for seeds cold stratified for 3 months. To our knowledge, this is the first report of T_b for a member of the Rhamnaceae. Constraining the linear regressions of each percentile for germination through the mean T_b improved the residual sum of squares and r^2 values; therefore, T_b can be used to describe the whole population response in *R. persicifolia* seeds, as previously reported for other species (e.g. Covell *et al.*, 1986; Ellis *et al.*, 1987; Pritchard and Manger, 1990; Orrù *et al.*, 2012).

Treatments for dormancy release clearly modified T_b in *R. persicifolia* seeds, and the widening of the range of temperatures for germination can be used as a surrogate for the efficient removal of dormancy. Chilling at 5 °C reduced T_b in *R. persicifolia* seeds by approx. 0·09 °C d^{-1} of chilling, such that T_b reached the chilling temperature after 68 d of stratification. A similar trend has been observed in *A. hippocastanum* seeds, with T_b reducing by 0·17 °C d^{-1} of chilling at 6 °C (Pritchard *et al.*, 1999). In both these species, the sequential removal of dormancy lowers T_b until the stratification temperature becomes permissive for germination growth *per se* (Pritchard *et al.*, 1999). However, the process is nearly twice as rapid in *A. hippocastanum* seeds, with T_b reducing by 1 °C for every 5·9 d of chilling compared with 11·1 d of chilling in *R. persicifolia*. Consequently, it is clear that the quantitative impacts of a shortened cold season as a result of climate change will be highly species-specific with respect to the efficiency of dormancy loss and the timing of germination.

The best model was obtained by fitting germination expressed in probit and log-normal (log °Cd) rather than normal distributed thermal times (°Cd), as previously reported for other herbaceous (Covell *et al.*, 1986; Ellis and Butcher, 1988) and tree species (Pritchard and Manger, 1990). Seeds of *R. persicifolia* vary in their thermal time estimates to reach θ_{50}, depending on treatment. Chilling increased the rate of accumulation of thermal units (°Cd) at any temperature in the sub-optimal range, leading to a reduction in θ_{50} values from 2·59 log °Cd (385 °Cd) for untreated seeds to about 2·18 log °Cd (150 °Cd) for cold-stratified seeds. Batlla and Benech-Arnold (2003) also detected a cold-induced decrease in θ_{50}, from 80 °Cd to 56 °Cd, for seeds of *Polygonum aviculare* stratified at 12 and 1·6 °C, respectively. Similarly, the thermal history of *V. vinifiera* subsp. *sylvestris* seed lots varying with maternal environment is known to affect θ_{50} (33·6 to 68·6 °Cd) for non-dormant, cold-stratified seeds (Orrù *et al.*, 2012).

Soil heat sum and thermal niche for in situ *seed germination*

Maximum germination of Mediterranean species is typically in the range 5–15 °C and is limited to autumn and winter, and usually decreases markedly above 20 °C (Thanos *et al.*, 1995; Luna *et al.*, 2012). *Rhamnus persicifolia* showed a typical germination phenology of temperate and alpine plants, where spring germination prevails due to temperatures being too low to stimulate emergence following autumn dispersal or due to a requirement for cold stratification over winter (Baskin and Baskin, 1998; Walck *et al.*, 2011; Mondoni *et al.*, 2012). However, under harsh Mediterranean climatic conditions, the topsoil in the mountains remains moistened for only a few weeks after snow-melt, such that adaptation for fast germination in the early spring is an advantage (Giménez-Benavides *et al.*, 2005; Mattana *et al.*, 2010). The dormancy breaking and thermal time requirements identified in this study, together with the recorded annual trends in soil temperature, allowed a model for the thermal niche of seed germination to be constructed and spring emergence to be predicted for *R. persicifolia* seeds. Soil temperatures of around 5 °C (i.e. the stratification temperature tested in the controlled conditions) from December to February for RO (approx. 75 d) and from December to March (approx. 95 d) for RC facilitate both a fall in T_b to approx. 3 °C and efficient germination of the seeds in March and April when the mean soil temperatures are approx. 10 °C.

Plant distribution and competitiveness are highly dependent on environmental envelopes or niches (Walck *et al.*, 2011; Bykova *et al.*, 2012). For *R. persicifolia* habitat, up to six temperature periods were identified throughout the year, which contribute to a better understanding of the field germination period in this and other species growing along Mediterranean mountain waterways; especially as there have, hitherto, been no historical series of monthly averages of temperatures and rainfall at altitudes higher

than approx. 1100 m a.s.l. in Sardinia. In each investigated site, seed germination of *R. persicifolia* was obtained after cold stratification, when the canopy was absent. Tree canopy seems therefore to have no influence on seed germination *sensu stricto*, but closure of the canopy could influence survival of newly established seedlings due to microclimate amelioration (moister and cooler) during the dry and hot Mediterranean summers (Valiente-Banuet *et al.*, 1991; Greenlee and Callaway, 1996; Gómez-Aparicio *et al.*, 2005). This was confirmed by the high germination percentages reached under controlled conditions by untreated and cold-stratified seeds (>80 %) when incubated under the alternating temperature regime (25/10 °C). The ecological significance of germination stimulation by alternating temperature can be interpreted as a season-sensing system for temperate plants because the diurnal fluctuation of the soil surface temperature is large in the spring before dense vegetation covers the ground of deciduous forest or grassland (Shimono and Kudo, 2003).

The ecology of germination identified in this study for *R. persicifolia* explains the present distribution of this species which is mainly limited to small 'temperate' refuge areas along mountain waterways (Mattana *et al.*, 2009), where the general lack of rainfall during summer is overcome by the moisture of the soil. These findings confirm the identification of *R. persicifolia* as a species with a relic distribution, as previously reported by Arrigoni (1977) and Bacchetta *et al.* (2011).

The quantification of thermal time for germination has been used in different studies to characterize changes in seed dormancy and subsequent germination in the field (i.e. Forcella *et al.*, 2000; Hardegree and Van Vactor, 2000; Steadman *et al.*, 2003; Chantre *et al.*, 2009). Recently, Orrù *et al.* (2012) used an environmental heat sum approach (using mean monthly temperatures) to predict germination timing under two simulated IPCC scenarios (+1·8 °C for B1 and + 3·4 °C for A2; IPCC, 2007) for *V. vinifera* subsp. *sylvestris* seeds. The B1 scenario of +1·8 °C would still adequately overcome dormancy for all the investigated populations, whereas under the A2 scenario with +3·4 °C the higher winter temperature would not allow seed dormancy loss in the lowest investigated population (Orrù *et al.*, 2012). The same altitude-related pattern of seed dormancy release and germination in response to global warming can be assumed for *R. persicifolia* seeds. An increase of +1·8 °C (B1) would not reduce the stratification period at 5 °C for the high RC population (approx. 90 d, leading to a T_b of approx. 3 °C), whereas it could affect that of the low RO population (approx. 21 d; T_b of approx. 9 °C). However, an increase of +3·4 °C (A2) would reduce the cumulative stratification time at 5 °C to only 50 (T_b of approx. 6·5 °C) and 17 d (T_b of approx. 9 °C) for RC and RO, respectively. According to the B1 scenario, these changes in T_b and the increased soil temperatures would affect the germination time, by anticipating field germination to February–March and March–April, for RO and RC, respectively. An increase of 3·4 °C (A2) could lead to germination in the field in autumn (November) in both sites. This phenological shift to germination in autumn is possible as seeds of this species may also germinate at temperatures ≥15 °C without any cold stratification. Therefore, warmer temperatures and a consequent reduction of the stratification period would not be detrimental *per se* for seed germination. However, seedling survival over winter might then become the limiting event for the natural regeneration of the species. Moreover, projections for Mediterranean mountains predict lower precipitations mainly during spring (Nogués-Bravo *et al.*, 2008), and the seedling growing season could also be shortened by a reduction in soil moisture and water availability.

Conclusions

In conclusion, Type 2 non-deep PD was identified for *R. persicifolia* seeds, the thermal niche requirements for dormancy release and germination were quantified, and predictions for germination were validated through field observations of emergence. Overall, the results confirm the value of using a soil heat sum approach to predict the effects of subtle changes in field temperature on germination performance. The soil heat sum model developed for seed germination in this species may have applicability to predictions of *in situ* regeneration of other species growing by Mediterranean mountain waterways and of PY species of temperate and alpine regions, where spring germination prevails due to a requirement for cold stratification over winter.

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Annexe II

GERMINATION NICHE OF SARDINIAN MOUNTAIN ENDEMIC SPECIES UNDER A CHANGING CLIMATE

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Central Northern Sardinia has been identified as one of the 52 putative glacial refugia in the Mediterranean region, whose existence implies the local long term persistence of a species or population, within a well-defined geographical range (e.g. mountain, gorge; Médail *et* Diadema, 2009). This region represents also a Southern European refugium (*sensu* Tzedakis *et al.*, 2002) for some temperate tree species, as detected for the Supramontes biogeographic sector (CE-Sardinia; Fenu *et al.*, 2010). The Supramontes region and the Gennargentu massif are two of the most interesting mountain territories of Sardinia. In these areas, vegetation among waterways is mainly constituted by *Alnus glutinosa* (L.) Gaertn. woods. Rare and threatened Sardinian endemic species belonging to genera typical of temperate climates (e.g. *Aquilegia* L., *Paeonia* L. and *Ribes* L.) grow under and close to the canopy of such riparian woods.
Environmental conditions during seed maturation influence germination, with temperature being the major environmental factor responsible for changes in dormancy states of seeds (Baskin *et* Baskin, 1998). Thermal time (θ, in °Cd), is commonly used as a mean to model population germination responses to temperature (Steadman *et al.*, 2003). It is based on the linear increase in germination rate that occurs as germination temperature is raised above the base temperature for germination (Covell *et al.*, 1986). The seed germination niche of *Aquilegia barbaricina* Arrigoni *et* Nardi, *Paeonia corsica* Sieber *ex* Tausch, *Rhamnus persicifolia* Moris, *Ribes multiflorum* Kit *ex* Roem *et* Schult. subsp. *sandalioticum* Arrigoni, and *Taxus baccata* L. is being characterized, with the aims of evaluating if these species show the same patterns of seed dormancy and germination, and understanding whether these habitats can act as a refuge under Mediterranean climate for these species.
In this work three study areas were selected: one in the Gennargentu massif and two in the Supramontes region (Table 1). Data-loggers have been buried in the natural populations in order to study and monitor the annual trend of temperatures and to detect differences in soil temperature underneath and outside the tree canopy. At the same time, seeds of the studied *taxa* have been sown in the field to investigate their *in situ* germination. The daily maximum, minimum, average temperature and ΔT (difference between daily maximum and minimum temperatures) is being analysed. Different environmental conditions (temperature and light) and several pre-treatments (cold and warm stratifications and combinations of them) are being tested on fresh seeds in laboratory, so that their optimal conditions and thermal requirements (thermal time approach; Pritchard *et al.*, 1996) for embryo growth, seed dormancy loss and germination can be revealed. All the investigated *taxa* belong to families characterized as

having oily (Corner, 1976), and endospermic seeds (Martin, 1946). Therefore, their seed oil content is being quantified, using the Supercritical Fluid Extraction with carbon dioxide (SFE-CO_2) methodology (Seal *et al.*, 2008), and thermal analysis of seeds and extracted oils being carried out using the Differential Scanning Calorimeter (DSC) to investigate ice crystallization and melting, so that seed freezing tolerance in the natural environment can be predicted.

Table 1 - Localities and geographic data of the study areas.

Study area	Locality	Coordinates	Altitude (m a.s.l.)	Substrate
Gennargentu	Rio Correboi (Vilagrande Strisaili, OG)	N 40°03' E 09°20'	1267	Metamorphytes
Supramontes	Monte Novo San Giovanni (Orgosolo, NU)	N 40°07' E 09°24'	1265	Limestones
	Rio Olai (Orgosolo, NU)	N 40°08' E 09°21'	947	Metamorphytes

The preliminary data on soil temperature (from June to December 2011; Fig. 1), underneath and outside the tree canopy, showed a difference on average temperatures and ΔTs. In Rio Correboi, the mean daily temperature underneath the canopy ranged from 17.17 ± 2.3°C in summer to 9.39 ± 2.60°C in autumn, with a maximum of 23.40°C on July and a minimum of 5.41°C on December, whereas outside the canopy these values ranged from 20.19 ± 4.23°C to 7.68 ± 2.43°C for summer and autumn, respectively and maximun values of 40.06°C on July and minimum of 2.74°C on December (Fig. 1). In Rio Olai, mean daily temperatures underneath the canopy ranged from 16.30 ± 1.13°C in summer to 10.33 ± 2.29°C in autumn, with a maximum of 22.75°C on August and a minimum of 3.59°C on December, whereas outside the canopy from 23.68 ± 3.11°C in summer to 10.56 ± 2.99°C in autumn, with maximum and minimum values of 47.16°C (July) and 2.82°C (December), respectively (Fig. 1). In Monte Novo San Giovanni mean daily temperatures underneath the canopy were 17.54 ± 2.33°C and 9.75 ± 2.80°C for summer and autumn, respectively with a maximum of 25.28°C on July and a minimum of 4.56°C on December, whereas outside the canopy it was 22.36 ± 2.9°C, ranging from a maximum of 39.86°C on July and a minimum of 4.01°C on December (Fig. 1).
High differences in ΔTs were also detected in summer for each locality, with the ΔTs calculated for outside being approximately five times higher than those obtained underneath the canopy (Fig. 1). In Rio Correboi, the average ΔTs were 2.57 ± 0.76°C and 12.24 ± 5.87°C, in Rio Olai 3.97 ± 1.03°C and 21.24 ± 6.47°C, and in Monte Novo San Giovanni 3.62 ± 1.02°C and 16.72 ± 3.86°C, underneath and outside the tree canopy, respectively (Fig. 1). In autumn, quite similar values were detected in each locality underneath and outside the tree canopy, with the

latter being only ca. two times higher: 1.57 ± 1.03°C and 3.82 ± 1.52°C for Rio Correboi, 3.30 ± 1.51°C and 6.95 ± 4.27°C for Rio Olai and 1.99 ± 1.34°C and 4.58 ± 2.50°C for Monte Novo San Giovanni (Fig. 1).

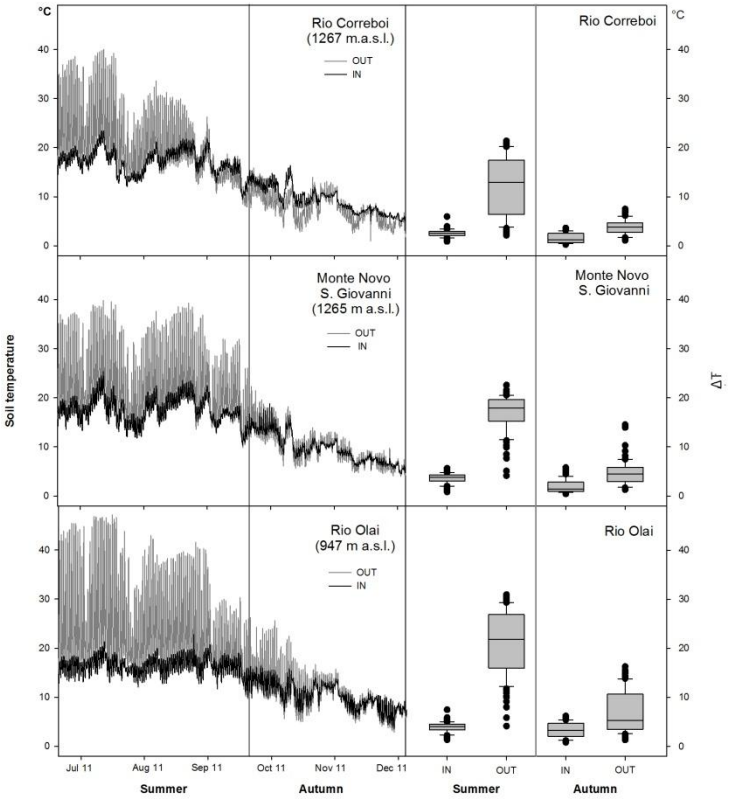

Figure 2 – On the left, the soil temperature trends recorded for summer and autumn by data loggers; on the right, the ΔTs calculated underneath (IN) and outside (OUT) the tree canopy for the two seasons

The high differences in ΔTs, as well as those highlighted in average temperatures, detected between seasons are due to the vegetation canopy that is well established in summer, generating

a different microhabitat respect to the autumn, when deciduous species such as *Alnus glutinosa* lose their leaves and the natural conditions underneath and outside the canopy are quite similar. Although preliminary, these data reveal new insights on the effect of vegetation canopy in the annual trend of soil temperatures in Mediterranean mountains and will be helpful to analyse and discuss the results of the experiments on seed dormancy, germination ecology and seed freezing tolerance in the natural environment that are still in progress. These approaches and the data generated will help us understanding of the potential impact of climate change on natural regeneration in this niche environments.

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