Università degli Studi di Cagliari

DOTTORATO DI RICERCA IN

BOTANICA AMBIENTALE ED APPLICATA

Ciclo XXV

Seed germination requirements and salt stress tolerance of coastal rare species in Sardinia

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Alla mia adorata nonna Vittoria,
ai miei splendidi genitori Giuseppe e Cettina
ed a tutte quelle persone che mi stimano e
nutrono per me affetto sincero

Andrea

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Summary

To survive to adverse factors that characterize coastal environments, plant species often require special physiological or metabolic adaptations to overcome environmental stresses. Stress may be physical (e.g. temperature) or chemical (e.g. salinity). Many communities comprise highly specialized species, which have comparatively restricted geographical distributions. The coastal species investigated in this Ph.D. program were chosen accordingly to their habitat: *Phleum sardoum* (Hackel) Hackel and *Rouya polygama* (Desf.) Coincy for sandy dunes; *Brassica insularis* Moris and *Lavatera triloba* L. ssp. *pallescens* (Moris) Nyman for coastal cliffs; *Lavatera triloba* L. ssp. *triloba* and *Halopeplis amplexicaulis* (Vahl) Ces., Pass. & Gibelli for ultra-saline environments. Moreover, seed germination ecology of *L. agrigentina* Tineo, a species growing in clayey-chalky plains of South Italy, was also investigated for a comparative study within the *Lavatera* genus.

For all the studied species, light and temperature requirements for seed germination were evaluated; their germination responses to salt stress (NaCl) and their germination recovery. Inter-population variability on germination patterns was also evaluated for *R. polygama*, *B. insularis* and *L. triloba* ssp. *triloba*. Salt spray tolerance on the vegetative growth and biomass production during the early seedling developmental stages was evaluated for *B. insularis*, *L. triloba* ssp. *pallescens*, *L. triloba* ssp. *triloba* and *H. amplexicaulis*.

Light did not affect germination percentages in any of the studied species enabling seed germination also under soil surface and highlighting that seeds were not photo-inhibited for germination. Seed germination of *P. sardoum* and *R. polygama*, as well as that of *L. agrigentina*, *L. triloba* ssp. *pallescens* and *L. triloba* ssp. *triloba*, reflected the optimal range of temperatures of "typical" Mediterranean species, suggesting germination in autumn-winter, when water

availability, soil moisture and rainfalls are high, and temperatures are not excessively prohibitive for germination and consequent seedlings establishment. *B. insularis* differed from other "typical" Mediterranean plants, for which germination at low temperatures is a widely extended trait, demonstrating that germination of this species may occur in a wide time window during the year. *H. amplexicaulis* seed germination was highly promoted by the daily fluctuation of temperatures, while germination at constant temperatures was sensibly lower.

Salinity tests showed higher germination percentages in the non-saline conditions, with seed mortality increasing proportionally with NaCl concentrations and temperatures. Salt tolerance limits varied among species, from a minimum of 100 mM NaCl for *P. sardoum* to 600 mM for *H. amplexicaulis* and *L. triloba* ssp. *pallescens*, without a clear habitat related pattern.

The species for which salt spray experiments were conducted showed different responses on seedling growth to salt aerosol tolerance, with these differences being related to the habitat of each species and their distance from the sea. Populations of *B. insularis* and *L. triloba* ssp. *pallescens*, growing in coastal cliffs highly influenced by wind and salt spray, showed the lowest seedling mortality. High inter-population variability in salt spray tolerance was detected for *B. insularis*, between a coastal and an inland population, with the latter resulting not adapted to this abiotic environmental factor. Seedling survival of the two inland species (*L. triloba* ssp. *triloba* and *H. amplexicaulis*) was inversely proportional to the increase of nebulization frequency, demonstrating a low adaptation to salt spray, likely due to their distance from the sea coast and/or to interposed vegetation that may determine a lower impact of marine aerosol.

The results of this study lead to a better knowledge on the autoecology of the investigated species and to their limits of tolerance to abiotic factors such as temperature, soil salinity and salt spray.

General introduction

Seed biology

A seed is the product of the ripened ovule of Gymnospermae and Angiospermae (Spermatophyta) which occurs after fertilization and some growth within the mother plant (Longo, 1997). The formation of the seed completes the process of reproduction in seed plants (which started with the development of flowers and pollination), with the embryo developed from the zygote and the seed coat from the integuments of the ovule. Seeds have been an important development in the reproduction and spread of Spermatophyta, respect to more primitive plants such as Bryophyta, Pteridophyta and Hepaticae, which do not have seeds and use other means to propagate themselves (Strasburger, 1992). This evolutionary success lead seed plants to dominate biological niches both in hot and cold climates (Strasburger, 1992; Baskin & Baskin, 1998).

A typical seed includes three basic parts: an embryo, a supply of nutrients for the embryo, and a seed coat (Longo, 1997; Figure 1). The embryo has one cotyledon in monocotyledons, two cotyledons in almost all dicotyledons and two or more in gymnosperms (Salisbury & Ross, 1994). The radicle is the embryonic root, while the plumule is the embryonic shoot. The embryonic stem above the point of attachment of the cotyledon(s) is the epicotyl while the embryonic stem below the point of attachment is the hypocotyl. The epicotyl will grow into the shoot, the radicle into the primary root, the hypocotyl connects the epicotyle and the radicle, the cotyledons form the seed leaves (Fenner & Thompson, 2005). Seeds of monocotyledons have also other structures, such as a coleoptile that forms the first leaf and connects to the coleorhiza which connects to the primary root and adventitious roots form from the sides. Within the seed, there usually is a store of nutrients for the seedling that will grow from the embryo. In angiosperms, the stored food begins as a tissue called endosperm, which is derived from the parent plant via double fertilization. The

usually triploid endosperm is rich in oils and proteins. The seed coat (testa) will develop from the tissue, the integument, originally surrounding the ovule (Baskin & Baskin, 1998). The seed coat in the mature seed can be a paper-thin layer or something more substantial and it protect the embryo from mechanical injury and from drying out. A scar also may remain on the seed coat, called hilum, where the seed was attached to the ovary wall by the funiculus (Strasburger, 1992; Fenner & Thompson, 2005).

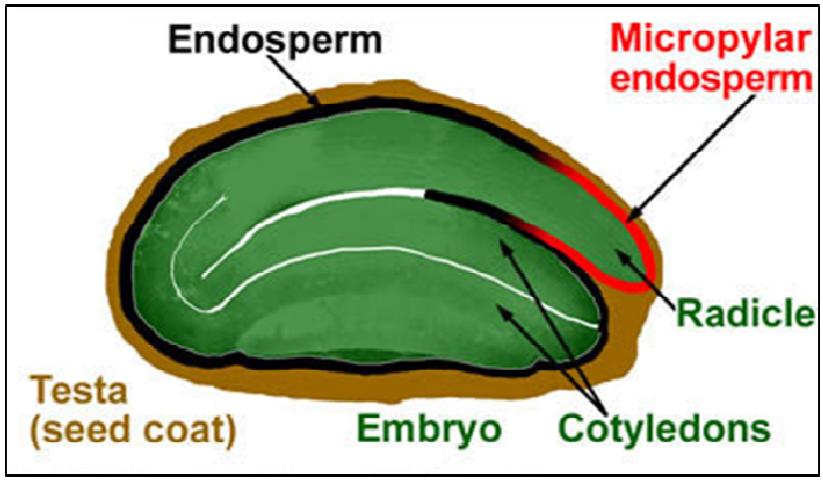

Figure 1 - *Lepidium sativum* **seed structure (from Leubner, 2008).**

Seed germination

Seed germination is the process by which a seed embryo develops into a seedling and it involves the reactivation of the metabolic pathways that lead to growth and the emergence of the radicle and plumule (Fenner & Thompson, 2005). The emergence of the seedling above the soil surface is the next phase of the plant's growth and it is called seedling establishment (Black & Halmer, 2006). Three fundamental conditions must exist before germination can occur: (1) the embryo must be alive; (2) any dormancy requirements that prevent germination must be overcome; (3) the

proper environmental conditions must exist for germination. Seed viability is the ability of the embryo to germinate and is affected by a number of different conditions. Predators and pathogens can damage or kill the seed while it is still in the fruit or after it is dispersed (Fenner & Thompson, 2005). Environmental conditions like flooding or heat can kill the seed before or during germination (Bewley, 1994). The age of the seed affects its health and germination ability: since the seed has a living embryo, over time cells die and cannot be replaced. Some seeds can live for a long time before germination, while others can only survive for a short period after dispersal before they die (Baskin & Baskin, 1998). Seed vigor is a measure of the quality of seed, and involves the viability of the seed, the germination percentage, germination rate and the strength of the seedlings produced. The germination percentage is the proportion of seeds that germinate from all seeds subject to the right conditions for growth. The germination rate is the length of time it takes for the seeds to germinate (Bewley, 1997). Germination percentages and rate are affected by seed viability, dormancy and environmental effects that impact on the seed and seedling. Three distinct phases of seed germination occur: water imbibition; lag phase; and radicle emergence (Figure 2). Germination commences with the uptake of water by imbibition by the dry seed, followed by embryo expansion. The uptake of water (or imbibition) is tri-phasic with a rapid initial uptake (phase I) followed by a plateau phase (phase II). A further increase in water uptake (phase III) occurs as the embryo axis elongates and breaks through the covering layers to complete germination. Cell elongation is necessary and is generally accepted to be sufficient for the completion of radical protrusion (visible germination). With few exceptions, radical extension through the structures surrounding the embryo is the event that terminates germination and marks the commencement of seedling growth (Bewley, 1997; Finch-Savage & Leubner-Metzger, 2006). Many factors influence germination process, such as temperature, light and water availability.

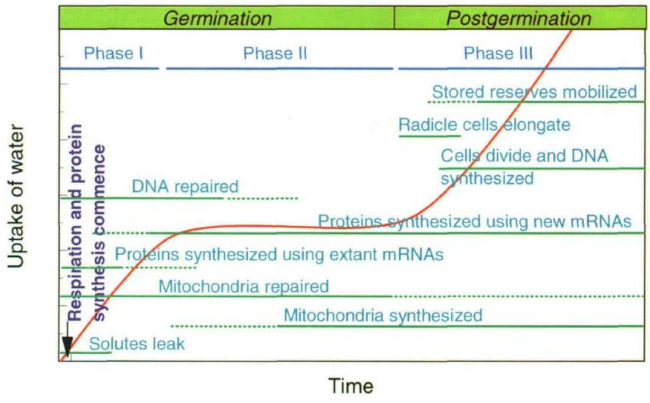

Figure 2 - Time course of major events associated with germination and subsequent post-germination growth. The time for events to be completed varies from several hours to many weeks, depending on the plant species and the germination conditions (from Bewley, 1997).

Temperature

Temperature has important effects on germination. In seasonal climates, temperature is of course a good indicator of the time of year and it is therefore implicated strongly in determining the timing of germination (Bewley, 1997). The low temperature limit for seed germination is unknown, but germination in many species may be prevented only by freezing. Because temperature requirements for germination are connected so intimately with germination timing, it is rarely possible to detect habitat specific effects. 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 (Fenner & Thompson, 2005).

Indeed, some studies have investigated germination of Mediterranean coastal species and a key feature of which is a rather low optimal temperatures for germination (Thanos *et al*., 1989, 1995). At the opposite extreme, arctic species need higher temperatures for germination (Baskin &

Baskin, 1998). Species of wide geographical distribution generally show the same intra-specific trend as that found between species. The reason for this slightly surprising trend is the gradual replacement of cold by drought as the main hazard for seedlings as we move south in Europe (Fenner & Thompson, 2005). In the Mediterranean, by far the least dangerous season for seedlings is the damp, cool but mostly frost-free winter. 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. In many species, germination is reduced, or does not occur at all, at constant temperatures, while may frequently to be increased by both the number and the amplitude of temperature alternations, and a response to temperature alternations seems to depend on the presence in the seed of at least a low level of the active form of phytochrome (Probert, 2000). The interaction between a requirement for light and for temperature alternations varies between species. Sometimes light can substitute entirely for alternating temperatures, while in other cases the effect of light is merely to reduce the amplitude of alternation necessary to stimulate germination (Bewley, 1997). A survey of germination responses to alternating temperatures revealed that stimulation of germination by alternating temperatures in the light is strongly habitat-dependent. Diurnal temperature alternations are known to decline with depth in soil and also to be much lower beneath an established canopy of insulating vegetation. Seeds that had been buried deeply responded to alternating temperature inexactly the same way as those from near the surface (Ghersa *et al*., 1992).

Light

The responses of seeds to light are important for preventing the occurrence of germination in places and at times that are unfavourable to seedling establishment. The ability to detect different aspects of the light environment enables the seed to have at least some control over where and

when germination takes place (Fenner & Thompson, 2005). The chances of successful establishment may be determined by whether the germinating seed is buried in the soil or is on the surface. If it is buried, then the precise depth is crucial for emergence. If it is on the surface, then the degree of shade (especially from surrounding vegetation) can be decisive. In some cases, day length plays a part in determining the timing of germination (Densmore, 1997). In all these situations, the ability to detect the intensity, quality or periodicity of the light provides the seed with information it requires about its environment (Fenner & Thompson, 2005). If a seed that is lying in darkness below the soil surface germinates, then its shoot may not be able to reach the surface. This hazard is greatest for small seeds, so the ability to detect light (or its absence) is of great survival value. Near the surface, the amount of light received diminishes rapidly with depth. Measurable quantities of light seldom penetrate more than a few millimetres (Bliss & Smith, 1985; Tester & Morris, 1987), though the presence of a high proportion of translucent particles such as quartz grains in sand may transmit light a little deeper. Not surprisingly, many small-seeded species are positively photoblastic (require light for germination) or are inhibited significantly by darkness. In a survey of 271 species, Grime *et al*. (1981) found that species with seeds weighing less than 0.1 mg were largely light-requiring, and that the incidence of light-dependence declined with increasing seed size. However, there is a phylogenetic component to the occurrence of photoblastism. Certain families such as the Fabaceae and Poaceae tend to germinate readily in the dark regardless of seed size, while seeds of Cyperaceae and Asteraceae are mostly light-requiring (Fenner & Thompson, 2005). In addition to the ability to detect the quantity and quality of light, the seeds of some species are sensitive to the photoperiod, i.e. the relative lengths of the light and dark periods corresponding to day and night (Isikawa, 1954; Cumming, 1963). Day-length detection is often highly dependent on the temperature regime, especially chilling (Black & Wareing, 1955; Stearns & Olsen, 1958). Photoperiod sensitivity is likely to increase in

importance with latitude because of the large seasonal variation in day length. Few wild species have been tested for sensitivity to day length, and its occurrence may be more widespread than the sparse literature would suggest. The published experiments do not always distinguish between the effects of total quantity of light received and the specific effect of the photoperiod. Some studies seem to indicate that elements of both light quantity and photoperiod are involved at the same time (Baskin & Baskin, 1976).

Water availability

Most seeds can maintain viability with a very low moisture content. In fact, the longevity of these "orthodox" seeds can be increased by desiccation in dry storage. In contrast, species with so called "recalcitrant" seeds require a high level of moisture to retain viability (Murdoch & Ellis, 2000). In a survey of 6919 species, 7.4% were classified as recalcitrant (Hong *et al*., 1996). The latter continue to metabolize actively and accumulate reserves right up to the point of shedding, after which they remain in a hydrated state and germinate straight away (Kermode & Finch-Savage, 2002). Seed-desiccation sensitivity is most frequent in non-pioneer evergreen rainforest trees, though even among these a large proportion are desiccation-tolerant (Tweddle *et al*., 2003) or have seeds in which partial dehydration may not always be fatal (Rodriguez *et al*., 2000). In a continuously warm wet climate, rapid germination may reduce predation risk. In addition to having a critical water content for the maintenance of viability, each species is thought to have a critical water content (or water potential) requirement for germination (Hunter & Erickson, 1952). The rate of imbibition is controlled by the permeability of the seed coat, the area of contact between the seed and the substrate, and the relative difference in water potential between the soil water and the seed (Bradford, 1995). A seed may become fully imbibed but remain ungerminated indefinitely if its dormancy-breaking or germination-inducing requirements are not met. The seeds

that form persistent seed banks may survive for many years in soils where they may be maintained (at least intermittently) in a fully imbibed state (Thompson, 2000). Germination may take many days or weeks, during which time the seed is likely to encounter a number of wet and dry periods. Numerous experiments have been carried out to determine the effect of cycles of hydration and dehydration on germination and the response varies with species. The length of the dry period has been found to reduce viability and germination speed in annual pasture legumes (*Trifolium* species; Jansen, 1994), but in other cases it has little or no effect (Vincent & Cavers, 1978). The response of the seeds of different species to the pattern of rainfall at the time of germination may determine which species will establish. A fast response to rain may be advantageous providing that the wet period is sufficiently long to allow the seedlings to grow to a size that enables them to withstand the subsequent dry period. A slow response, in which germination can occur cumulatively even if interrupted by periods of drought, can be of advantage where the rain events are of short duration (Fenner & Thompson, 2005).

Seed dormancy

Seed dormancy is an innate seed property that defines the environmental conditions in which the seed is able to germinate (Baskin & Baskin, 1998). It is determined by genetics with a substantial environmental influence which is mediated, at least in part, by the plant hormones abscisic acid and gibberellins (Fenner & Thompson, 2005). The dormancy status is not only influenced by the seed maturation environment, but also continuously changing with time following shedding in a manner determined by the environment (Bewley, 1997). As dormancy is present throughout the higher plants in all major climatic regions, adaptation has resulted in divergent responses to the environment (Baskin & Baskin, 2003; Fenner & Thompson, 2005). Through this adaptation,

germination is timed to avoid unfavourable weather for subsequent plant establishment and reproductive growth (Finch-Savage & Leubner-Metzger, 2006).

A dormant seed is one that will not germinate under any combination of normal physical environmental factors (temperature, light/dark, etc.) that otherwise is favourable for its germination, after the seed becomes non-dormant (Fenner & Thompson, 2005). A freshly-matured dormant seed is said to have primary dormancy (Baskin & Baskin, 2003). A completely non-dormant seed has the capability to germinate over the widest range of normal physical environmental factors possible for the genotype (Baskin & Baskin, 1998, 2004). The seed will germinate when the appropriate combination of environmental conditions is within its range of requirements for radicle emergence, providing it has not entered secondary (Baskin & Baskin, 2003).

There are three fundamentally different types of seed dormancy, at least two of which have evolved on several separate occasions (Baskin & Baskin, 1998). These dormancy types are morphological, physical and physiological. Moreover morpho-physiological and combinational dormancy are known.

Morphological dormancy (MD)

In seeds with MD, the embryo is either small (underdeveloped) and undifferentiated or underdeveloped and differentiated, i.e., cotyledon(s) and hypocotyl-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 (radical protrusion). The dormancy period is the time required for completion of embryo growth, after which the radicle emerges (Baskin & Baskin, 2003). 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 (Fenner & Thompson, 2005).

Physiological dormancy (PD)

Following Nikolaeva (1977), three levels of PD are recognized: deep, intermediate, and non-deep. PD is the most abundant form and is found in seeds of gymnosperms and all major plants. PD can be divided into three levels: deep, intermediate and non-deep (Baskin & Baskin, 2004). Based on patterns of change in physiological responses to temperature, five types of non-deep PD can be distinguished (Figure 3). 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 or from high to lower temperature (type 2). In addition, the sensitivity of the seeds to light and GA increases as non-deep PD is progressively released. In type 3, a dormant seed germinate only at average temperatures and after dormancy release its germination can occur from low to high temperatures, while in type 4 and 5, after dormancy release the seed can germinate only at high temperatures (type 4) or low temperatures (type 5).

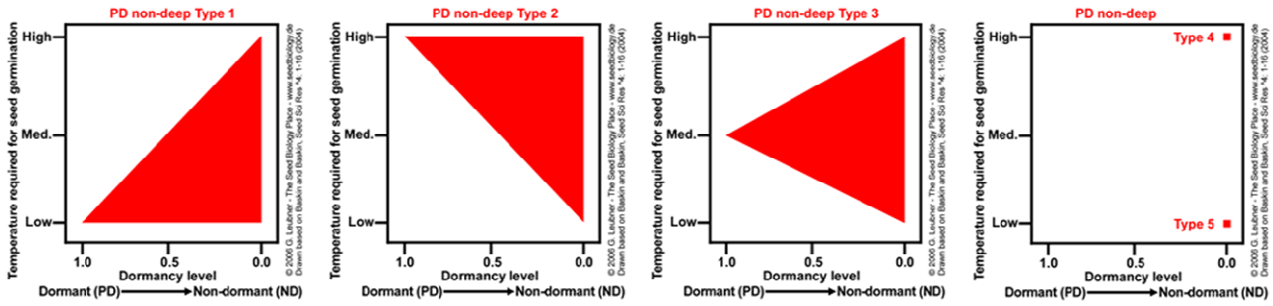

Figure 3 - Five types of non-deep physiological seed dormancy (PD) according to Baskin and Baskin (2004), (from Leubner, 2008).

Morpho-physiological dormancy (MPD)

Seeds with this kind of dormancy have an underdeveloped embryo (MD) that has also a physiological component of dormancy. There are eight known levels of the MPD class based on the protocol for seed dormancy break and germination (see Baskin & Baskin, 1998). These seeds therefore require a dormancy-breaking treatment, for example a defined combination of warm and/or cold stratification which in some cases can be replaced by GA application.

Physical dormancy (PY)

Physical dormancy is caused by (a) water-impermeable layer(s) of palisade or palisade-like cells in the seed or fruit coat (Baskin *et al*., 2000). Dormancy-break in seeds with PY under both natural and artificial conditions typically has been assumed to involve the formation of an opening ("water gap") in a specialized anatomical structure on the seed (or fruit) coat through which water moves to the embryo (Baskin *et al*., 2000; Baskin & Baskin, 2003; Fenner & Thompson, 2005).

Combinational dormancy (PY + PD)

Combinational dormancy (PY + PD) is observable in seeds whose seed coat is water-impermeable and the embryo is physiologically dormant. The physiological component appears to be at the non-deep level in all examples with which we are familiar (Baskin and Baskin, 1998). Embryos of freshly matured seeds of some *taxa* of winter annuals have a low amount of conditional dormancy and will come out of dormancy (after-ripening) in dry storage, or in the field, within a few weeks after seed maturity, even while the seed coat remains impermeable to water (Finch-Savage & Leubner-Metzger, 2006). Embryos in such genera as *Cercis* and *Ceanothus* are more deeply dormant (but still non-deep), and thus the seeds require a few weeks of cold stratification, i.e., after PY is broken and seeds imbibe water, before they will germinate (Baskin & Baskin, 1998).

Germination patterns under the Mediterranean climate

The typical climate of the Mediterranean Basin is a particular variety of that temperate and it is commonly named “Mediterranean climate”; it is characterized by warm to hot, dry summers and mild to cool, wet winters (Doussi & Thanos, 2002). This climate is characterized by a considerable unpredictability of temperature and precipitations (Thanos *et al*., 1995). Water availability, in particular, is extremely variable, both within a single growing season and among consecutive years. Therefore, lower germination rate and the requirement of a narrow range of cool temperatures, typically 5-15°C, as highlighted by Thanos *et al*. (1989, 1995), suggest that field germination of Mediterranean coastal species is tuned to take place well into the rainy season, in early winter, and provided that a quite lengthy, uninterrupted water supply is ensured (Doussi & Thanos, 2002). This pattern is generally known as “Mediterranean germination syndrome” and such a “delay mechanism” is often considered an advantageous ecological adaptation towards the unpredictable rainfall pattern during the start of the rainy period under the pluviseasonal Mediterranean climate (Thanos *et al*., 1995). In Mediterranean coastal environments, as reported by Bell *et al*. (1995), light generally inhibits germination (photo-inhibition), highlighting a surface avoiding mechanism and especially so in small seeded species, germination on the surface of a rapidly drying soil might be especially detrimental for the seedlings of small-seeded species (Thanos *et al*., 1991, 1994, 1995).

Sardinia

Sardinia is situated in the Western Mediterranean basin and it is the second largest island in the Mediterranean after Sicily. It features a surface of 23,821 km² (24,089 km² together with the minor islands administratively belonging to the Sardinian region; Bacchetta *et al*., 2009). According to Rivas-Martínez (2008), the Sardinian bioclimate can be described as oceanic on the

basis of the continentality index and principally set in the semi hyperoceanic, the euoceanic and in the semicontinental subtype, while concerning bio-climate the most representative is the Mediterranean pluviseasonal oceanic (MPO). Three thermotypes characterize the MPO, the upper Thermomediterranean, the lower and upper Mesomediterranean and the lower Supramediterranean. At the end, concerning the ombrotype, in Sardinia there are at least the upper and lower dry, the lower and upper subhumid and the lower humid ones (Bacchetta *et al*. 2009).

The Checklist of the Italian Vascular Flora (Conti *et al*., 2005; 2007) ascribes to the Sardinian flora 2,494 *taxa* while, after the latest floristic researches, the number of *taxa* has raised approximately up to 3,000 (Bacchetta *et al*., personal coomunication). Bacchetta *et al*. (2005) have listed 347 endemic *taxa* (including narrow endemics, Sardinian endemics, Corso-Sardinian endemics, Corso-Sardinian-Balearic endemics). Its isolation and high geological diversity have created a wide range of habitats, with high levels of endemism, especially on its mountain massifs, where there are conditions of ecological insularity (Médail & Quézel, 1997). Many natural habitats that characterize the Sardinian territory and its important plant areas (IPAs) are still not under protection. Among them, the sandy coastal dunes, the halophithyc marshes, the coastal cliffs, the orofitic habitats, the grasslands of the *Thero-Brachypodietea* class, the Mediterranean temporary ponds, the mining environments and the riparian woods are those which better characterize the richness of the Sardinian flora. Up to now 34 IPAs have been pinpointed in the Sardinian territory, covering approximately 18% of the regional area and 10% of the total Italian surface that has been considered remarkable for plant conservation (Blasi *et al*. 2010). Notwithstanding, many of these IPAs currently are not under any kind of protection or, at least, not for the entirety of their area but they are under severe threat due to the overexploitation of natural resources, grazing and tourism.

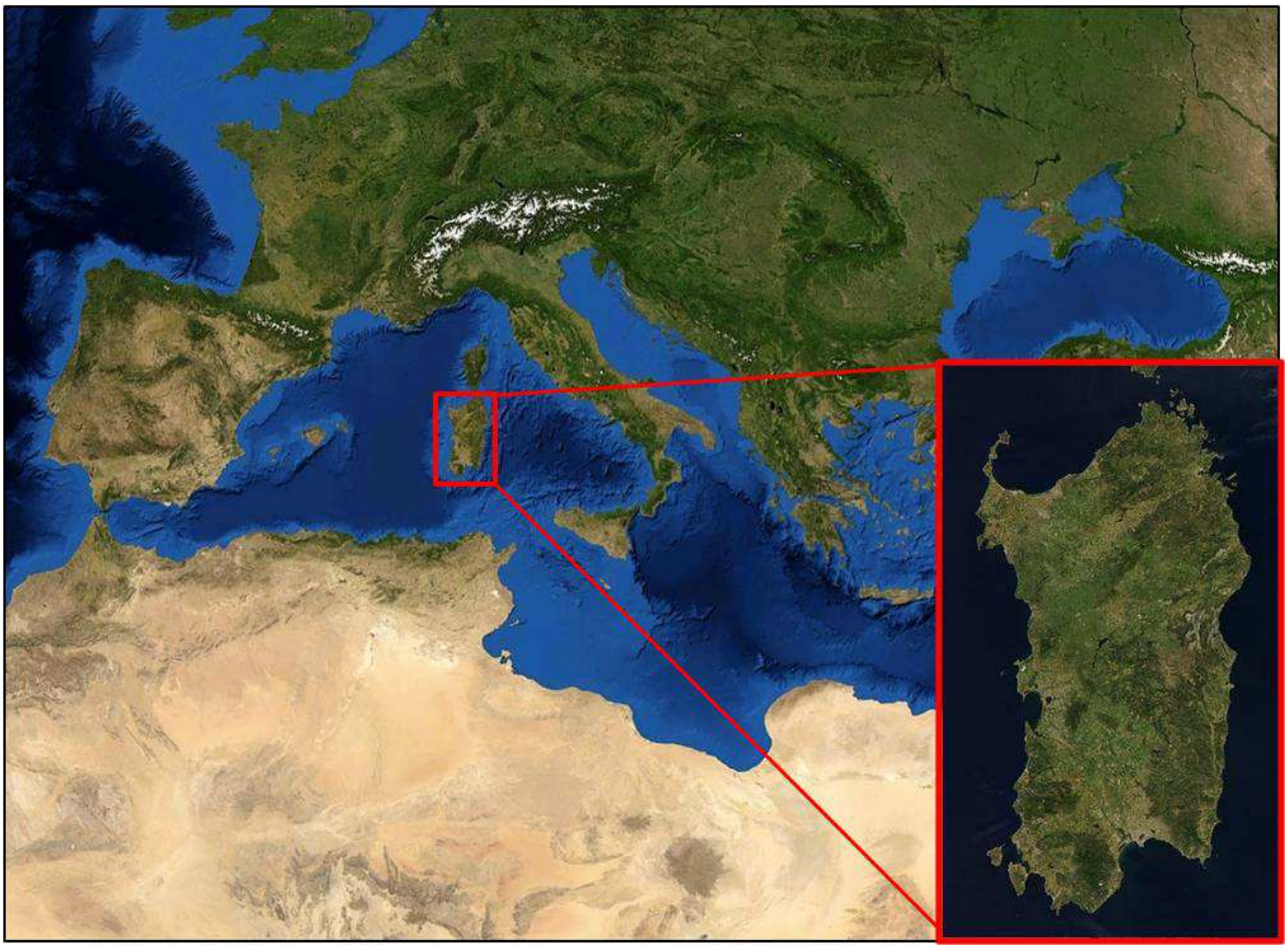

Figure 4 - The west part of the Mediterranean Basin, with Sardinia in red square.

Coastal environments and factors influencing seed germination and plant growth

Understanding the processes and products of interaction in coastal environments is rarely simple. Most physical coastal changes are associated with the movement of sediments. Interactions between organisms and their environment may be achieved at a number of hierarchical levels of which populations, communities and ecosystems are the most relevant. Many coastal communities are species-poor, e.g. coastal wetlands are characterized by relatively few plant species. To survive to adverse factors that characterize coastal environments, organisms often require special

physiological, metabolic or behavioral adaptations to overcome environmental stress. Stresses may be physical (e.g. wave forces, temperature, tidal inundation, etc.) or chemical (e.g. salinity, deoxigenation, etc.). Many communities comprise highly specialized species, which have comparatively restricted geographical distributions (Carter, 1988).

The soil water content is one of the most important limiting factors in plant growth. Sandy soils have high porosity and after a rain most of the water is drained away from the habitat because of the large interstitial spaces between soil particles and the low capability of sand to retain water (Figure 5). Evaporation in open dune systems also removes substantial quantities of water (Maun, 2009). Major problems faced by seeds are sand accretion and soil salinity, both of which have positive and negative effects on seed germination. Burial to an appropriate depth is beneficial for seed germination because it provides intimate soil contact, maintains high humidity around the seed, improves imbibition of moisture, protects it from surface predation, decreases evaporative seed surface and reduces chances of desiccation from heat and light (Maun, 2009). Even if seeds germinate on the sand surface their chances of seedling establishment are very low because their radicles are unable to affect speedy penetration of the sand surface and anchor the seedlings. To penetrate the soil the roots must exert greater pressure than the resistance offered by the soil. Most of these seedlings die of desiccation and exposure to high light intensity. In sandy dunes, seed germination is strongly related to available moisture. Very small seeds produced high germination at shallow depths of only 0.5 cm because they require small amounts of moisture for imbibition (Stairs, 1986; Maun & Perumal, 1999; Maun, 2009). In contrasts large seeds exhibited lowest germination at shallow depths of 2 cm because they require prolonged hydration and greater amounts of moisture for imbibition.

Figure 5 - Coastal sandy dunes of Su Giudeu with psammophytes (S - Sardinia)

Soil salinity above a certain level also has a strong negative effect on seed germination (Maun, 2009). Seedling emergence is related to the energy contained in the endosperm or cotyledons of a seed rather than the embryo. As soon as soil temperatures rise to the optimum for seed germination of a species, seedlings start to emerge. Similarly, seedling emergence in autumn coincides with a decrease in temperatures and increase in rainfalls. The establishment of the seedling is probably the most hazardous period in its life history. The timing of germination is synchronized with the best period of emergence and growth (Maun, 2009). In sandy dune systems, the survivorship, establishment and growth of seedlings is influenced by a number of physical and abiotic factors such as predation, disease, desiccation, competition, salt spray, nutrient deficiency, high soil surface temperatures and burial by sand (Maun, 2009). Warming up of surface layers has both useful and deleterious effects. In early spring, the sand surface warms up faster thus allowing a rise in temperature to the optimum for seed germination of plant species. Seedling emergence period coincides with the highest available soil moisture levels. Salt spray and soil salinity may,

under certain environmental conditions, exert an influence on seed germination, seedling emergence and their establishment (Maun, 2009).

Rupestrian habitats as coastal cliffs are characterized by harsh conditions, such as high sun exposition, strong winds, daily thermal variations (Giulietti *et al*. 1997; Ribeiro & Fernandes, 2000) and strong water deficit during dry months (Oliveira Silveira *et al*., 2012). Moreover, high quantities of marine aerosol as well as the direct waves splashing invest halophytic plant species, resulting inevitable abiotic factors, often limiting plant growth in these habitats (Figure 6). With the high insolation and temperatures typical of summer period, the considerable water quantity present in these habitats tends to become salt crusts on the rocks, reaching salinity values even higher than sea water (ca. 35-37 ‰) (Ungar, 1982, 1995).

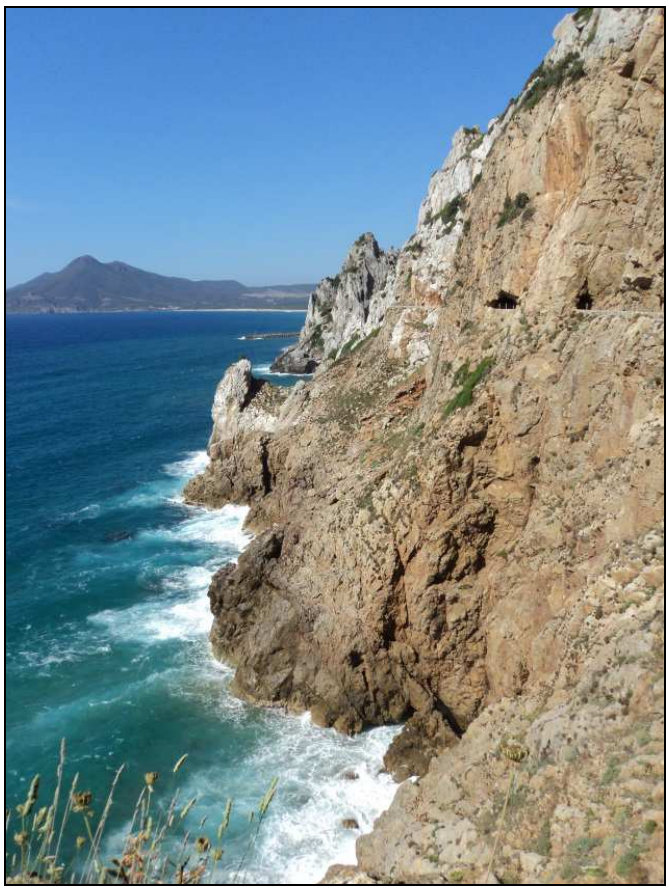

Figure 6 - Coastal cliffs of Planu Sartu - Buggerru (SW - Sardinia).

Salt marshes in the Mediterranean area are important for biodiversity conservation but have been severely degraded due to human pressure (Costanza *et al*., 1997). Coastal salt marshes are characterized by fine sediments and halophytic vegetation and are formed through a combination of physical (i.e., sediment deposition/erosion) and biological (i.e. vegetation) processes (Figure 7). The existence of spatial-temporal gradients of soil salinity and moisture traditionally has been considered one of the most important physical factors in the plant zonation of salt marshes (Chapman, 1974). These soil-plant relationships are particularly interesting in a Mediterranean climate, where the areas farthest from the coast are not always those with the lowest soil salt concentration (Callaway *et al*., 1990). Alternating periods of rainfall, during which salts are leached towards the deepest soil horizons, and periods of drought when they are brought to the surface horizons, bring about an important variation in salinity, both in regard to the quantity and type of salt (Chapman, 1974, Alvarez Rogel *et al*., 1997). Because of the relationship between salinity and electrical conductivity (Richards, 1974), the latter is commonly used to estimate the concentration of salts across edaphic gradients (Porta *et al*., 1980).

Effect of soil salinity in coastal environments

Substrate salinity can act as a major selective force in seed germination and seedling emergence. In spring, seeds of annual and perennial species may be exposed to different levels of soil salinity because of salt spray deposition and periodic inundation by seawater during winter months. For these reasons, plants growing in habitats with a high influence of salinity have evolved specific mechanisms of resistance of plants to saline environments.

Several traits of avoidance and tolerance are prevalent in strand species, such as (Maun, 1998, 2009):

1) hypertrophy: the abnormal enlargements of cells, is a common occurrence in some annual and perennial species of seashores by which they rely on high ion uptake to maintain cell turgor under conditions of low water potential. These plants also accumulate salt in their vacuoles and keep the concentration of Na^+ and Cl^- in the cytoplasm at low levels;

2) annual habit: some annual species although susceptible to injury from salt spray thrive because they complete their short life cycles between storms, are able to survive in protected habitats, have higher relative growth rates, reproduce prolifically and have high phenotypic plasticity;

3) prostrate growth habit: several species of sea coasts, although they rate low to extremely low in salt resistance, occur regularly in the high salt spray zone. They grow at an elevation where the amount of salt spray is below their level of tolerance and is only a fraction of what is received by a species with an erectile canopy (Maun, 2004, 2009);

4) reduced uptake: some grass species respond to high salt spray concentrations by limiting the influx of ions into the leaves because of low wetting properties of cuticular surfaces, through beading especially during rain and then rolling the seawater droplets off the plant leaves. The presence of sclerenchyma surrounding the parenchyma in grasses may also reduce the amount of chlorides reaching the parenchyma cells;

5) loss of salt from roots: some species move salt from the shoots to the roots and then leach it into the soil;

6) shedding of old leaves: salt resistance is acquired by sequestering of salt into old leaves and then shedding them.

Figure 7 - Salt marsh at "Molentargius Saline" (S - Sardinia).

Salt spray and its influence on coastal vegetation

Salt spray is an important abiotic stress that affects plant and other biotic communities in the proximity of sea coasts. The salt crystals act as condensation nuclei in the air and damage plants by abrasion during wind storms. However salt spray may also be beneficial because it improves plant growth by providing some essential nutrients. Three factors, wind speed, wave amplitude and wind direction, influence the formation of salt spray (Maun, 2009). Along seashores salt spray acts as a strong environmental stress on plants of coastal sandy dunes. High waves also inundate part of the seashore, thus increasing the salinity of the soil. Salt spray is formed by the bursting of bubbles that eject droplets of seawater into the air which are carried inland by wind. Because of a marked relief in sand dunes, salt deposition varies at different elevations above the sand surface and different distances from the sea coasts (Boyce, 1954). Usually dune ridges receive higher amounts of salt spray than low-lying areas such as the slacks. Seawater is a weak nutrient solution

that contains salts of Na^{+}, Ca^{++}, K^{+}, Mg^{++}, Cl^{-} and S with only minute quantities of all other essential elements (Maun, 1994, 2009). Symptoms of salt spray injury to plants manifest themselves as necrosis that begins on leaf tips, progresses to upper margins of leaves and eventually develops into an inverted V-shaped pattern. Trees and shrubs on sea coasts exhibit asymmetric growth forms because the seaward twigs are killed by salt spray while leeward twigs grow normally (Cheplick & Demetri, 1999). Some strand species respond to salt spray by completing their growth during periods of low salt spray storms or by making metabolic adjustments to either avoid or tolerate salt stress (Boyce, 1954). In laboratory, salt spray experiments are usually realized through two ways. The first method consists in the nebulization of different solutions with the same frequency, as reported by Sànchez-Blanco *et al*. (2003), while through the second method the same solution is nebulized with different frequencies on seedlings (Cheplick & Demetri, 1999; Griffiths & Orians, 2003). In both cases, the control condition is realized through a no spray treatment. In this study, we applied the same method used by Cheplick and Demetri (1999), which, in our opinion, can better reproduce in laboratory the natural spray episodes along coastal environments. Therefore, we applied on seedlings of the investigated *taxa*, a salt spray solution (35‰, i.e. 600 mM) to mimic sea water, with three different weekly frequencies (one, two and three days/week) for a total experiment duration of two months.

Species selection

The coastal species investigated during this Ph.D. program were chosen according to their habitat: *Phleum sardoum* (Hackel) Hackel and *Rouya polygama* (Desf.) Coincy for sandy dunes; *Brassica insularis* Moris and *Lavatera triloba* L. ssp. *pallescens* (Moris) Nyman for coastal cliffs; while *Lavatera triloba* L. ssp. *triloba* and *Halopeplis amplexicaulis* (Vahl) Ces., Pass. & Gibelli for environments with high salt concentrations in the substrate. Moreover, seed germination ecology

of *L. agrigentina* Tineo, a species growing in clayey-chalky plains and distributed in South Italy (Sicily and Calabria) was investigated for a comparative study within the *Lavatera* genus.

Plan of the work

The present study was structured in five chapters, each of which examining the germination ecology of one of the investigated species (except for the chapter four, which included togheter the three *Lavatera taxa*). Therefore, the first chapter was about *Phleum sardoum*, while the second on seed ecology of *Rouya polygama* and in both chapter the salt stress tolerance of these psammophylous species were investigated. Moreover, in the third chapter the germination ecology and salt stress response in seed germination of the rupestrian species *Brassica insularis* and its tolerance to salt spray on seedling development were evaluated. In the fourth chapter, *Lavatera triloba* ssp. *pallescens* seed germination requirements and salt stress tolerance were compared with those of *L. triloba* ssp. *triloba* and *L. agrigentina*. In the fifth chapter, *Halopeplis amplexicaulis* seed germination and salt stress response were investigated. In a conclusive chapter the comparison among the different investigated *taxa* were evaluated, in order to identify specific patterns related to their habitat.

The aims of this Ph.D. research program were to:

- investigate seed germination ecology and to provide new data for these previously unstudied or poorly studied species;
- evaluate the effects of several NaCl concentrations (0, 100, 200, 300, 400, 500, 600 mM) on seed germination of these species, with particular focus on the interaction temperature/salinity;

- evaluate the effects of salt spray on the vegetative growth and biomass production during the early seedling developmental stages.

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Chapter I - Light, temperature, dry after-ripening and salt stress effects on seed germination of *Phleum sardoum* (Hackel) Hackel

Abstract

Phleum sardoum is an endemic psammophylous species of Sardinia, growing exclusively on coastal sandy dunes. The effect of glumes on seed germination, germination requirements at constant (5-25°C) and alternating (25/10°C) temperatures, both in the light (12/12 h) and in the dark were evaluated, as well as the effect of a dry after-ripening period (90 days at 25°C), the salt stress effect (0-600 mM NaCl) and its recovery on seed germination. The presence of glumes reduced final germination percentages. For fresh naked seeds, high germination percentages were observed at 10°C. Dry after-ripening increased germination rate at low temperatures, but did not affect final germination percentages. NaCl determined a secondary salt-induced dormancy which recovery interrupted only partially. Our results highlighted that this species has its optimum of germination during autumn-winter when, under a Mediterranean climate, water availability is highest and soil salinity levels are minimal.

Keywords: dry after-ripening; glumes; NaCl; psammophylous species; recovery.

Introduction

High temperatures on soil surface characterize coastal sandy dunes habitats, particularly under the Mediterranean climate, and a delay of germination in early winter is considered an advantageous ecological adaptation towards an unpredictable rainfall pattern (Thanos *et al*., 1991). Moreover, photo-inhibition of seed germination has been reported for several species growing in Mediterranean sea coasts (Thanos *et al*., 1989), acting as a surface-avoiding mechanism for seedling establishment (Thanos *et al*., 1991). Seed drying under warm temperatures (dry after-ripening) is a natural mechanism that controls dormancy in dry climates (Finch-Savage *et al*., 2007) and coastal plants are exposed to frequent fluctuations of salinity levels in relation to seasons, also depending on their distance from the sea (Weber & D'Antonio, 1999). An increase in salinity stress can induce physiological secondary dormancy, determining a delay in the onset of the germination process and a reduction in the percentage of germinating seeds (Baskin & Baskin, 1998). When salinity stress is reduced, partial to complete germination recovery has been observed for various species (Khan, 2003).

Phleum sardoum (Hackel) Hackel (Poaceae) is a psammophylous species endemic to Sardinia (Camarda, 1980). The only data available on seed germination of this species is reported in the Seed Information Database, which reports high germination percentages (> 90%) at 15°C and 20°C, with 8 h of irradiance per day, after removing seed covering structures (Royal Botanic Gardens Kew, 2008). However, no factorial germination experiments were carried out on seeds of this species to determine the key factors in stimulating germination. Seeds of *Phleum* L. species, as well as of many other Poaceae, are reported to have a basal lateral embryo (Martin, 1946) and Finch-Savage and Leubner-Metzger (2006) described seeds of Poaceae as non dormant (ND) or physiologically dormant (PD).

The aims of this study were (1) to characterize seed germination of *P. sardoum*, by investigating the effect of the removal of covering structures (lemma and palea, hereafter glumes), identifying its germination requirements in terms of light and temperature, to evaluate the effect of (2) a dry after-ripening period and (3) NaCl on its seed germination.

Materials and methods

Study species

Phleum sardoum (Hackel) Hackel (Poaceae) is an endemic caespitose therophyte, 10-50 mm high (Figure 1), flowering from April to May and fruiting in May and June (Camarda, 1980). It grows exclusively in Sardinia in only two populations (Is Arenas - Arbus, South-West Sardinia and Rena Majore - Aglientu, North Sardinia), with a disjointed distribution area (Camarda, 1980). It has been found on coastal sandy dunes in gaps of *Juniperus oxycedrus* L. subsp. *macrocarpa* (Sibth. *et* Sm.) woods and in the back dunes.

Figure 1 - *Phleum sardoum* **(Hackel) Hackel on sandy substrate.**

Seed lot detail

Infructescences of *P. sardoum* were collected in the sandy dune system of Is Arenas - Arbus at 1-15 m a.s.l. (Figure 2 and Figure 3). Seeds were cleaned through sieves and a seed mass of 0.24 ± 0.01 mg (mean ± 1 standard deviation) calculated by weighing ten replicates of 50 seeds each.

Figure 2 - Psammophylous vegetation on sandy dune systems of Is Arenas - Arbus.

Germination tests

The effect of light, temperature and dry after-ripening on germination was evaluated on seeds collected in May 2010, on 1% water agar substrate, in plastic Petri dishes of 60 mm diameter (Figure 4). For all the experiments, the criterion for germination was visible radical protrusion. When no additional germination occurred for two consecutive weeks, the viability of any remaining seeds was checked by a cut-test and the final germination percentage calculated on the basis of the total number of filled seeds. All tests were started in two weeks after collecting.

A preliminary test was carried out to evaluate the effect of the presence of glumes on seed germination, by incubating three replicates of 20 intact and three of 20 naked seeds (without glumes) at 10°C with an irradiance of 12 h per day. Three replicates of 20 naked seeds each were incubated in a range of constant temperatures (5-25°C) and at an alternating temperature regime (25/10°C), both in the light (12 h of irradiance per day) and in the dark. Darkness was achieved by wrapping dishes in two aluminium foils. Seeds incubated in the light were scored daily and germinated seeds discarded, while dark-incubated seeds were scored only once at the end of the test to avoid any exposure to irradiance.

Figure 3 - Some *P. sardoum* individuals in "Is Arenas - Arbus" dunes.

Dry after ripening

A sub-lot of freshly collected seeds was subjected to a period of dry after-ripening, by putting it in a sealed polyethylene envelope, together with two microbags containing silica gel (0.5 g each) and incubated in a growth chamber at constant 25°C for three months, with an irradiance of 12 h per

day. The envelope was stored in a hermetic 2000 ml glass jar, with granular brown silica gel, to maintain low level of humidity. After three months, seeds were moved to germination conditions, and incubated as above specified.

NaCl stress and recovery on seed germination

To evaluate the effect of salt stress, three replicates of 20 naked seeds each, collected in the same natural population in May 2011, were sown in 1% water agar substrate, with different NaCl concentrations (0, 100, 200, 300, 400, 500, 600 mM) and incubated in a range of constant temperatures (5-15 °C), with an irradiance of 12 h per day (Figure 5). After two weeks without additional germination under control conditions (NaCl 0 mM), non-germinated seeds were washed with distilled water and then sown in new Petri dishes containing 1% water agar substrate (recovery phase).

The low number of replicates (3) and of seeds per replicate (20) used in all experiments were due to a limited seed availability, resulting from this species being rare with small populations and were choosen in order to allow testing a wide range of germination conditions.

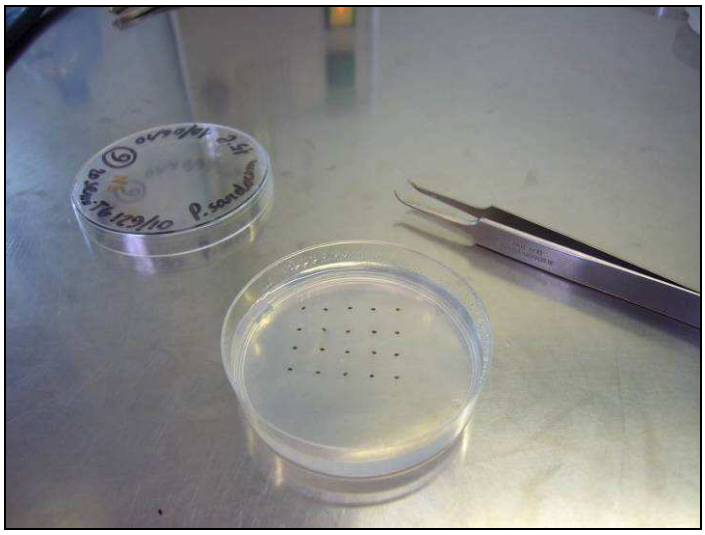

Figure 4 - Germination tests on *Phleum sardoum* **seeds.**

Data analysis

Final germination percentage at each temperature was calculated as the mean of the three replicates (± 1SD). The rate of germination was estimated by using a modified Timson's index (TI) of germination velocity: $TI = \sum G/t$, where G is the percentage of seed germination at two-days intervals and t is the total germination period (using this index, higher the value more rapid is the germination; Khan & Ungar, 1984). For NaCl experiments, the recovery percentages (RP) were calculated according to the following equation: $RP = \{[(a-b)/(c-b)] \times 100\}$, where a is the total number of seeds germinated after being transferred to distilled water, b is the total number of seeds germinated in the saline substrate, and c is the total number of seeds (Khan & Ungar, 1984).

When ANOVA assumptions were satisfied for original or arcsin-transformed germination percentages, one- or two-way ANOVA, with subsequent Fisher's LSD *post hoc* test, were used to evaluate the effect of temperature, light and pretreatment. When these assumptions were not satisfied, the non-parametric Kruskal-Wallis test was carried out. TI were calculated for both fresh and dry after-ripened (hereafter DAR) seeds, only for seeds germinated in the light and analysed by non-parametric Kruskal-Wallis and Mann-Whitney *U-test*. All the analyses were carried out using R v. 2.15.1 (R Development Core Team 2011).

Results

The glumes removal increased the final germination percentage (91.6 ± 2.8 %, mean ± 1SD) compared to that of intact seeds (68.3 ± 2.8%); these differences were statistically significant, with $p < 0.001$ by one-way-ANOVA. Naked seeds also germinated significantly ($p < 0.05$ by Mann-Whitney *U-test*) faster (TI: 4.16 ± 0.13) than intact ones (TI: 2.44 ± 0.10).

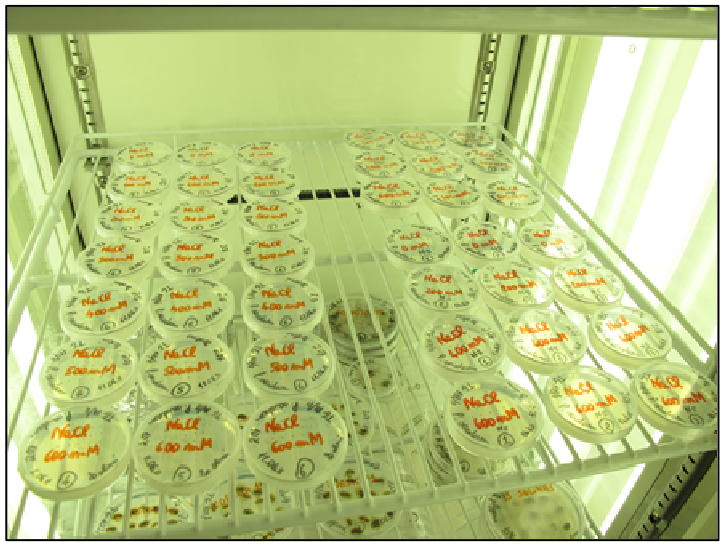

Figure 5 - Inside of a growth chamber with NaCl germination tests of *Phleum sardoum*.

For fresh seeds, the Kruskal-Wallis test showed a significant effect of temperature (T; $p < 0.0001$) but not for light (L; $p > 0.05$) on germination. The optimum germination temperature for fresh seeds was 10°C (96.7 ± 2.8% and 100 ± 0%, for light- and dark-incubated seeds, respectively). Germination percentages decreased at 5°C (46.7 ± 11.5% and 83.3 ± 5.77%, for light- and dark-incubated seeds, respectively) and at 15°C (40.0 ± 5.0% and 30.0 ± 0.0%, for light- and dark-incubated seeds, respectively) while at higher temperatures, germination percentages were lower than 35%, both in the light and in the darkness. Significant differences ($p < 0.01$) were detected among TI at different temperatures, with a significantly ($p < 0.05$) most rapid germination velocity detected at 10°C (TI: 3.2 ± 0.1).

For DAR seeds, the two-way ANOVA showed that temperature (T) significantly affected germination ($p < 0.001$), but not light (L; $p > 0.05$), whereas their interaction (T x L) was significant ($p < 0.001$). DAR seeds showed higher germination percentages at 5°C and 10°C in the light (80.0 ± 5.0 % and 98.3 ± 2.9 %, respectively), than in darkness (60.0 ± 21.8 % and 85.0 ± 8.7 %, respectively). Also at 15°C germination showed high values, but less in the light than in the darkness (13.3 ± 12.6 % and 78.3 ± 11.5 %, respectively). Germination decreased, both in the light

and in the dark, at 20°C (5.0 ± 5.0%, in both conditions) and 25°C (1.7 ± 2.9% and 3.3 ± 5.8%, for light- and dark-incubated seeds, respectively). Seeds incubated at the alternating temperature regime (25/10°C) reached 46.7 ± 15.2% and 25.0 ± 5.0 % germination, in the light and in the darkness, respectively. Significant differences ($p < 0.05$) were detected among TI at different temperatures, with TI being significantly higher ($p < 0.05$) at 10°C (TI: 3.5 ± 0.1 and 6.1 ± 0.2, respectively).

Considering the not statistically significant effect of the light detected for both fresh and DAR seeds, a two-way ANOVA was carried out for final germination percentages of fresh and DAR seeds sown in the light, in order to detect the effect of the DAR pretreatment (Figure 6). The two-way ANOVA showed a not significant effect of the pretreatment (P) on germination ($p > 0.05$), while temperature (T; $p < 0.001$) and their interaction (P x T; $p < 0.01$) were highly significant. The DAR pre-treatment determined an increase of germination ($p < 0.05$) at 5 and 10°C, whereas proved to be indifferent ($p > 0.05$) at warmer temperatures (Figure 6).

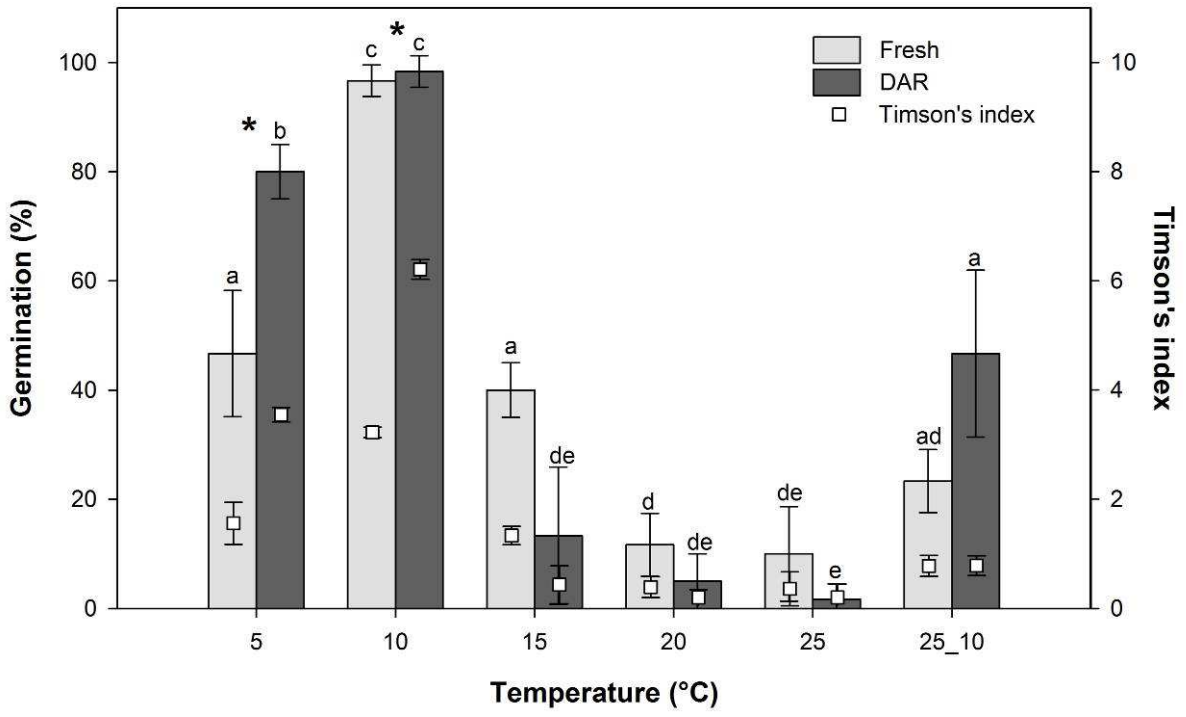

Figure 6 - Final germination percentage and Timson's index (TI) at each temperature regime for fresh and dry after-ripened (DAR) seeds in the light. Pretreatment (P): $p > 0.05$, temperature (T): $p < 0.001$ and (P x T): $p < 0.01$, by two-way ANOVA. Bars with the same letters are not significantly different at $p < 0.05$ (by *post hoc* Fisher's LSD test). * indicates significant differences ($p < 0.05$) between TI at the same temperature of the two treatments, by Mann-Withney *U-test*. Data, are the means (± 1 SD) of three replicates, for each treatment.

A one-way ANOVA was conducted in order to detect the effect of temperature among germination percentages under control condition (0 mM NaCl) in the salt stress experiment. The maximum germination percentage was detected at 10°C (100.0 ± 0.0%) while in the other two tested temperatures it was always lower than 55% (53.3 ± 5.8 and 50.0 ± 8.7% at 5 and 15°C, respectively). These differences were statistically significant at $p = 0.057$ by Kruskal-Wallis test. NaCl totally inhibited germination of this species at the tested temperatures, although 1.7 ± 2.9% of germinated seeds was observed at 100 mM, 10°C (Figure 7). At the end of the experiments, independently of the tested NaCl concentrations, no more than 20% of died seeds were observed (data not shown) and ungerminated viable seeds were moved to the recovery phase.

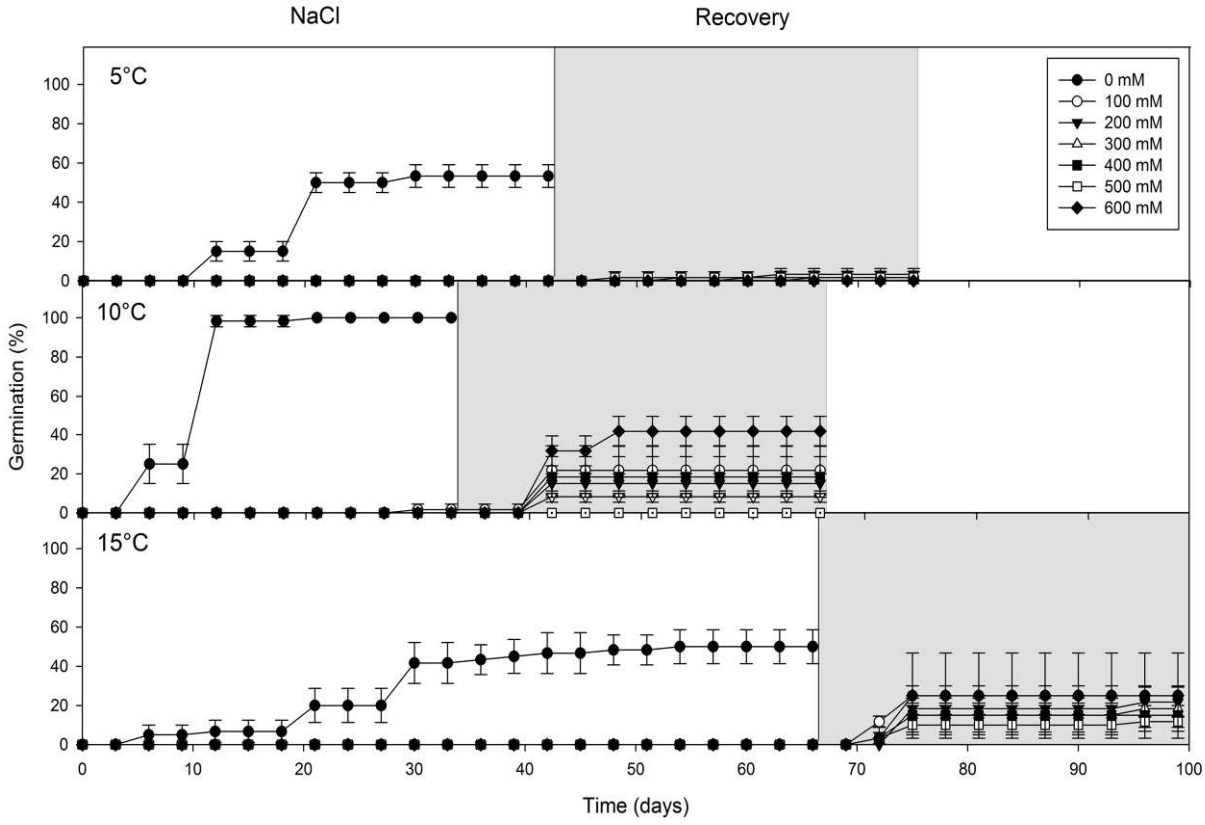

Figure 7 - Cumulative germination percentages under different conditions (5-15°C and 0-600 mM NaCl) and following transfer to distilled water (recovery, indicated by the shaded area in the graph). Each point represents the mean (± 1 SD) of three replicates.

No effect of temperature and NaCl concentration on RP was detected by the Kruskal-Wallis test ($p > 0.05$). At 5°C RP were not higher than 3.3 ± 2.9% at 200 mM (Figure 7 and Table 1). At 10°C RP reached the highest value (48.3 ± 7.7%) at 600 mM respect to those of all the other salinities (ranging from ca. 3 to 20%; Table 1). At 15°C, the highest value of RP was detected at 100 mM (28.3 ± 5.8%) respect to those at 200, 300, 400 and 600 mM (21.7 ± 7.7%, 21.7 ± 10.4%, 15.0 ± 0.0 and 25.0 ± 21.8%, respectively), except for that at 500 mM (11.66 ± 2.88%; Table 1).

Table 1 - Germination and recovery (RP) percentages at different conditions (5-15°C and 0-600 mM NaCl). A Kruskal-Wallis test was conducted in order to detect the effect of temperature among germination percentages at 0 mM (p = 0.057) and the effect of temperature and NaCl concentration on RP (p > 0.05). Data are the means (± 1 SD) of 3 replicates.

Temperature (°C)	Percentage (%)	NaCl concentration (mM)						
		0	100	200	300	400	500	600
5	Germination	53.3 ± 5.8	-	-	-	-	-	-
	Recovery (RP)	-	1.67 ± 2.9	3.33 ± 2.9	0.00 ± 0.00	1.7 ± 2.9	1.7 ± 2.9	0.0 ± 0.0
10	Germination	100.0 ± 0.0	1.66 ± 2.9	-	-	-	-	-
	Recovery (RP)	-	20.5 ± 10.7	11.7 ± 2.88	8.3 ± 2.9	18.3 ± 10.4	3.3 ± 2.9	48.3 ± 7.6
15	Germination	50.0 ± 8.7	-	-	-	-	-	-
	Recovery (RP)	-	28.3 ± 5.8	21.7 ± 7.6	21.7 ± 10.4	15.0 ± 0.0	11.7 ± 2.9	25.0 ± 21.8

Discussion

The glumes may constitute a physical barrier for the optimal imbibition of seeds and various studies showed the presence of substances in lemma and palea of Poaceae that inhibit germination (Huarte & Garcia, 2009). The positive effect of the removal of covering structures on seed germination reported by Royal Botanic Gardens Kew (2008), was confirmed in this study by a considerable increase of TI and an increase of germination percentage.

The optimum germination temperature of 10°C, for *P. sardoum* and the significant decrease of germination at higher temperatures (20°C and 25°C) reflects the optimal range of temperatures of Mediterranean species, between 5°C and 15°C (Thanos *et al*., 1989).

Phleum sardoum seeds achieved high germination percentages both in the light and in the dark, therefore they are not photo-inhibited for germination, contrary to other Mediterranean maritime plants (Thanos *et al*., 1989, 1991), enabling seed germination also under the harsh conditions of the soil surface (Figure 8). Probert (1992) suggested that responding to alternating

temperatures represents an adaptation of small-seeded species which ensure that germination occurs only close to the soil surface. The indifference of *P. sardoum* seeds to germinate both in the light and in the dark, and the lack of an alternating temperature requirement highlighted their capability to germinate also when deeply buried under sand surface and, therefore, suggest the lack of a permanent soil seed bank. In addition, with a maximum depth for seedling emergence of only ca. 15 mm (according to the allometric correlation between maximum depth of seedling emergence and seed mass, elaborated by Bond *et al*., 1999) germination in the dark may allow seeds to germinate even when they are buried too deep in the soil, leading to the death of the seedlings (Figure 9).

Figure 8 - Particular of a naked seed of *Phleum sardoum* **germinated (few hours after seed germination).**

The range of optimal temperatures for germination was not widened after the application of the dry after-ripening treatment, as reported for other annual species (Schütz *et al*., 2002). Therefore,

according to Baskin and Baskin (2004), seeds of this species are non dormant (ND), as reported for other Poaceae (Finch Savage & Leubner-Metzger, 2006).

The presence of NaCl in the substrate, highly inhibited germination of *P. sardoum* and induced a secondary salt-induced dormancy which recovery interrupted only partially, highlighting a physiological secondary dormancy (Baskin & Baskin, 1998). Seeds did not show the same recovery at all tested temperatures, in particular, the recovery performance was highly inhibited at 5°C. Various studies demonstrated that these variations in recovery responses may be related to the difference in the temperature regime to which seeds are exposed, especially to the lowest and highest temperatures (Khan & Ungar, 1999).

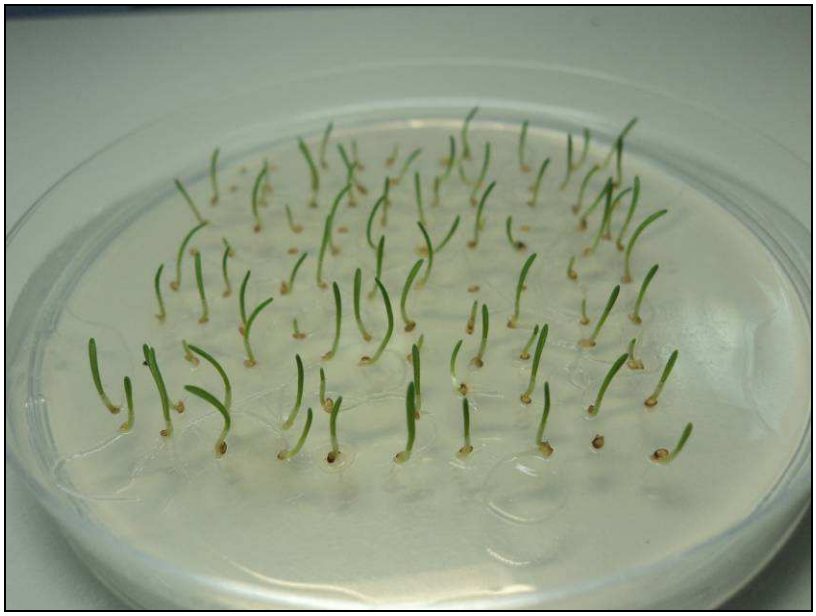

Figure 9 - Ten days seedlings of ***Phleum sardoum*****, transplanted from seed germination tests.**

The results of this study highlighted that while low temperatures have a significant effect on seed germination of this species, light and dry after-ripening did not affect final germination percentages. In addition, although seed viability was not affected by NaCl, salt induced secondary dormancy. These findings are coherent with a delayed field germination in early winter, when

temperatures and salinity concentrations in the soil are low and moisture in the soil is high, representing an advantageous ecological adaptation towards the unpredictable Mediterranean rainfall pattern (Thanos *et al*., 1989, 1991).

Acknowledgements

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Chapter II - Variability in seed germination and salt stress response of *Rouya polygama* (Desf.) Coincy

Abstract

Rouya polygama is an endangered Mediterranean species of great phytogeographical and ecological interest, growing on coastal sandy dunes. Germination requirements at constant (5-25°C) and alternating (25/10°C) temperatures, in the light (12/12 h) were evaluated among six populations from Sardinia and Corsica. The salt stress effect (0-600 mM NaCl) and its recovery on seed germination and inter-population variability in NaCl tolerance were also investigated. Seeds were non-dormant and germinated with high percentages (ca. 80%) in a wide range of temperatures (10-20°C), similarly to other Mediterranean coastal species. *R. polygama* showed the capability to germinate up to concentration of 200 mM NaCl, while higher salinities totally inhibited germination. Salt affected seed viability and recovery response decreased, proportionally with salinity increase and temperature. Inter-population variability and highest sensitivity for both Corse populations to NaCl was detected. Our results indicate the capability of seeds to germinate in autumn, when water availability is high and sandy salt concentration level is low. The significantly different germination behavior and the highest sensibility in salt stress response of Corse populations respect to Sardinian ones, may be imputable to their shorter distance from the sea and to their peripheral position in areal of distribution and to independent evolutionary divergence processes.

Keywords: Apiaceae, Mediterranean flora, NaCl, psammophyte, recovery, sandy dunes.

Introduction

Coastal dune environments are complex, vulnerable and characterized by close interactions between abiotic and biotic components and they have been recognized as stressful and frequently disturbed habitats, especially in the Mediterranean area (Del Vecchio *et al*., 2012; Fenu *et al*., 2012). Coastal sandy dunes have been identified as being particularly susceptible to destabilization through visitor pressure, which has increased dramatically in the last 50 years (Curr *et al*., 2000; Acosta *et al*., 2007). Coastal plant communities play an important role in dune system maintenance since they create an efficient protective barrier for backdune vegetation and for human infrastructures (Davies *et al*., 1995; Jolicoeur & O'Carrol, 2007; Maun, 2009; Del Vecchio *et al*., 2012).

In the Mediterranean sea, Sardinia and Corsica are considered part of the Thyrrenian hotspot (Medail & Quezel, 1999). In both islands, coastal sandy dunes are threatened by tourism, urbanization and reduction of habitat (Blasi *et al*., 2007). In these ecosystems occurs *Rouya polygama* (Desf.) Coincy (Apiaceae), an endangered species of great phytogeographical and ecological interest distributed in Sardinia, Corsica, Tunisia and Algeria (Bacchetta, 2001).

Under the Mediterranean climate, many adverse factors for plant survival characterize these ecosystems, such as high temperatures on soil surface, low soil moisture, nutrition and water deficiency, salinity of substrate and salt spray on plant surface (Thanos *et al*., 1991, 1994). Several key factors are known to influence seed germination, including light, temperature and salinity (Khan & Ungar, 1984; Baskin & Baskin, 1998). Light plays a crucial role in optimizing the time of seed germination (Baskin & Baskin, 1998; Franklin & Whitelam, 2005). Absence of light inhibits the seed germination either completely (Benvenuti *et al*., 2004), or partially (Zia & Khan, 2004), or may have no effect (Zheng *et al*., 2005; Wei *et al*., 2008). Temperature can also interact with light and other parameters and modify the sensitivity of seeds to light (Sugahara & Takaki,

2004). In psammophylous habitats, sand burial play also an important role in seed germination, and it is probably one of the most important physical stress in these ecosystems, because burial imposes a strong stress on production by altering normal growth conditions and exposing plants to extreme physiological limits of tolerance (Maun, 2009).

Salt tolerance to salinity is another important trait for species of coastal environments (Necajeva & Ievinsh, 2008), with higher salinity levels usually reducing or delaying germination of many species (Ungar, 1995; Tlig *et al*., 2008). Salinity tolerance during germination depends on temperature, being higher at low temperatures (Khan & Ungar, 1996; Gulzar *et al*, 2001; El-Keblawy *et al*., 2007; El-Keblawy & Al-Shamsi, 2008). For seeds that are unable to germinate at high salinity levels, it is essential to survive during exposure and maintain the ability to germinate later (recovery), when salinity may decrease due to various environmental events (Baskin & Baskin, 1998; Zia & Khan, 2004; Necajeva & Ievinsh, 2008). The ability of seeds to still remain viable would give ecological advantage in harsh climatic conditions like the playa habitat due to considerable variability of environmental factors (Khan & Gul, 2006). Seeds of several species treated with high salinity levels recovered their germination following transfer to distilled water, but variations in recovery percentages was attributed to differences in the temperature regime to which they were exposed (Badger & Ungar, 1989; Pujol *et al*., 2000; Gulzar *et al*, 2001; El-Keblawy *et al*., 2007). Greater ability to recover germination has been reported only at cold temperatures (15°C) for *Salsola imbricata* (El-Keblawy *et al*., 2007) and *Haloxylon salicornicum* (El-Keblawy & Al-Shamsi, 2008) and only at warm temperatures (25°C) for *Limonium stocksii* (Zia & Khan, 2004, 2008), while in *Panicum turgidum*, optimum recovery of germination was observed at moderate temperatures (20°C) and decreased at both lower and higher temperatures (15°C and 40°C, respectively; El-Keblawy *et al*., 2010). Several studies highlighted the presence of intra-specific variation (inter-population variability) in germination and dormancy (Neuffer &

Hurka, 1988; Pérez-García *et al*., 1995; González-Melero *et al*., 1997; Andersson & Milberg, 1998; Baskin & Baskin, 1998; Christal *et al*., 1998; Keller & Kollman, 1999; Qaderi & Cavers, 2000a, b, c). The inter-population variability in germination can be due to environmental differences or to genetic variations (Fenner, 1991; Gutterman, 1992; Kigel, 1995; Wulf, 1995; Degreef *et al*., 2002) while inter-population variability in seed dormancy can serve as an adaptive strategy in unpredictable environments (Cohen, 1968; Cruz *et al*., 2003). Various studies investigated also inter-population variability in salt tolerance for coastal species (Baskin & Baskin, 1998; Megdiche *et al*., 2007; Atia *et al*., 2011; Del Vecchio *et al*., 2012a) demonstrating that factors as provenance, seed size, distance from sea, local adaptations and climate may influence intra-specific differences in salt tolerance in some species.

Seeds of Apiaceae are reported to have orthodox seeds (Hong *et al*., 1998) and Martin (1946) described three typologies of embryo for this family: rudimentary, spatulate and linear axile, with an endosperm usually firm but watery-fleshy. Vandelook *et al*. (2012) investigated the factors driving the evolution of the relative embryo length in Apiaceae and indicated that it may have evolved as an adaptation to habitat and life cycle, whereas dormancy was mainly related to temperature at the sampling sites. Finch-Savage and Leubner-Metzger (2006) reported seeds of Apiaceae as morphologically dormant (MD) or morpho-physiologically dormant (MPD). However, no information is available about *Rouya polygama* seed germination and on the key factors stimulating germination, the response to salinity and recovery and inter-population variability for this species.

The aims of this study were (1) to characterize seed germination of *R. polygama*, by identifying its germination requirements in terms of light and temperature, (2) to evaluate the effect of NaCl and recovery on its seed germination and interactions of salinity with temperature, (3) investigating inter-population variability in seed germination and in salt stress tolerance.

Materials and Methods

Study species

Rouya polygama is a psammophylous and heliophylous species, growing on coastal sandy dunes of Sardinia, Corsica, Algeria and Tunisia and it is inserted in the "Washington Convention" (CITES), in the Annex I of the "Berna Convention" and in the Annex II of the "Habitat Directive 92/43/EEC" (Bacchetta, 2001). It is a scapous hemicryptophyte, 15-30(50) cm high, with ascending and flexuous stems. Lower leaves are two-pinnate, with 3-fid or pinnatepartite segments of second order, 5-10 mm long, acute, hairless on upper surface. Umbels have 10-20 rays; bracts are numerous, often 3-fid and downward; petals are white (Figure 1). Fruit is a schizocarp (consisting of 2 mericarps) of 8-9-mm, with undulate wings 2 mm long, (Pignatti, 1982; Tutin *et al*., 1993; Pozzo di Borgo & Paradis, 2000; Bacchetta, 2001; Gamisans & Jeanmonod, 2007). Flowering occurs from June to July while fruiting starts in September (Pignatti, 1982; Tutin *et al*., 1993; Bacchetta, 2001).

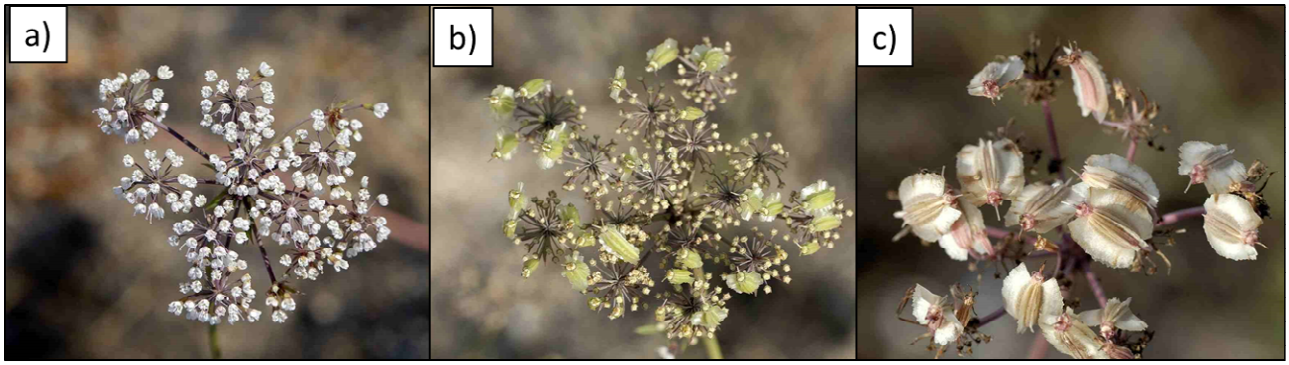

Figure 1 - *R. polygama* **inflorescence (a) and progressive fruiting stages (b and c) (photos from www.malachia.it).**

Seed lot details

Schizocarps (hereafter seeds) were collected in Sardinia and Corsica, in their natural populations (Figure 2 and Table 1), at the time of natural dispersal (Table 2). Seeds collections in Sardinia were carried out after obtaining permits from the "Ministero dell'Ambiente e della Tutela del Territorio e del Mare", as required by the European and national laws for the species listed in the appendices of the Habitat Directive 92/43 EEC, following articles 9 and 10 of DPR 357/97 modified by DPR 120/03, while seed material from Corsica was provided from "Conservatoire Botanique National de Corse", institution authorized by the Office de l'Environment de la Corse and the Ministry of the Environments of France. Seeds were selected by hand through sieves (5 mm). A mean seed mass value (± 1SD) for each population was calculated by weighing 10 replicates of 20 seeds each (Table 2).

Table 1 - Population data.

Locality	Island	Code	Coordinates	Altitude (m a.s.l)	Slope (°)	Aspect	Distance from the sea (m)
Portoscuso (CI)	Sardinia	SA1	39°12' N 08°23' E	1	5	-	570-1260
Is Solinas - Masainas (CI)	Sardinia	SA2	39°01' N 08°34' E	2-4	10-40	S	120-310
Porto di Arbatax (OG)	Sardinia	SA3	39°56' N 09°41' E	1	5-20	NE	42
Lido di Orrì (OG)	Sardinia	SA4	39°54' N 09°40' E	0.5	0-5	E	50
Port de Portovecchio	Corsica	CO1	41°35' N 09°17' E	1	0	-	80
Punta di Benedetto	Corsica	CO2	41°36' N 09°19' E	1	5	-	20

Table 2 - Seed lot details. In the column "Experimental trials" the different experiments carried out for each population are reported (L = Light; T = Temperature; NaCl = Salinity tests).

Code	Data of collection	Seed mass (mg ± SD)	Experimental trials
SA1	30/09/2010	7.91 ± 0.95	T, NaCl
SA2	30/09/2010	7.55 ± 0.63	L, T, NaCl
SA3	23/10/2010	8.45 ± 0.72	T, NaCl
SA4	23/10/2010	7.13 ± 0.35	T, NaCl
CO1	20/10/2010	8.33 ± 0.46	T, NaCl
CO2	20/10/2010	7.60 ± 0.69	T, NaCl

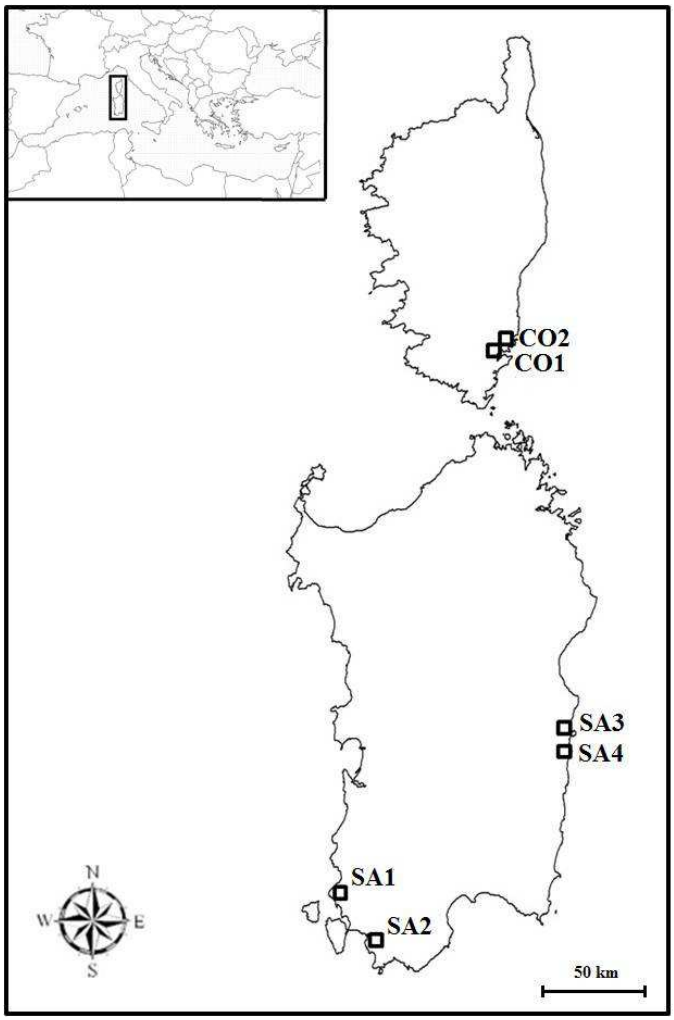

Figure 2 - Seed provenances of the six accessions of *R. polygama* investigated in this study in Sardinia and Corsica. See Table 1 for the explanation of the population codes.

Germination tests

Seeds were sown on 1% water agar substrate, which provided a solid, non-sterile medium for germination, in plastic Petri dishes of 90 mm diameter and then incubated in growth chambers (SANYO MLR-351) at different temperatures regimes both in the light and in the dark as specified below for the different experiments. For each condition, 3 replicates of 20 seeds each were used. The low number of replicates and of seeds per replicate used in all experiments were due to a limited seed availability, resulting from this species being endangered and rare and were chosen in order to allow testing a wide range of germination conditions. The criterion for germination was visible radical protrusion. When no additional germination occurred for two consecutive weeks, tests were stopped and the viability of any remaining seeds was checked.

Effect of light

A preliminary test was carried out in order to evaluate the effect of light on seed germination for seed lot SA2 due seed availability (see Table 2). Three replicates of 20 seeds each were incubated in the light (12 h of irradiance per day) and in the dark at 10°C, 15°C and 20°C. These temperatures were chosen because under the Mediterranean climate many coastal species have their range of optimum germination, between 5°C and 20°C (Thanos *et al*., 1989, 1995; Doussi & Thanos, 2002). Darkness was achieved by wrapping dishes in two aluminum foils. Seeds incubated in the light were scored daily and germinated seeds discarded, while seeds incubated in the dark were scored only at the end of the test to avoid any exposure to irradiance (Baskin *et al*., 2006). When no additional germination occurred in the light for two consecutive weeks, tests were stopped both in the light and in the dark and the viability of any remaining seeds checked.

Effect of temperature

Germination tests were started in May 2011 on seeds from each population (see Table 2). Three replicates of 20 seeds each were incubated in a range of constant temperatures (5, 10, 15, 20 and 25°C) and at an alternating temperature regime (25/10°C) in the light (12 h of irradiance per day) in growth chambers. In the alternating temperature regime, the higher temperature period coincided with the light period (Baskin *et al.*, 2006).

Effect of NaCl on seed germination and recovery

To evaluate the effect of salt stress on seed germination, seeds from population SA2 (see Table 2) were sown with different NaCl concentrations (0, 100, 200, 300, 400, 500, 600 mM) and incubated at a range of constant temperatures (10, 15, 20°C), in the light. To evaluate the variability in salt stress response among populations, germination with these NaCl concentrations (0, 200, 400, 600 mM) was also tested at 15°C for seeds from each population (Table 2 and Figure 3).

After two consecutive weeks without additional germination under control conditions (0 mM NaCl), non-germinated seeds were washed with distilled water and then sown in new Petri dishes containing 1% water agar substrate for additional 30 days (recovery phase) at the same incubation temperatures (Figure 4).

Figure 3 - Petri dishes with NaCl at different concentrations to test salinity response of *R. polygama* seeds.

Data analysis

Final germination percentages were calculated as the average of the three replicates (± 1 standard deviation) on the basis of filled seeds. The rate of germination was estimated by using a modified Timson's index (TI) of germination velocity:

$$TI = \sum G/t \,,$$

where G is the percentage of seed germination at two-days intervals and t is the total germination period (Khan & Ungar, 1984). Using this index, higher the value, more rapid is the germination.

For NaCl experiments, the recovery percentages (RP) were calculated according to the following equation (Khan & Ungar, 1984):

$$RP = \{[(a-b)/(c-b)] \times 100\},$$

where a is the total number of seeds germinated in salt solutions plus those that recovered to germination in the fresh water, b is the total number of seeds germinated in saline solutions, and c is the total number of seeds.

Germination percentages were analysed by non-parametric Kruskal-Wallis test, followed by a Mann-Whitney *U-test*. All the analyses were carried out using the software Statistica 8.0 for Windows.

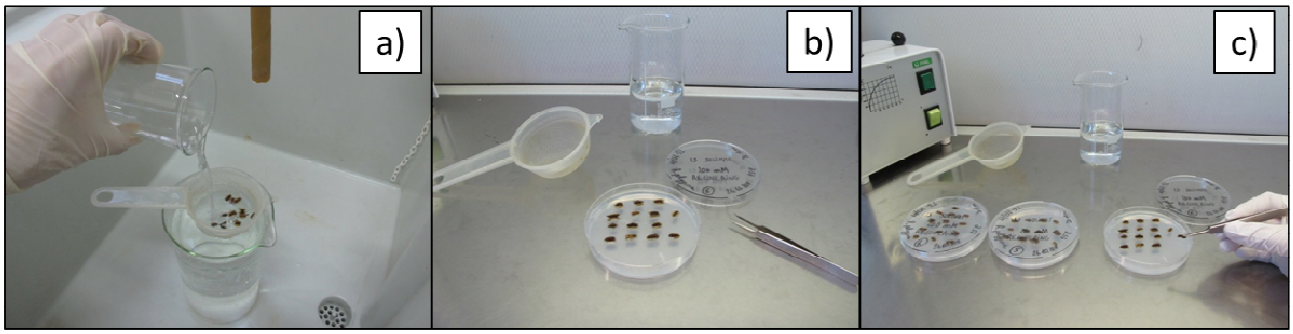

Figure 4 - Progressive phases of recovery experiment: seed washing with distilled water (a) and consequent sown in new Petri dishes with 1% agar substrate (b and c).

Results

Germination tests

Effect of light

Light did not affect seed germination at each tested temperature ($p > 0.05$). At 10°C, 65.0 ± 10.0% of seeds germinated in the light and 76.7 ± 14.4% in the dark; at 15°C, germination percentages were 81.5 ± 16.1% and 76.7 ± 16.1% for light and dark-incubated seeds, respectively, while at 20°C, 75.0 ± 5.0% of seeds germinated in the light and 73.3 ± 10.4% in the dark. Therefore all subsequent germination tests were conducted in the light (12/12 h).

Effect of temperature

At 5°C final germination differed among populations, in particular the higher germination percentages were detected for SA1, SA2 and SA4 (66.7 ± 7.6%, 70.0 ± 10.0% and 58.3 ± 16.1%, respectively) and these values were significantly different ($p < 0.05$) from that of all other populations, with the exception of SA4, whose values were statistically similar ($p > 0.05$) to that of SA3 (30.0 ± 15.0%). Low germination percentages were detected for CO1 and CO2 (16.7 ± 12.6% and 10.0 ± 13.2%, respectively) and these values did not differ only with the SA3 (Fig. 5).

At 10°C, no differences ($p > 0.05$) were detected among populations, with percentages ranging from 55.0 ± 5.0% (CO2) to 78.3 ± 11.5% (SA4; Fig. 5). At 15°C, the four Sardinian populations (SA1: 70.0 ± 5.0%; SA2: 81.5 ± 7.3%; SA3: 75.0 ± 5.0%; SA4: 78.3 ± 2.9%) differed significantly ($p < 0.05$) from the two Corse populations (CO1: 58.3 ± 7.6%; CO2: 28.3 ± 10.4%; Fig. 5). At 20°C, differences among populations and the higher germination percentages were detected for SA4 (78.3 ± 5.8%), whose values were statistically similar ($p > 0.05$) with that of SA1 and SA2 (73.3 ± 2.9% and 75.0 ± 5.0%, respectively), while the lower germination percentages were detected for CO2 (38.3 ± 12.6%), which did not show significant differences ($p > 0.05$) only with SA3 and CO1, with 60.0 ± 8.7% and 58.3 ± 12.6%, respectively. Germination percentages of CO1 were also statistically similar ($p > 0.05$) to that of SA1 and SA2 (Fig. 5).

At 25°C, germination percentages of the four Sardinian populations were not statistically different ($p > 0.05$) among themselves (ranging from 61.7 ± 12.6% to 75.0 ± 5.0% for SA3 and SA4, respectively). CO1 showed statistically similar values ($p > 0.05$) only with SA3 (ca. 50.0 %), while significantly lower germination percentages were detected for CO2 (18.3 ± 2.9%; Fig. 5). At the alternating temperature regime of 25/10°C, SA4 showed significantly higher germination percentages (83.3 ± 7.6%) than all the other populations except for SA1 (71.7 ± 7.6%). The lowest values were detected for CO2 and CO1 (26.7 ± 12.6 and 43.3 ± 10.4%, respectively; Fig. 5).

For the four Sardinian populations and the Corse CO1, no significant differences were detected among germination percentages at the different temperatures. For CO2, the highest germination percentages were detected at 10°C (55.0 ± 5.0%) and 20°C (38.3 ± 12.6%), whereas the lowest were observed at 5°C (10.0 ± 13.2%) and 25°C (18.3 ± 2.9%; Fig. 5).

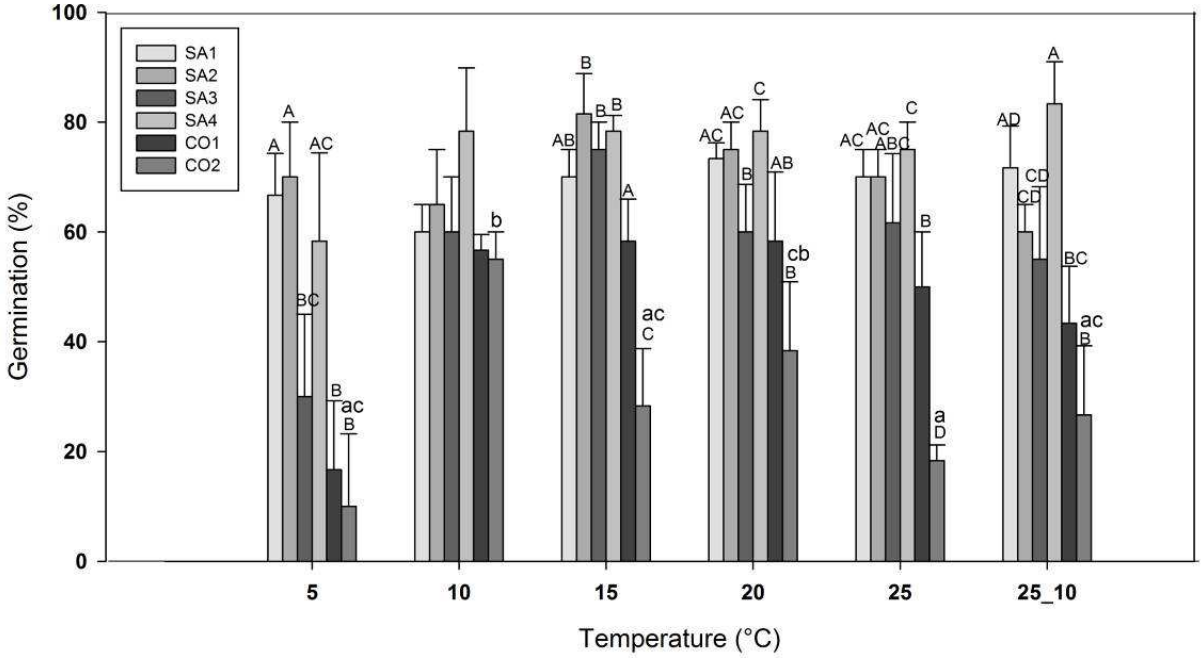

Figure 5 - Germination percentages in the light (12/12 h) at constant (5-25°C) and alternating temperature regime (25/10°C) for the six populations of *R. polygama* investigated in this study. Kruskal-Wallis tests were conducted to detect the effect of different populations at the same temperature (capital letters, by Mann Whitney *U-test*) and that of different temperatures for the same population (lower-case letters, by Mann Whitney *U-test*). Values with different letters were used to indicate significant differences at p < 0.05 (Mann Whitney *U-test*). Data, are the means (± 1 SD) of three replicates. See Table 1 for the explanation of the population codes.

NaCl stress and recovery on seed germination

Effect of temperature

Under control condition (0 mM NaCl) no differences were detected among temperatures, with percentages ranging from 65.0 ± 13.2% (15°C) to 75.0 ± 5.0% (20°C; Table 3 and Figure 6). At all tested NaCl concentrations (from 100 to 600 mM), temperature did not influence significantly ($p > 0.05$) germination percentages (see Table 3). At 10°C, final germination differed among concentrations, although values at 0 mM and 100 mM were statistically similar ($p > 0.05$) between themselves (66.7 ± 12.6% and 45.0 ± 10.0%, respectively) and germination at NaCl concentrations higher than 100 mM was totally inhibited (see Table 3). At 15°C, differences were detected among germination percentages, although statistically similar ($p > 0.05$) germination percentages were reached at 0 mM and 100 mM (ca. 70.0%, see Table 3). Germination at 200 mM (10.0 ± 10.0%) showed significant differences ($p < 0.05$) from that at NaCl concentrations lower than 100 mM, whereas at concentrations above 200 mM, germination resulted totally inhibited (see Table 3). At 20°C, germination percentages differed among concentrations and values at 0 mM and 100 mM NaCl were significantly different ($p < 0.05$) from that detected at concentrations above 100 mM, but not among themselves (ca. 65.0%; see Table 3). At 200 mM, only 3.3 ± 2.9% of germinated seeds were observed, while at NaCl concentrations above 200 mM germination was totally inhibited (Table 3).

No differences were detected among RP at 100, 200, 400, 500 mM NaCl, with exceptions of 300 and 600 mM NaCl (Table 3). In particular, at 300 mM, RP at 10°C (53.3 ± 10.4%) differed significantly ($p < 0.05$) from that at 15°C and 20°C (10.0 ± 0.0% and 6.7 ± 2.9%, respectively), while at 600 mM, all RP values differed significantly (10°C: 45.0 ± 8.7%; 15°C: 10.0 ± 0%; 20°C: 1.7 ± 2.9%; Table 3). At 10°C, RP values differed among NaCl concentrations, with the higher values at 200 and 300 mM (71.7 ± 10.4% and 53.3 ± 10.4%, respectively), being significantly ($p < 0.05$) different from all other values (see Table 3), although values at 300 mM were statistically

similar ($p > 0.05$) with that at 600 mM (45.0 ± 8.7%). RP values detected at 100, 400, 500 and 600 mM were without statistical differences ($p > 0.05$) among themselves, ranging from 16.7 ± 14.4% (400 mM) to 45.0 ± 8.7% (600 mM). At 15°C, no significant differences were detected among RP, ranging from 3.3 ± 5.8% (400 mM) to 66.8 ± 1.9% (200 mM). At 20°C, RP differed among NaCl concentrations, and the higher values were detected at 100 and 200 mM (47.6 ± 26.9% and 64.6 ± 12.7%, respectively), while RP were significantly lower ($p < 0.05$) at NaCl concentrations higher than 200 mM, ranging from 0.0 % (400 mM) to 6.7 ± 2.9% (300 mM, see Table 3).

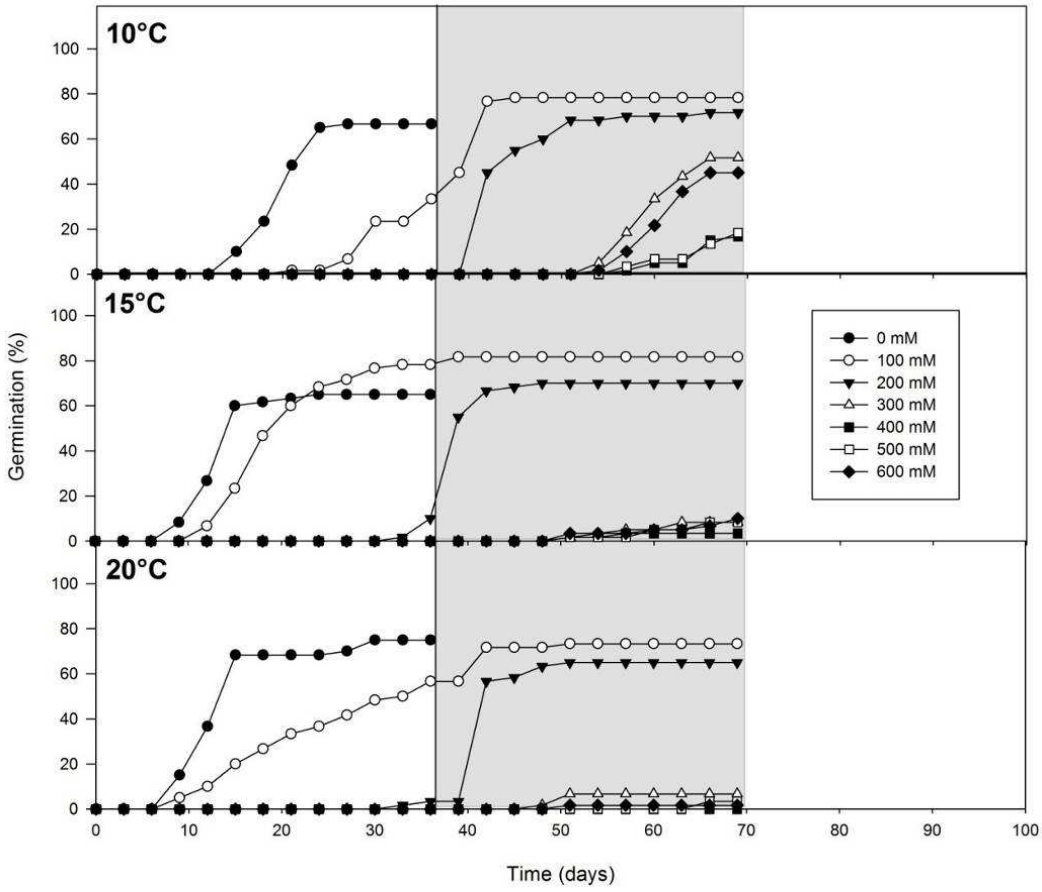

Figure 6 - Cumulative germination percentages at the tested temperatures (10-20°C), under different salt concentrations (0-600 mM NaCl) and following transfer to distilled water (recovery, indicated by the shaded area in the graph) for *R. polygama* (population SA2). Each point represents the mean (± 1 SD) of three replicates.

Table 3 - Germination and recovery (RP) percentages at the tested temperatures (10-20°C), at different saline concentrations (0-600 mM NaCl) for *R. polygama* (population SA2). Kruskal-Wallis tests were conducted to detect the effect of the same temperature on germination percentages and RP and that of the same salinity on germination percentages and RP; [p values were considered not significant (p > 0.05, ns) and significant (p < 0.05, *; p < 0.01, **), by Kruskal-Wallis test]. Capital letters in columns are related to the same salinity, while lower-case letters in rows to the same temperature. Values with different letters were used to indicate significant differences at p < 0.05 (by Mann Whitney *U-test*). Data are the means (± 1 SD) of three replicates. See Table 1 for the explanation of the population code.

Temperature (°C)	Percentage (%)	NaCl concentration (mM)							
		0	**100**	**200**	**300**	**400**	**500**	**600**	
10	**Germination**	66.7 ± 12.6^{a}	45.0 ± 10.0^{a}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	****
	Recovery (RP)	-	33.7 ± 8.9^{a}	71.7 ± 10.4^{b}	53.3 ± 10.4^{bcA}	16.7 ± 14.4^{a}	18.3 ± 14.4^{a}	45.0 ± 8.7^{acA}	*
15	**Germination**	65.0 ± 13.2^{a}	78.3 ± 7.6^{a}	10.0 ± 10.0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	****
	Recovery (RP)	-	16.7 ± 28.9	66.8 ± 1.9	10.0 ± 0.0^{B}	3.3 ± 5.8	8.3 ± 5.8	10.0 ± 0.0^{B}	ns
20	**Germination**	75.0 ± 5.0^{a}	56.7 ± 27.5^{a}	3.3 ± 2.9^{b}	0^{b}	0^{b}	0^{b}	0^{b}	**
	Recovery (RP)	-	47.6 ± 26.9^{a}	64.6 ± 12.7^{a}	6.7 ± 2.9^{bB}	0^{b}	3.3 ± 5.8^{b}	1.7 ± 2.9^{bC}	*
	Germination	ns	ns	ns	ns	sn	ns	ns	
	Recovery (RP)	-	ns	ns	*	ns	ns	*	

Seed Mortality

Figure 7 shows the estimate of the relationship between NaCl concentration and seed mortality percentages at different temperatures. At 10°C and 20°C, the regression lines showed the significant increase ($p < 0.05$ and $p < 0.001$, respectively) of seed mortality with increasing NaCl concentrations (with r^2 values of 0.63 and 0.95, respectively), while at 15°C, despite seed mortality increased with NaCl concentration, regression was not significant ($p > 0.05$), and the p value detected may be due of the anomalous mortality percentages at 300 mM at this temperature, probably due to the low number of seeds and replicates which were used. At 20°C the increase of seed mortality velocity was much greater than that detected at 10°C (with angular coefficient values of straight line of 0.12 and 0.06, for 20°C and 10°C, respectively; Fig. 7).

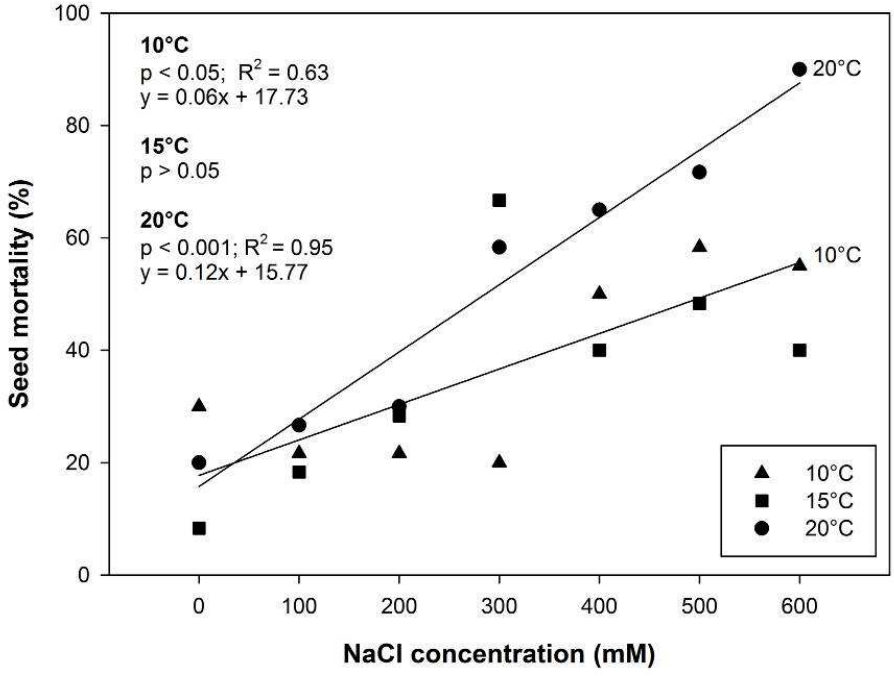

Figure 7 - Mortality of seeds of *R. polygama* at the tested NaCl concentrations (0-600 mM) and different temperatures (10, 15, 20°C). Black lines indicate linear regressions for each temperature. Each symbol is the mean of three replicates.

Inter-population variability

Under control condition (0 mM NaCl), final germination differed among populations and the four Sardinian populations showed statistically similar ($p > 0.05$) values among themselves (ca. 65.0%, see Table 4), but significantly different from both the two Corsican populations (55.0 ± 5.0% and 33.3 ± 17.5%, for CO1 and CO2, respectively). CO1 showed also statistically similar ($p > 0.05$) values with that of populations SA2 and SA3 (see Table 4). At 200 mM NaCl, germination percentages considerably decreased, if compared with 0 mM and differed among populations (Table 4). Germination percentages of Sardinian populations did not differ significantly ($p > 0.05$) among themselves, ranging from 10.0 ± 10.0% (SA2) to 16.7 ± 2.9% (SA4), but were significantly ($p < 0.05$) different from that of the two Corsican populations (1.7 ± 2.9% and 0.0% for CO1 and CO2, respectively; Table 4), with the exception of SA2, which showed statistically similar ($p > 0.05$) values with all populations (Table 4). CO1 did not show significant differences with populations SA1 and SA2. Germination was totally inhibited at NaCl concentrations above 200 mM, for both Sardinian and Corsican populations (Table 4).

RP values were higher at 200 mM than at higher NaCl concentrations and these differences were statistically significant for each population, except for populations SA1 and SA3, for which no difference was observed among RP at different NaCl concentrations (Table 4). At 200 mM, differences were detected among RP values and the two Corsican populations showed the lowest values (20.0 ± 5.0% and 15.0 ± 5.0%, for CO1 and CO2, respectively), which were statistically similar ($p > 0.05$) only with that of SA1 (37.2 ± 18.6%; Table 4). The highest RP values were found for SA2 and SA4 (66.8 ± 1.9% and 70.5 ± 20.2%, respectively; $p > 0.05$) and these values were significantly ($p < 0.05$) different from all others. At 400 mM, no differences were detected among RP of Sardinian populations, ranging from 3.3 ± 5.8% (SA2 and SA4) to 15.0 ± 15.0% (SA1) and recovery was totally inhibited for both Corse populations. At 600 mM, the highest RP

values were detected for SA1 (26.7 ± 5.8%) and differed significantly ($p < 0.05$) from all other values (Table 4). The remaining three Sardinian populations showed similar RP values among themselves (ca. 15.0%), while both Corsican populations did not have recovery at this NaCl concentration (see Table 4).

Table 4 - Inter-population variability of *R. polygama* in response to NaCl for germination and recovery percentages (RP) at 15°C. Kruskal-Wallis tests were conducted to detect the effect of the same population on germination percentages and RP and that of the same salinity on germination percentages and RP; [p values were considered not significant ($p > 0.05$, ns), significant ($p < 0.05$, *; $p < 0.01$, **) and highly significant ($p < 0.001$, *), by Kruskal-Wallis test]. Data are the means (± 1 SD) of three replicates. Capital letters in columns are related to the same salinity, while lower-case letters in rows to the same temperature. Values with different letters were used to indicate significant differences at $p < 0.05$ (Mann Whitney *U-test*). See Table 1 for the explanation of the population codes.**

Population code	Percentage (%)	NaCl concentration (mM)				
		0	200	400	600	
SA1	Germination	75.0 ± 8.7^{aA}	10.0 ± 5.0^{bAC}	0^{c}	0^{c}	*
	Recovery (RP)	-	37.2 ± 18.6^{AC}	15.0 ± 15.0	26.7 ± 5.8^{A}	ns
SA2	Germination	65.0 ± 13.2^{aAB}	10.0 ± 10.0^{bABC}	0^{b}	0^{b}	*
	Recovery (RP)	-	66.8 ± 1.9^{aB}	3.3 ± 5.8^{b}	10.0 ± 0^{bB}	*
SA3	Germination	61.7 ± 5.8^{aAB}	13.3 ± 5.8^{bC}	0^{c}	0^{c}	*
	Recovery (RP)	-	42.4 ± 8.4^{C}	21.7 ± 2.9	15.0 ± 8.7^{AB}	ns
SA4	Germination	70.0 ± 5.0^{aA}	16.7 ± 2.9^{bC}	0^{c}	0^{c}	*
	Recovery (RP)	-	70.5 ± 20.2^{aB}	3.3 ± 5.8^{b}	16.7 ± 7.6^{bAB}	*
CO1	Germination	55.0 ± 5.0^{aBC}	1.7 ± 2.9^{bAB}	0^{b}	0^{b}	*
	Recovery (RP)	-	20.0 ± 5.0^{aA}	0^{b}	0^{bC}	*
CO2	Germination	33.3 ± 17.5^{aC}	0^{bB}	0^{b}	0^{b}	*
	Recovery (RP)	-	15.0 ± 5.0^{aA}	0^{b}	0^{bC}	*
	Germination	*	*	ns	ns	
	Recovery (RP)	-	*	ns	*	

Discussion

The capability of *R. polygama* seeds to germinate at high percentages in a wide range of temperatures suggest that they are non-dormant (*sensu* Baskin & Basin, 2004) (Fig. 8). The highest germination percentages in the range 10-20°C, and decrease of germination at higher temperatures (25°C) reflects the optimal range of temperatures typical of Mediterranean coastal species (Thanos *et al*., 1989, 1995; Doussi & Thanos, 2002; Kadis & Georghiou, 2010). This pattern suggests germination in autumn-winter, when water availability, sand moisture and rainfalls are high, and temperatures are not excessively prohibitive for germination and consequent seedlings establishment (Thanos *et al*., 1995; Maun, 2009; Kadis & Georghiou, 2010) (Fig. 9). In sandy dune systems, the survivorship, establishment and growth of seedlings are influenced by a number of physical and biotic factors such as predation, disease, desiccation, competition, salt spray, nutrient deficiency, high soil surface temperatures and burial by sand, therefore germination in these particular habitats may occur in the period when all these adverse factors are minimal or absent (Maun, 2009).

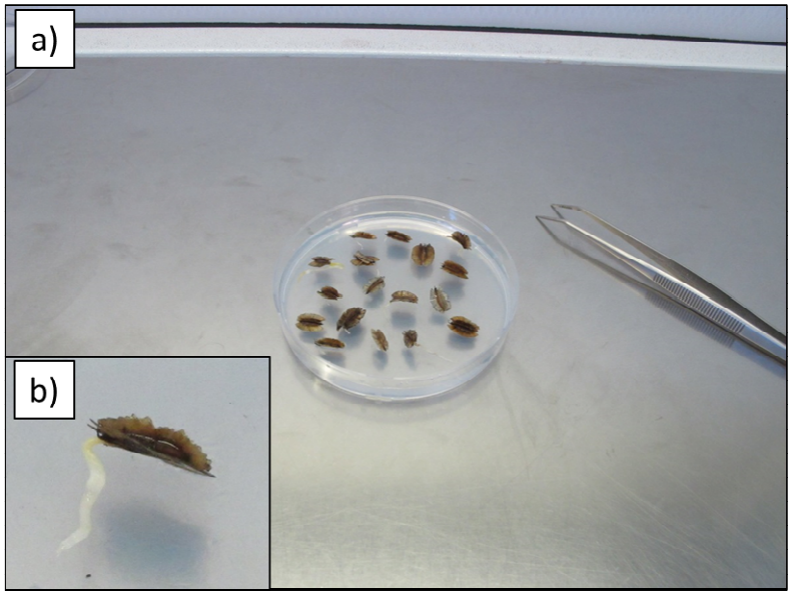

Figure 8 - Germinated seeds of *R. polygama* in Petri dish (a) and particular of a germinated seed (b).

R. polygama seeds achieved high germination percentages both in the light and in the dark, therefore they are not photo-inhibited for germination, contrary to other Mediterranean psammophylous species, e.g., *Brassica tournefortii*, *Cakile maritima*, *Otanthus maritimus* (Thanos *et al*., 1991), *Matthiola tricuspidata*, (Thanos *et al*., 1994), *Pancratium maritimum* (Keren & Evenari, 1974), *Crucianella maritima* (Del Vecchio *et al*., 2012). Germination may occur in a seasonal period when solar radiation is low, sand moisture is high due the high rainfalls and temperatures are not prohibitive. This germination pattern was confirmed by the seed mass of *R. polygama* (ca. 7.5 mg) as species with seed < 0.1 mg in weight are largely light-requiring for germination and the incidence of light-dependence declines with increasing seed size (Grime *et al*., 1981; Pearson *et al*., 2002).

Probert (1992) suggested that responding to alternating temperatures represents an adaptation of small-seeded species which ensure that germination occurs only close to the soil surface. Light is only able to penetrate 4-5 mm into the soil in physiologically significant quantities (Tester & Morris, 1987). The indifference of *R. polygama* seeds to germinate both in the light and in the dark, and the lack of an alternating temperature requirement highlighted their capability to germinate also when deeply buried under sand surface. In addition, with a maximum depth for seedling emergence of ca. 55 mm (according to the allometric correlation between maximum depth of seedling emergence and seed mass, elaborated by Bond *et al*., 1999) germination in the dark may allow seeds to germinate even when they are buried too deep in the soil, leading to the death of the seedlings, in fact seedling emergence declined with depth of burial and this may be caused by a lack of seed reserves to elongate the epicotyl sufficiently to reach the soil surface (Pearson *et al*., 2002). However, *R. polygama* seeds, with their wings are adapted to anemocory and rarely could reach high depths and so do not germinate.

R. polygama seeds germinated with NaCl in the substrate up to 200 mM of concentration, although in salt substrate were observed lower germination percentages, in comparison with that higher under control conditions (0 mM NaCl) and temperature did not influence germination under salt stress. Many studies reports that percentages of germination decreased with increased salinity stress and highest germination occurs in absence of NaCl in the substrate (Khan & Ungar, 1984, 1996; El-Keblawy *et al*., 2010; Keiffer & Ungar, 1997, Li, 2008; Khan *et al*., 2000; Pujol *et al*., 2000; Vallejo *et al*., 2010; Gulzar *et al*., 2001; Del Vecchio *et al*., 2012a). At concentration higher than 200 mM, germination was totally inhibited. The limit of tolerance to salt vary among different species (Ungar, 1995). For examples, the limit of tolerance was 200 mM NaCl in *Halopyrum mucronatum* and *Sporolobus arabicus* (Gulzar *et al*., 2001), 310 mM in *Brixa maxima* (Lombardi *et al*., 1998), 344 mM in *Puccinellia nuttalliana* (Macke & Ungar, 1971) and *Hordeum vulgare* (Badger & Ungar, 1989), 400 mM in *Diplachne fusca* (Morgan & Myers, 1989), 500 mM in *Urochondra setulosa* (Gulzar *et al*., 2001) and up to 1712 mM NaCl for *Kochia americana* (Clarke & West, 1969). For *R. polygama*, recovery showed a good performance only at lower salinity concentrations (≤ 200 mM), independently from temperature, while for seeds exposed, in the previous NaCl experimental phase, at NaCl concentrations higher than 200 mM, recovery was unsuccessfull or minimal. Only at the low temperature of 10°C, also seeds under high salinities (> 200 mM) showed their recovery capability, with performances inversely proportional to concentration which seeds were exposed. The highest tested temperature (20°C) interfered with seeds recovery and amplified the deleterious effect of salinity in their capability to recover from saline conditions as previously detected by Guma *et al*. (2010) for *Salsola vermiculata*. Salinity-temperature interactions may have significant eco-physiological implications in terms of time of germination under field conditions (Ungar, 1995). At high temperatures, salinity exposure could result in a loss of viability and consequently, poor recovery response. Tolerance and recovery

from salinity and temperature stress is also species specific (Khan & Ungar, 1997; Song *et al*., 2005). However, seeds of some species did not recover or showed little recovery response when subjected to high salinity and temperature stress (Khan & Gul, 2006).

R. polygama seed mortality was influenced by temperature, in particular at 600 mM NaCl, the highest tested temperature of 20°C amplified the negative effect of salinity on seed viability (with mortality > 95%). At each tested temperature, seed mortality increased proportionally with NaCl concentration and the increase of temperature increased seed mortality velocity affecting irreversibly seed viability, probably through ion toxicity. Several studies reported that salt stress negatively affected seed germination, with consequent seed mortality, either osmotically (through reduced water absorption) or ionically (through the accumulation of Na^+ and Cl^-), causing an imbalance in nutrient uptake and toxicity effect (Baskin & Baskin, 1998; Ungar, 1995; Li, 2008; Shokohifard *et al.*, 1989; Vallejo *et al*., 2010).

Intra-specific variability in germination patterns has been reported for several species and investigated in various studies (Bischoff *et al*., 2006; Kremer *et al*., 2009; Bischoff & Müller-Schärer, 2010); for example, depending on seed provenance, *Cakile maritima* and *Polypogon monspeliensis* showed inter-population variability in salt stress tolerance (Megdiche *et al*., 2007; Atia *et al*., 2011). Differences in salt stress response were showed also among populations of *Panicum turgidum* seeds (El-Keblawy *et al*., 2010). For *R. polygama*, different behavior in germination and recovery capability were detected among populations (Table 4), confirming the ability of *R. polygama* seeds to germinate at low NaCl concentrations in the substrate, the occurrence of inhibition of germination when seeds were exposed to highest concentrations (> 200 mM), the limited capability of seeds to have recovery of germination and the deleterious effect of salinity increase on seed viability. In particular, high differences were detected among Sardinian and Corsican populations: for both Corsican populations were shown, both under control

conditions than in NaCl (200 mM), lowest germination capability and seed viability, respect to that from Sardinia. In particular, CO1 population was totally inhibited at 200 mM NaCl, highlighted a higher sensibility of seeds of this population also to lowest salinity. Also recovery response of both Corse populations was considerably lower from that of Sardinian ones, showing highly significant differences, in particular both Corse populations did not show recovery response at NaCl concentrations above 200 mM. Seeds of all populations tested in this study were collected in the same period of natural dispersal and in the same habitat typology for all populations. Salt effects tend to decrease with the distance from the coastline (Maun, 2009), so the more deleterious effect of NaCl detected for both Corse populations, may be related to their highest sensibility, depending also from their short distance from sea, on average less than that of Sardinian populations, although nearest of these to the coastline (SA3 and SA4) resulted less sensitive and more adapted to this environmental adverse factor.

Figure 9 - *R. polygama* **seedlings at the Botanical Garden of Cagliari**

The different germination behavior and higher sensibility to salinity of Corse populations, observed in this study, may be related also to their peripheral distribution, and to independent

evolutionary divergence processes which would be differentiating Corse populations of *R. polygama* from other populations in the rest of areal. Several plant species are characterized by a disjunct distribution in which peripheral populations can be found isolated from the main home range. These have long attracted the attention of many researchers (Lutz *et al*., 2000; Gargano *et al*., 2007; Abeli *et al*., 2009), interested in understanding historical causes of distribution patterns, ecological and evolutionary relationships across the species range, and models of genetic variations related to isolation and divergence processes (Mosseler *et al*., 1992). Both the two Corse populations are smaller, as individual size, respect to Sardinian populations and is known that small and isolated populations are particularly sensitive to different threats (Bucci *et al*., 1997; IUCN, 2001) and are always at risk of local extinction, so the limited populational individual size, with limited gene flow, may be a factor influencing germination behavior response.

Our results highlighted the absence of primary dormancy for *R. polygama* seeds and the capability to germinate in a wide temperature range, with light which did not affect final germination percentages. Germination was totally inhibited when seeds were exposed to highest salt concentrations. *R. polygama* seeds showed the capability to germinate up to concentration of 200 mM NaCl, salt affected seed viability and recovery response decreased, proportionally with salinity and temperature increase. These results indicate the capability of *R. polygama* seeds to germinate in a seasonal period of autumn or spring, when water availability, need for seed imbibition, is high, sandy salt concentrations level are low because high rainfalls and the dry summer period, fateful for seedlings establishment, is far. The significantly different germination behavior and the highest sensibility in salt stress response of Corse populations respect to Sardinian populations, may be imputable to their shorter distance from sea and to their peripheral position at the limit of areal of distribution and to independent evolutionary divergence processes.

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Chapter III - Effect of provenance on seed germination and salt stress tolerance of *Brassica insularis* Moris heteromorphic seeds

Abstract

Brassica insularis is a perennial plant, growing both on coastal and inland cliffs. Germination requirements at constant (5-25°C) and alternating (25/10°C) temperatures were evaluated among three Sardinian populations, as well as the effect of a dry after-ripening period (90 days at 25°C). Seeds were analyzed through an image analysis system. The salt stress effect (0-600 mM NaCl) and its recovery on seed germination and inter-population variability in NaCl tolerance were also evaluated. Seedling salt spray tolerance was investigated between a coastal and an inland population. *B. insularis* seeds were non-dormant and germinated in a wide range of temperatures (5-25°C), differently to other Mediterranean species. Light and dry after-ripening did not affect final germination percentages. The morpho-colorimetric analysis, clearly identified seeds from different populations and discriminated three chromatic categories for seeds belonging to the Isola dei Cavoli population. *B. insularis* seeds showed the capability to germinate up to concentration of 200 mM NaCl, while higher salinities totally inhibited germination. Salt affected seed viability and recovery response decreased with increases of salinity and temperature. Inter-population and inter-annual variability on seed germination and seedling salt spray tolerance were detected. The heteromorphy phenomenon, for the first time observed in this species, may be due to independent evolutionary divergence processes of Isola dei Cavoli population. Inter-population variability in seedlings salt spray tolerance may be environmentally inducted, while inter-annual variability in

germination behavior may be a survival strategies for species growing under unpredictable environmental conditions, such as under Mediterranean climate.

Keywords: Brassicaceae, heteromorphy, NaCl, recovery, salt spray, Sardinia.

Introduction

The rupestrian flora is particularly interesting because of the great number of endemic plants, highly specialized from an ecological viewpoint (Bacchetta *et al*., 2007). Harsh conditions characterize rupestrian habitats, such as high sun exposition, strong winds, daily thermal variations (Giulietti *et al*., 1997; Ribeiro & Fernandes, 2000) and strong water deficit during dry months (Oliveira Silveira *et al*., 2012).

Coastal plants growing on cliffs are exposed to frequent fluctuations of salinity levels, in relation to seasons, depending on their distance from the sea (Weber & D'Antonio, 1999). An increase in salinity stress can determine a delay in the initiation of the germination process and a reduction in the percentage of germinating seeds (Baskin & Baskin, 1998). Saline conditions could: 1) completely inhibit germination (induced secondary dormancy) at salinities beyond the tolerance limit of species, 2) delay seed germination at salinities that cause some stress to seeds but do not prevent germination, 3) cause loss of viability of seeds due to high salinity and temperature (Khan & Gul, 2006).

When salinity stress is reduced, partial to complete germination recovery has been observed for various species (Khan, 2003). The ability of seeds to remain dormant at extremely low water potentials and to also have recovery germination after inhibition under hypersaline conditions, indicates that they may be more salt tolerant than actively growing plants (Ungar,

1982). Salt spray is another important abiotic stress that affects plants in the vicinity of sea coasts and it may, under certain environmental conditions, exert an influence on seedling emergence and their establishment. The salt crystals can damage plant epigeal parts by abrasion during wind storms, however, salt spray may also be beneficial because it improves plant growth by providing some essential nutrients (Maun, 2009).

Several key factors are known to influence seed germination, including light and temperature (Khan & Ungar, 1984; Baskin & Baskin, 1998). Light plays a crucial role in optimizing the time of seed germination (Baskin & Baskin, 1998) and non-dormant seeds of many species germinate equally well in light and darkness, those of others germinate to higher percentages in light than in darkness (Grime *et al*., 1981), and those of a relatively few germinate to higher percentages in darkness than in light (Bullowa *et al*., 1975; Hilton, 1982; Maze & Whalley, 1992), because light can prevent the germination of negatively photoblastic (light-inhibited) seeds (Thanos *et al*., 1989, 1991, 1994). Seed drying under warm temperatures (dry after-ripening) is a natural mechanism that controls dormancy in dry climates (Finch-Savage *et al*., 2007). A period of usually several months of dry storage at room temperature of freshly harvested, mature seeds is a common method used in laboratory to mimic this mechanism and release seed dormancy (Bewley, 1997).

Seed heterogeneity or heteromorphy, defined as the production of different types of seeds by a single individual, appears in many different species of angiosperms (Imbert, 2002; Matilla *et al*., 2005). Morphological heteromorphy may occur in seed size, shape, and also color (Baskin & Baskin, 1998) and this phenomenon may affect physiological properties, including dormancy (Duran & Retamal, 1989), germination (Puga-Hermida *et al*., 1997) and longevity behavior (Diederichsen & Jones-Flory, 2005). Morphological and colorimetric characterization of the seeds

as well as other structural parts, are performed applying computer vision techniques that allow to execute very accurate and repeatable measurements.

Seeds of Brassicaceae are reported to have orthodox seeds (Hong *et al*., 1998; Royal Botanic Gardens Kew, 2008) and Martin (1946) described for this family an axile slightly folded embryo, without endosperm. Finch-Savage and Leubner-Metzger (2006) reported seeds of Brassicaceae as physiologically dormant (PD) or non dormant (ND). In the genus *Brassica*, seed heteromorphy is largely documented for various species: e.g. *B. campestris* (Chen & Heneen, 1992), *B. carinata* (Getinet & Rakow, 1997), *B. alboglabra* (Heneen & Brismar, 2001), *B. napus* (Shirzadegan, 1986; Rahaman *et al*., 2001; Liu *et al*., 2005), *B. juncea* (Vera & Woods, 1982; Anand *et al*., 1985), *B. rapa* (Stringam, 1980; Hawk, 1982).

Maselli *et al*. (1996) investigated *Brassica insularis* Moris seed germination of eight years stored seeds (+ 5°C) from Corsica, Sardinia and Tunisia and, for Sardinian populations, showed that germination percentages ranged from 10% to 94%, at 25°C, in the light (16 h of irradiance per day). The Seed Information Database (Royal Botanic Gardens Kew, 2008) reports high germination percentages (100%) for *B. insularis* at the alternating regime of 25/10°C, in the light (8 h of irradiance per day), without pre-treatments or after seed chipping with scalpel. However, no factorial germination experiments were carried out on seeds of this species to determine the key factors in stimulating germination, their response to salinity and recovery and no information is available about the effects of sea salt spray on seedlings of this species.

The aims of this study were to (1) apply image analysis techniques to investigate seed inter-population variability of *B. insularis*; (2) characterize seed germination of this species; (3) evaluate the effects of NaCl and recovery on its seed germination; (4) to evaluate the effects of salt spray on seedlings development; and (5) to investigate inter-population variability in in salt stress tolerance and salt spray response.

Materials and Methods

Study species

B. insularis Moris is a perennial plant, 40-100(180) cm high, with erected-ascending stems, branched in distal third. Leaves are 10-20(35) x 5-12(15) cm long, glabrous, of green-glaucous colour, alternate, fleshy and with robust petiole. Terminal inflorescences are in raceme, with 50-100 flowers, with peduncles (8)12-24(30) mm long and calyx with erected and deciduous green sepals, 9-13 mm long; petals are four for each flower, ovate-spatulate and of white colour, 10-16 mm long (Fig. 1); stamens are six, with white filaments 1-1.2 mm long and yellow anthers (3)3.5-4.5 mm long. Fruits are lomentaceous siliques, (3)4-6 x 30-70(90) mm long, with circular section, and fructiferous pedicels (12)20-30(40) mm and beak (3)5-20(23) mm long.

Figure 1 - *Brassica insularis* **at the Botanical Garden of Cagliari.**

Spherical seeds are in number of 30-70 per siliqua, with diameter 1-2 mm, generally of a dark-brown colour (Fig. 3) (Pignatti, 1982; Tutin *et al.*, 1993; Bacchetta, 2001). It is a rupestrian,

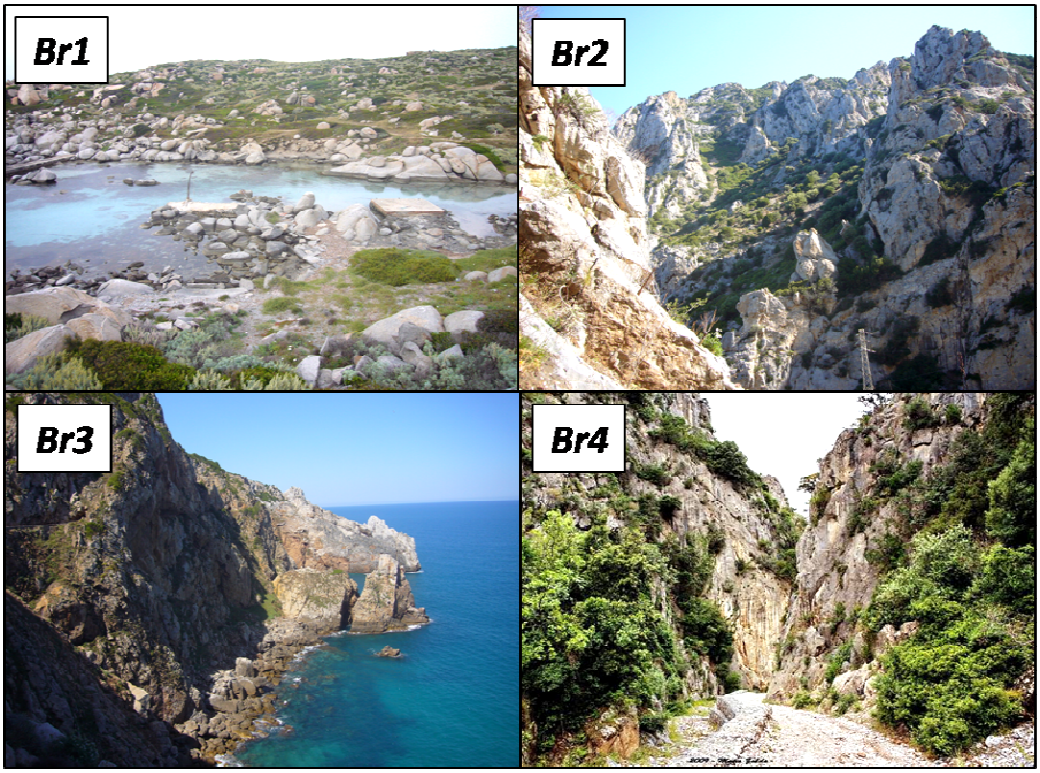
xerophylous, heliophylous and indifferent edaphic species, that occurs in coastal areas, under the influence of wet marine flows, less frequently in inland areas, on slopes, cliffs and vertical walls, at altitudes between 0 m and 1200 m a.s.l. and it is distributed in Sardinia, Corsica, Tunisia and Algeria (Bacchetta, 2001). *B. insularis* is a member of the *B. oleracea* L. group and it is inserted in the Annex II of the "Habitat Directive 92/43/EEC".

Seed lot details

Seeds of *B. insularis* were collected in their natural populations (Table 1 and Figure 2) at the time of natural dispersal (Table 2). Seeds were cleaned by hand and stacking up circular steel sieves, with mesh of various sizes (1-5 mm), in order to separate seeds from siliquas. Mean seed mass (± 1SD) for each seed lot was calculated by weighing 10 replicates of 20 seeds each (Table 2).

Table 1 - Population data.

Locality	Code	Coordinates	Substrate	Altitude range (m a.s.l)	Slope (°)	Aspect	Distance from sea (m)
Isola dei Cavoli - Villasimìus (CA)	*Br1*	39°05' N 09°31' E	Granites	5-22	20-45	N-NW	15
Masùa - Iglesias (CI)	*Br2*	39°19' N 08°26' E	Limestones	180-199	55-90	N	1500
Planu Sartu - Buggerru (CI)	*Br3*	39°23' N 08°23' E	Limestones	71-82	40-90	W	40
Gutturu Cardaxius - Iglesias (CI)	*Br4*	39° 22' N 08°27' E	Limestones	162-359	90	N	6220



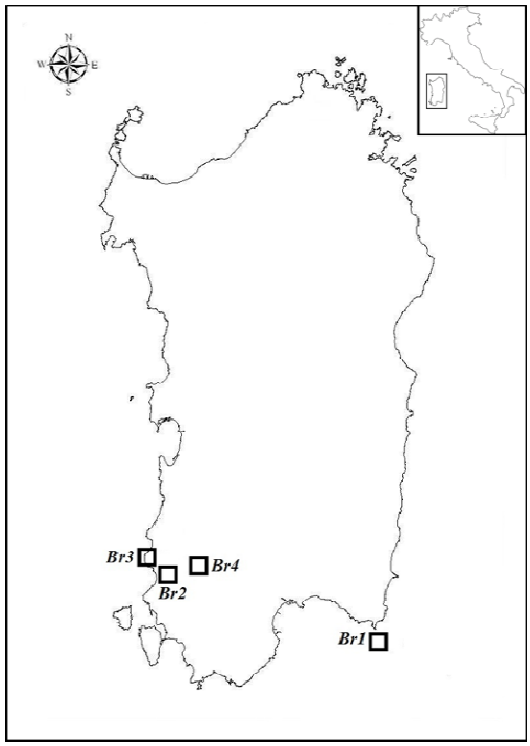


Figure 2 - Stations of seed collection for the four populations of *B. insularis* in Sardinia. For each population, the code is the same of Table 1.

Table 2 - Seed lot details. In the column "Experimental trials" the different experiments carried out for each seed lot are reported (L = Light; T = Temperature; DAR = Dry after-ripening; MC = Moisture content; NaCl = Salinity tests; Spray = Salt spray experiments).

Population code	Seed lot	Data of collection	Seed mass (mg ± SD)	Experimental trials
Br1	38/10	28/05/2010	5.61 ± 0.75	L; T; DAR; MC
	CAV/11	02/08/2011	4.22 ± 0.15	NaCl
Br2	79/10	24/06/2010	6.04 ± 0.90	T; DAR
	33/11	20/06/2011	4.46 ± 0.30	NaCl
Br3	M.O. 400/09	11/07/2009	6.20 ± 0.50	Spray
	81/10	24/06/2010	7.62 ± 0.33	T; DAR
	32/11	20/06/2011	5.85 ± 0.60	NaCl
Br4	322/06	05/09/2006	3.39 ± 0.40	Spray

Figure 3 - Siliquas and seeds of ***B. insularis*****.**

Morpho-colorimetric analysis

Digital images of seed samples were acquired using a flatbed scanner (Epson GT-15000) with a digital resolution of 200 dpi and a scanning area not exceeding 1024×1024 pixel. Image acquisition was performed before drying the seeds at 15°C to 15% of R. H. to avoid spurious variation in dimension, shape and colour. The scanner was calibrated for colour matching following the protocol of Shahin & Symons (2003) before image acquisition, as suggested by Venora *et al.* (2007). Samples consisting of 100 seeds, randomly disposed on the flatbed tray, were acquired and used for the digital image analysis (Fig. 4). For accessions of fewer than 100 seeds, the analysis was executed on the whole batch.

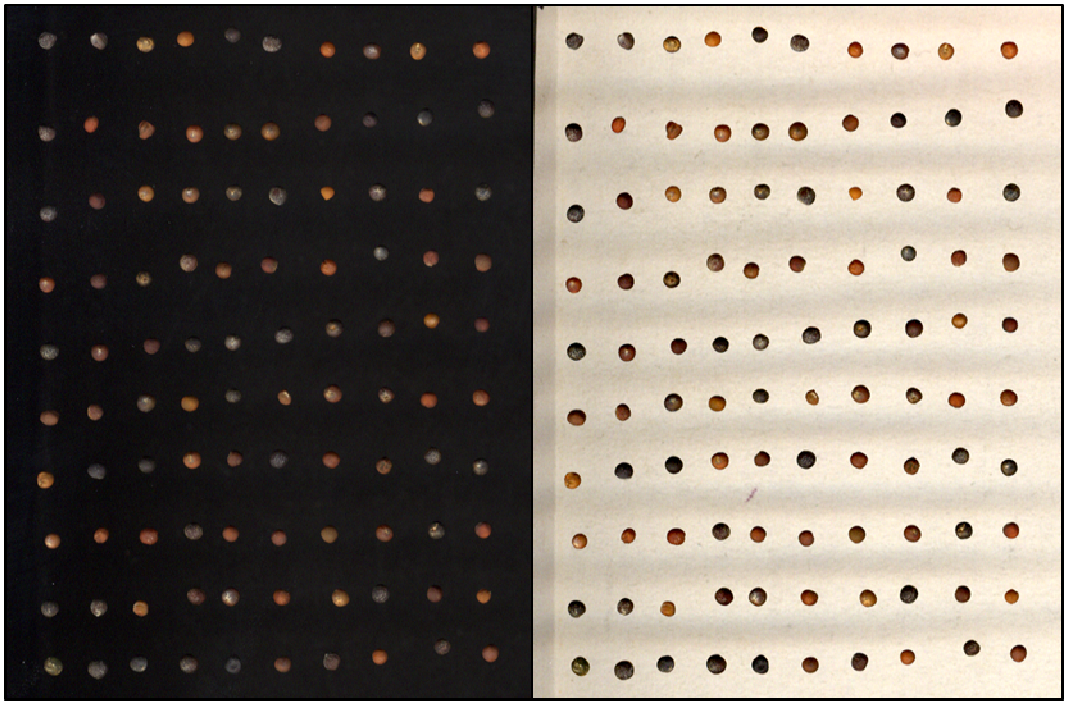


Figure 4 - Image acquisitions of *B. insularis* seeds, on black and white background.

Digital images of seeds were analysed using the software package KS-400 V. 3.0 (Carl Zeiss, Vision, Oberkochen, Germany). The accuracy and speed of measurements was maximised by running an automated macro developed specifically for the characterization of wild seeds

(Bacchetta *et al*., 2008; Mattana *et al*., 2008; Grillo *et al*., 2010). In order to increase the number of discriminant parameters the Elliptic Fourier Descriptors (EFD) method was also applied as described by Orrù *et al*. (2012). A total of 114 morpho-colorimetric quantitative variables describing seed size, shape, colour and texture were measured by computer vision (Appendix 1).

Germination tests

Effect of light

A preliminary test was carried out in order to evaluate the effect of light on seed germination for Br1 seeds (see table 2). Seeds collected in 2010 were sown on 1% water agar substrate, which provided a solid, non-sterile medium for germination, in plastic Petri dishes of 90 mm diameter. Three replicates of 20 seeds each were incubated in the light (12 h of irradiance per day) and in the dark, in growth chambers (SANYO MLR-351) at 15°C. This temperature was chosen because under the Mediterranean climate many species have their optimum germination, between 5°C and 20°C (Thanos *et al*., 1995; Doussi & Thanos, 2002). Darkness was achieved by wrapping dishes in two aluminum foils. The criterion for germination was visible radical protrusion. Seeds incubated in the light were scored daily and germinated seeds discarded, while seeds incubated in the dark were scored only at the end of the test to avoid any exposure to irradiance (Baskin *et al*., 2006). When no additional germination occurred in the light for two consecutive weeks, test were stopped both in the light and in the dark and the viability of any remaining seeds was checked by a cut-test.

Effect of temperature

Germination tests were started in June 2010 on seeds of Br1, Br2 and Br3 (see table 2). Three replicates of 20 seeds each were incubated in a range of constant temperatures (5, 10, 15, 20 and 25°C) and at an alternating temperature regime (25/10°C) in the light (12 h of irradiance per day

(Figure 5). In the alternating temperature regime, the higher temperature period coincided with the light period (Baskin *et al.*, 2006).

Effect of dry after-ripening

A sub-lot of freshly collected seeds from Br1, Br2 and Br3 (see table 2), was placed in 2010 in a dry room (15°C and 15% R.H.) for a dry after-ripening period (hereafter DAR). The advancement of drying, was monitored by measuring the activity water (aw) by the hygrometer Hygropalm Aw1 (Rotronic), equipped with the AW-DIO probe. When seeds reached aw = 0.180, they were closed in a sealed transparent polyethylene envelopes, together with two microbags containing silica gel (0.5g each) within a hermetic 2000 ml glass jar (mod. Fido, Bormioli Rocco S.p.a), with granular brown silica gel (diameter 2-5 mm), to maintain low level of humidity. The jar was then incubated at 25°C in a growth chamber and after three months, seeds were sown in Petri dishes in the light (12 h of irradiance per day) to the above specified germination conditions.

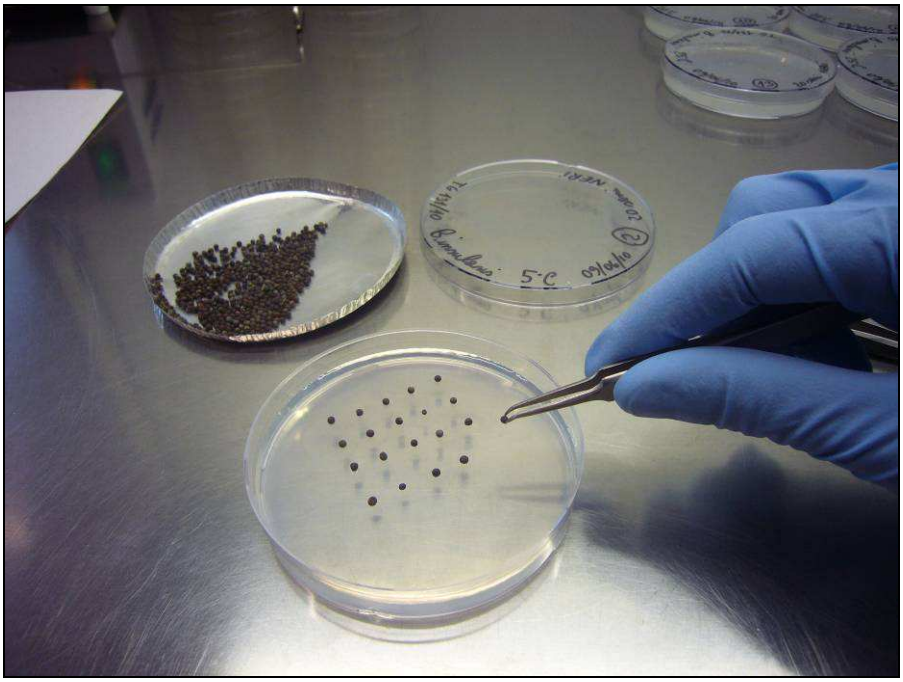


Figure 5 - Germination test on *B. insularis* seeds.

Seed heteromorphy

Seed lot from Br1 collected in 2010 (Table 2) showed heterogeneous seeds in colour (orange, brown and black), therefore seeds were separated into three sub-lots, according to the results obtained by morpho-colorimetric analysis. The seed moisture content (MC) of each chromatic category was determined by drying at 103 ± 1°C for 17 hours (ISTA, 2006). Three replicates of 100 seeds each were used for the test. To identify germination requirements of each chromatic category, three replicates of 20 seeds each were incubated in a range of constant temperatures (5, 10, 15, 20 and 25°C) and at an alternating temperature regime (25/10°C), in the light (12 h of irradiance per day).

Effect of NaCl on seed germination and recovery

Three replicates of 20 seeds each, from Br1 (see Table 2) were sown in 2011 in 1% water agar substrate, with different NaCl concentrations (0, 100, 200, 300, 400, 500, 600 mM) and incubated in a range of constant temperatures (5, 10, 15, 20 and 25°C) and at an alternating temperature regime (25/10°C), in the light. To evaluate the inter-population variability, germination in four NaCl concentrations (0, 200, 400, 600 mM) at 15°C and 25/10°C, was tested for seed lots Br2 and Br3 of the same year (Table 2). After two consecutive weeks without additional germination under control conditions (NaCl 0 mM), non-germinated seeds were washed with distilled water and then sown in new Petri dishes containing 1% water agar substrate for additional 30 days (recovery phase) at the same incubation temperatures. The low number of replicates (3) and of seeds per replicate (20) used in all experiments were due to a limited seed availability, resulting from this species being endangered with small populations and were chosen in order to allow testing a wide range of germination conditions.

Effect of salt spray on seedling development

A solution of NaCl was applied in 2011 on early seedlings by spraying. Seeds from Br3 and Br4 (see Table 2), were sown on 1% water agar substrate in plastic Petri dishes of 90 mm diameter. In order to obtain the number of seedlings required for the start of the experiments, five replicates of 100 seeds each were incubated in the light (12 h of irradiance per day) at constant temperature of 20°C, in a growth chamber. One week after seed germination, seedlings were sown in polyethylene pots (70 x 70 x 90 mm) in number of four for each, but only one seedling per pot was kept for the experiment. Before the use, all pots were disinfected by immersion in a solution of NaClO (860 mM) per two hours and then washed in distilled water. Pots were filled by a substrate, constituted by turf (55%), perlite (35%) and coconut fiber (10%), sterilized at 80°C per five hours in an oven. Four replicates of 13 seedlings each per condition, were inserted in a phytotron (8 m^3) at the alternating regime 20/10°C, with 12 h of irradiance per day (the higher temperature period coincided with the light period) (Fig. 6). Conductivity (conductometer microCM200, Crison) and pH (pH-meter GLP 21, Crison) values of the substrate were measured at the end of experiments. Humidity values inside the phytotron were monitored for all the duration of the experiments by a humid bulb hygrometer and they ranged from 73% (during the light period) to 91% (in the dark period). For eight weeks, four replicates were sprayed with a 600 mM NaCl solution (to mimic sea water) at a distance of 200 mm, with different frequencies (1 day/week, 2 days/week and 3 days/week) (Cheplick & Demetri, 1999), while other four replicates did not get any spraying (control). The temperature of the salt spray solution was 15°C and all epigeal parts of each seedling were equally exposed to the solution. The number of dead seedlings was annotated weekly. After eight weeks, at the end of the experiment, the length of epigeal and hypogeal parts for each survived seedling was measured by a digital caliper and the dry weight (Fig. 18) calculated by drying in oven at 103°C per 17 hours.

Figure 6 - Replicates of *B. insularis* seedlings for salt spray experiments.

Data analysis

Statistical analyses of the morpho-colorimetric data were performed with the software SPSS release 15 (SPSS Inc. 1999), applying the same stepwise Linear Discriminant Analysis (LDA) algorithm proposed by Grillo *et al*. (2012). This approach is commonly used to classify/identify unknown groups characterized by quantitative and qualitative variables (Fisher 1936, 1940) finding the combination of predictor variables with the aim of minimizing the within-class distance and maximizing the between-class distance simultaneously, thus achieving maximum class discrimination (Hastie *et al*., 2001; Holden *et al*., 2011). A cross-validation procedure was applied to test the performance of the classifiers, as reported by Bacchetta *et al*. (2011). Following

this approach, statistical classifiers were developed in order to distinguish sample clusters referred to the sampled populations, the harvest years and the colour of the seed accessions.

Final germination percentage was calculated as the mean of the three replicates (± 1SD) on the basis of the total of filled seeds. The rate of germination was estimated by using a modified Timson's index (TI) of germination velocity:

$$TI = \sum G/t ,$$

where *G* is the percentage of seed germination at two-days intervals and *t* is the total germination period (Khan and Ungar, 1984). Using this index, higher the value, more rapid is the germination. Moisture content (MC) was calculated using the following equation (Beena Anto & Jayaram, 2010):

$$MC = [(W_i - W_f)/W_f] \times 100,$$

where W_i is the fresh weight of seeds or initial weight, W_f is the dry weight of seeds or final weight. For NaCl experiments, the recovery percentages (RP) according to the following equation (Pujol *et al*., 2000):

$$RP = \{[(a-b)/(c-b)] \times 100\},$$

where *a* is the total number of seeds germinated in salt solutions plus those that recovered to germination in the fresh water, *b* is the total number of seeds germinated in saline solutions, and *c* is the total number of seeds. For salt spray experiments, a value of dry weight (mean ± SD) was calculated by weighing 13 seedlings of each treatment after eight weeks from the beginning of experiments. Germination percentages of light experiment and inter-population variability, MC and mean seed mass values for chromatic categories were analysed by ANOVA, followed by a Fisher LSD *post hoc* test when $p < 0.05$. Germination percentages of the three chromatic

categories, germination in NaCl, RP, mortality percentages, conductivity and pH values were analysed by a non-parametric Kruskal-Wallis, followed by a Mann-Whitney *U-test*. For salt spray experiments, dry weight and length of epigeal and hypogeal part of seedlings were analysed by one-way ANOVA, followed by a Fisher LSD *post hoc* test when $p < 0.05$. All the analyses were carried out using the software Statistica 8.0 for Windows.

Results

Morpho-colorimetric analysis

Comparing the results achieved by the stepwise LDA of *B. insularis* seed lots belonging to the three studied populations collected in 2010, an overall cross-validation percentage of correct identification of 97.6% was reached, with performances ranging between 95.0% and 98.7%, for Br3 and Br1, respectively (Figure 7).

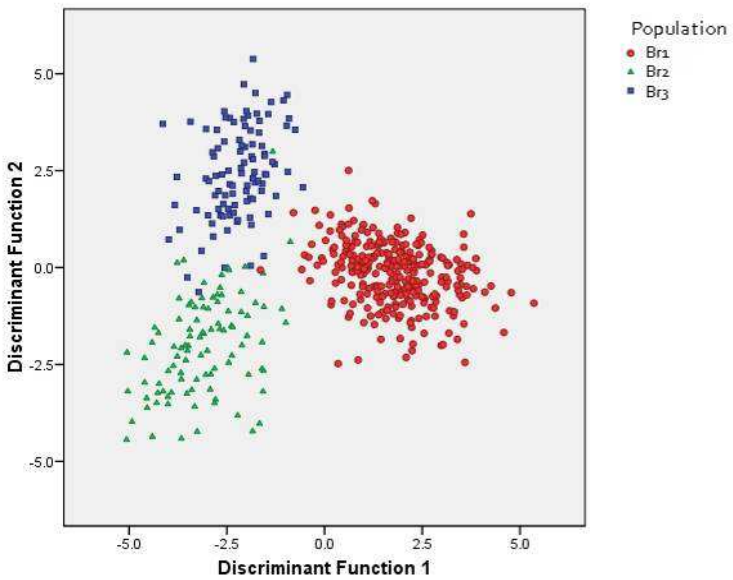


Figure 7 - Graphic representation of the discriminant function scores for the three populations of *B. insularis* **collected in 2010.**

Germination tests

Effect of light

For the preliminary light experiment, the one-way ANOVA showed the indifference of light on seed germination ($p > 0.05$) at the tested temperature of 15°C. Germination percentages were 35.0 ± 13.2% and 21.7 ± 5.8% for light and dark-incubated seeds, respectively. Therefore all subsequent germination tests were conducted in the light (Fig. 8).

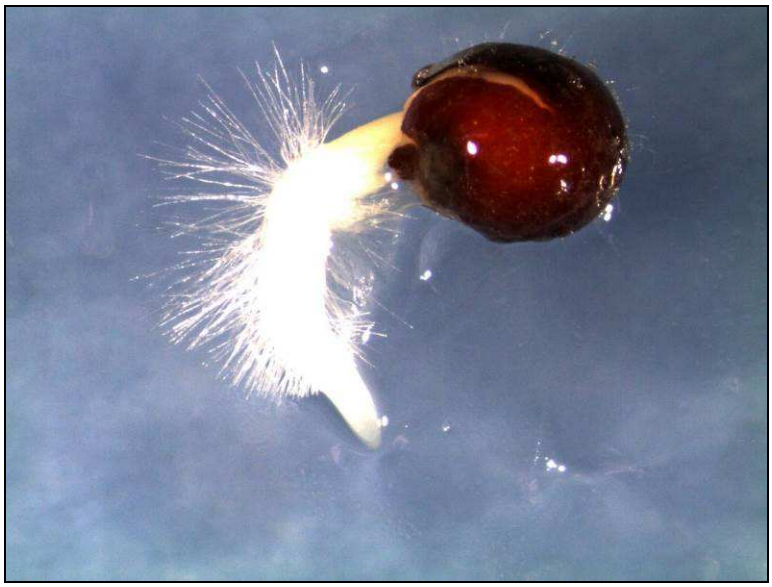


Figure 8 - Germinated seed of *B. insularis* in the light.

Effect of temperature and dry after-ripening

The three-way ANOVA (Table 3) showed a highly significant effect of temperature (T) on germination ($p < 0.001$) as well as for population (Po), but not of dry after-ripening (hereafter DAR) pretreatment (Pr), ($p > 0.05$). The interactions population and pretreatment (Po x Pr) and population and temperature (Po x T) were both highly significant ($p < 0.001$), whereas that of

pretreatment and temperature (Pr x T) was not significant ($p > 0.05$) as well as the three factors interaction (Po x Pr x T; Table 3).

For Br1 fresh seeds, the highest germination percentage was detected at 5°C (55.0 ± 5.0%) and was significantly ($p < 0.05$) different from all other values, which did not show differences ($p > 0.05$) among themselves (ca. 35.0%) (see Figure 9). For DAR seeds, germination percentages at 5°C, 10°C, 15°C and 25/10°C showed values (ca. 27.0%) without significant differences ($p > 0.05$), while the lowest value was detected at the highest temperature (25°C; 8.3 ± 5.8 %). Significant ($p < 0.05$) decreases between fresh and DAR seeds germination percentages (ca. 30%) were detected at 5°C, 20°C and 25°C (Figure 9).

For Br2 fresh seeds, the highest germination percentages was detected at the alternating regime of 25/10°C (48.3 ± 7.6) and was significantly different ($p < 0.05$) from all other values, whereas the lowest percentages was at 5°C (6.7 ± 7.6%; Figure 9). For DAR seeds, the highest, although not statistically significant ($p > 0.05$), values were at 15°C and 25/10°C (ca. 40%; Figure 9). The lowest germination percentage was detected at 5°C (6.7 ± 2.9%) and was without significant differences ($p > 0.05$) with values at 10°C, 20°C and 25°C. No significant differences ($p > 0.05$) were observed between fresh and DAR seeds at all tested temperature.

For fresh seeds of population *Br3*, the lowest germination percentage was detected at 20°C (16.7 ± 12.6%) and was significantly ($p < 0.05$) different only with that achieved at 25/10°C (36.7 ± 10.4%; Fig. 9). For DAR seeds, the highest value was detected at 25/10°C (76.7 ± 7.6%) and was significantly different ($p < 0.05$) from all germination percentages detected at all other temperatures (ranging from 35.0 ± 20.0% at 5°C to 48.3 ± 5.8% at 15°C). Significant increases ($p < 0.05$) between fresh and DAR seeds (ca. 32%) were detected at 20°C and 25/10°C (see Figure 9).

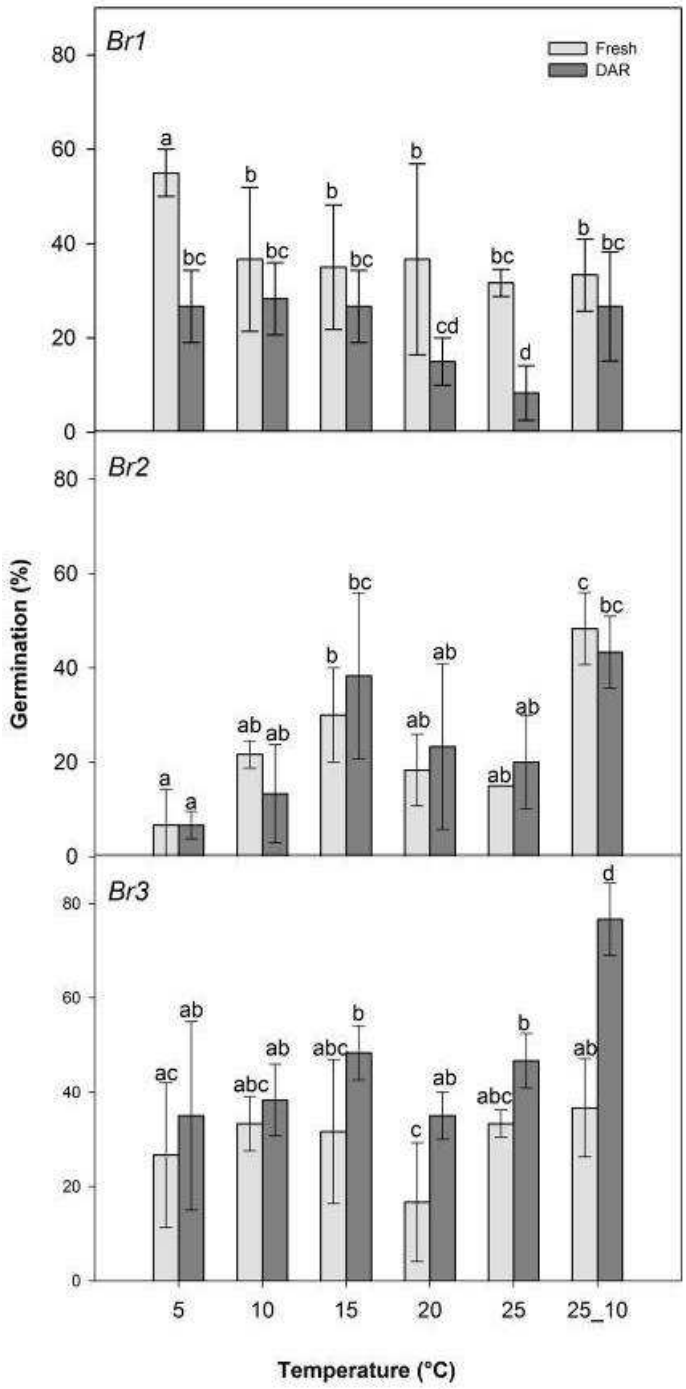


Figure 9 - Germination percentages in the light (12/12 h) at each temperature regime for fresh and dry after-ripened (DAR) seeds of the three populations (*Br1*, *Br2* and *Br3*) of *B. insularis*. **A three-way ANOVA was conducted among germination percentages in order to detect the effect of population (Po), dry after-ripening pretreatment (Pr), temperature (T), and their interactions (Po x Pr; Po x T; Pr x T; Po x Pr x T). A Fisher's LSD** *post hoc* **test was conducted to identify significant differences at $p < 0.05$, for each population. Data are the mean (± 1 SD) of three replicates. For each population, the code is the same of Table 1.**

Table 3 - Effect of the population (Po), dry after-ripening pretreatment (Pr), temperature (T) and their interactions on germination percentages for *B. insularis*; [p values were considered not significantly (p > 0.05, ns) and highly significant (p < 0.001, *) different, by three-way ANOVA].**

Factor	SS	Degr. of freedom	MS	F	p
Population (Po)	3778.2	2	1889.1	17.7413	***
Pretreatment (Pr)	8.3	1	8.3	0.0783	ns
Temperature (T)	5249.1	5	1049.8	9.8591	***
Po x Pr	4918.1	2	2459.0	23.0935	***
Po x T	5410.6	10	541.1	5.0813	***
Pr x T	813.9	5	162.8	1.5287	ns
Po x Pr x T	1309.7	10	131.0	1.2300	ns
Error	7666.7	72	106.5		

Seed heteromorphy

Morpho-colorimetric analysis

Comparing the results achieved by the statistical analysis of *B. insularis* heteromorphic seeds collected in 2010 from Br1 population, an overall cross-validation percentage of correct identification of 90.0% was reached, with performances included between 80.0% (brown seeds) and about 95.0% (orange and black seeds). No misattributions were found between orange and black seeds (Figure 10).

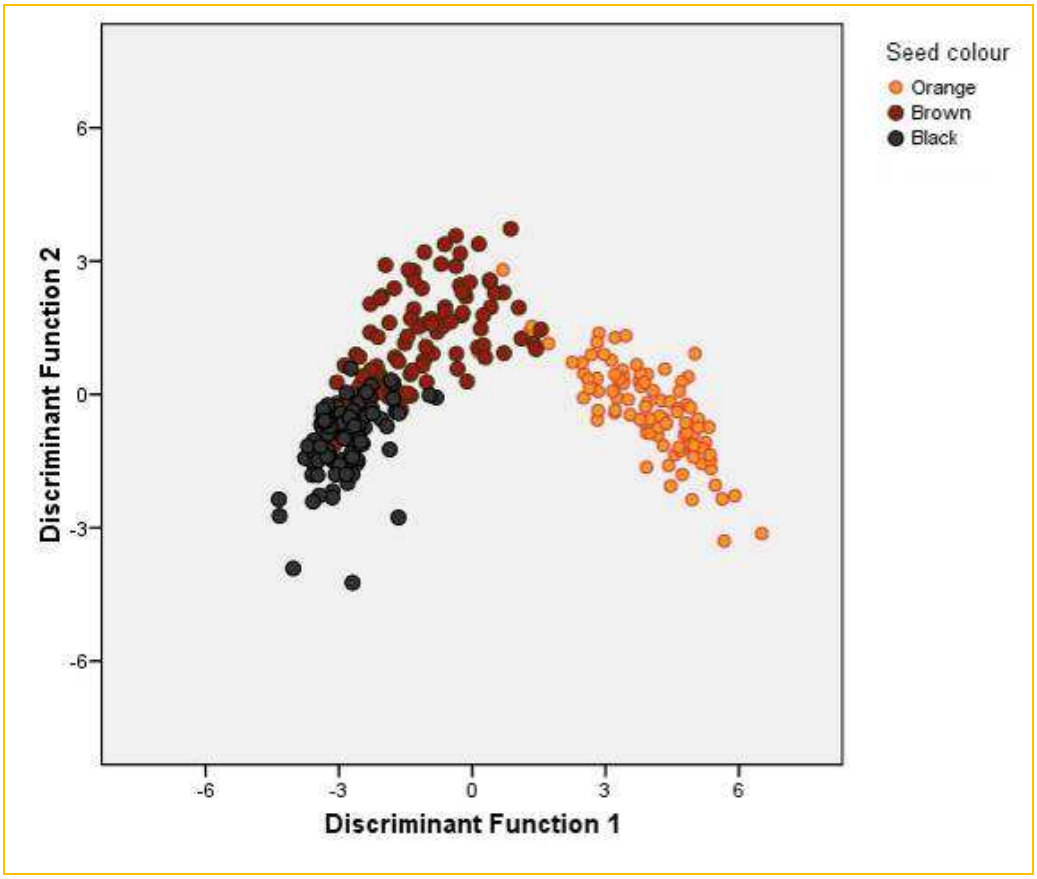


Figure 10 - Graphic representation of the discriminant function scores for the *B. insularis* heteromorphic seeds of Br1 population collected in 2010.

Seed mass

For the mean seed mass of the three chromatic categories, the one-way ANOVA showed significant differences ($p < 0.05$) among colours. Mean seed mass of orange seeds (3.9 ± 0.6 mg) was significantly ($p < 0.05$, by Fisher's LSD *post hoc* test) lower than that of both black (5.0 ± 0.6 mg) and brown seeds (5.4 ± 0.3 mg; Figure 11).

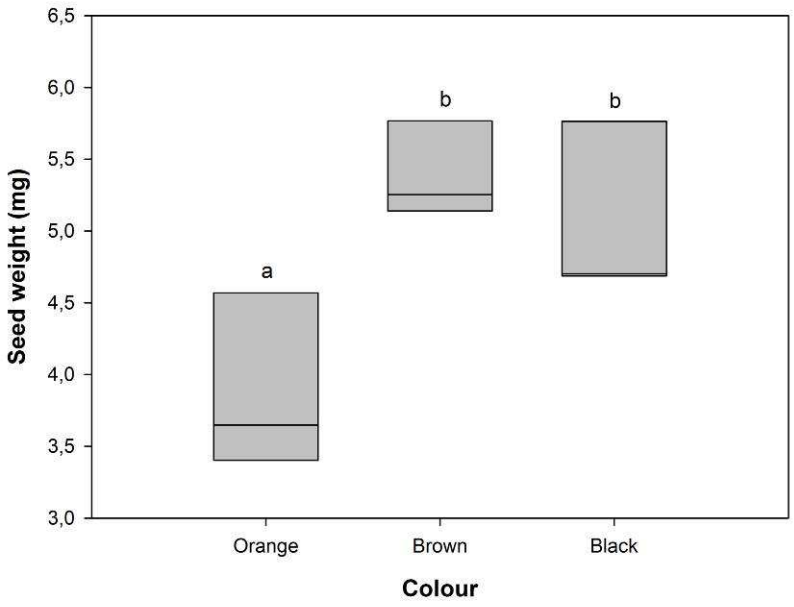


Figure 11 - Mean seed mass (mg), for each chromatic category. A one-way ANOVA was conducted in order to identify differences among colours. Values with the same letters are not significantly different at p > 0.05 (Fisher's LSD *post hoc* **test).**

Moisture content measure

For the moisture content measure, the one-way ANOVA showed the indifference of colour in moisture content (MC) among the three seed chromatic categories ($p > 0.05$). For black and orange seeds, MC was 6.7 ± 0.1%, while a value of 6.3 ± 0.2% were detected for brown seeds (Fig. 12).

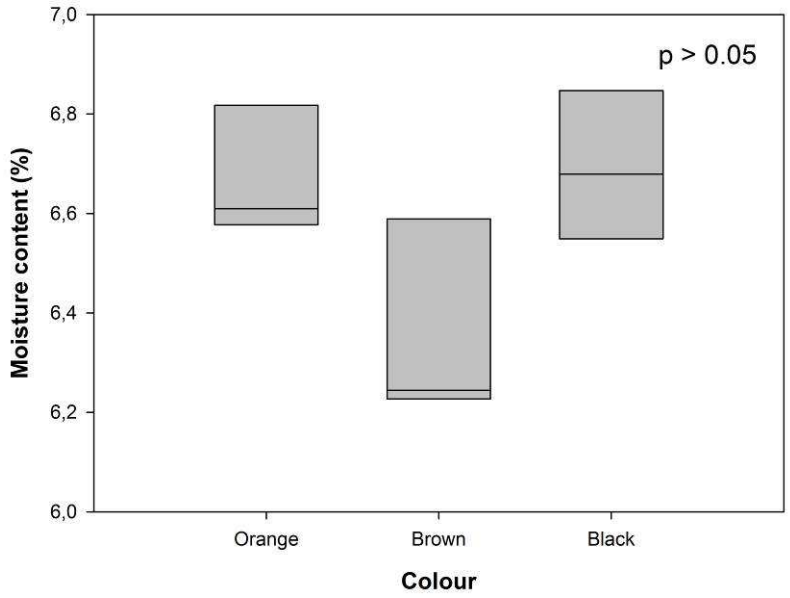


Figure 12 - Mean moisture content (MC), calculated by weighing three replicates of 100 seeds each for each chromatic category. A one-way ANOVA was conducted in order to identify differences among colours. Values were not significantly different at p > 0.05.

Seed germination

For both black and orange seeds, no differences were detected among germination percentages at different temperatures. For brown seeds, temperature was significantly different among temperatures (Figure 13). The highest germination percentage was detected at 5°C (53.3 ± 18.9%) and this value was without statistically significant differences ($p > 0.05$) only with that at the alternating temperature regime of 25/10°C (36.7 ± 15.3%; Figure 13). The lowest germination percentages were detected at 15°C, 20°C and 25°C (ca. 20.0%) and did not show significant differences ($p > 0.05$) among themselves. Germination at 10°C (25.0 ± 5.0%) was not statistically different from that detected at 15°C, 25°C and 25/10°C (Figure 13).

At 5°C, no differences were detected on seed germination among colours, with percentages ranging from 46.7 ± 2.9% to 56.7 ± 23.1% for black and orange seeds, respectively (Figure 13). At 10°C, final germination differed among chromatic categories and germination percentages of

orange seeds (38.3 ± 7.6%) was significantly different ($p < 0.05$) from that of black seeds (11.7 ± 10.4%) but statistically similar ($p > 0.05$) with that detected from brown seeds (25.0 ± 5.0%). At 15°C, no differences were detected among germination percentages of different colours, ranging from 11.7 ± 5.8% to 33.3 ± 15.3% for black and orange seeds, respectively (Figure 13). At 20°C, germination percentages differed among colours and the higher values were detected for orange seeds (30.0 ± 5.0%), significantly different ($p < 0.05$) from those of other two chromatic categories (ca. 15.0%, for both black and brown seeds; Figure 13). At 25°C, no differences were detected among colours, with percentages ranging from 11.7 ± 7.6% to 21.7 ± 11.5%, for black and brown seeds, respectively. At the alternating temperature regime of 25/10°C, significant differences ($p > 0.05$) were not detected among germination percentages of black (10.0 ± 5.0%), orange (28.3 ± 7.6%) and brown (36.7 ± 15.3%) seeds (Fig. 13 and 14).

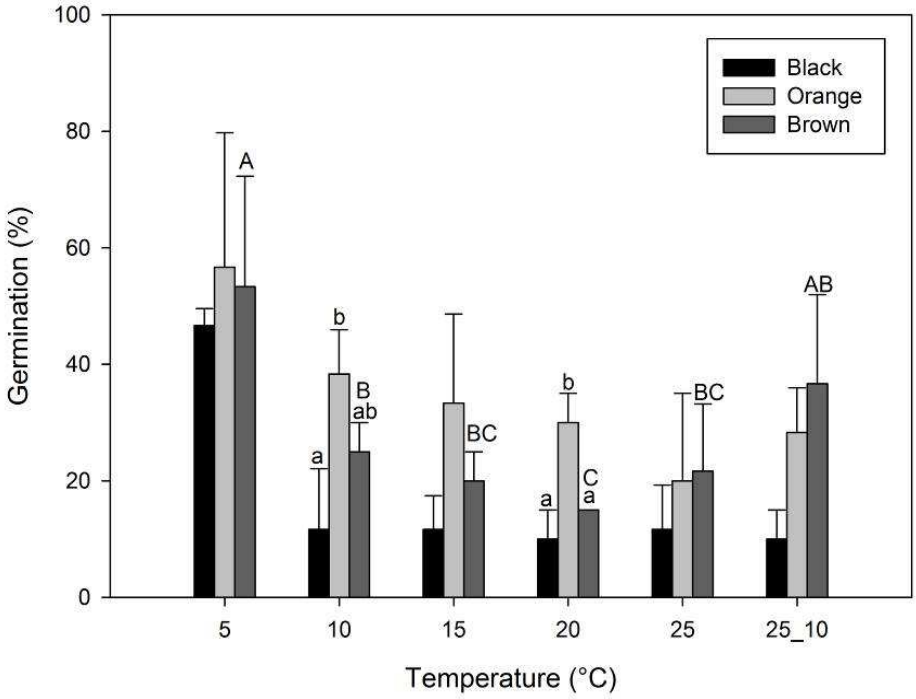


Figure 13 - Final germination percentages at each temperature regime for fresh seeds in the light (12/12 h) for the three chromatic categories of seed lot Br1 collected in 2010. Kruskal-Wallis tests were conducted to detect the effect on germination of the same colour at different temperatures (capital letters, by Mann Whitney U-test) and of temperature among colours (lower-case letters, by Mann Whitney U-test). Values with different letters were used to indicate significant differences at $p < 0.05$ (Mann Whitney U-test).

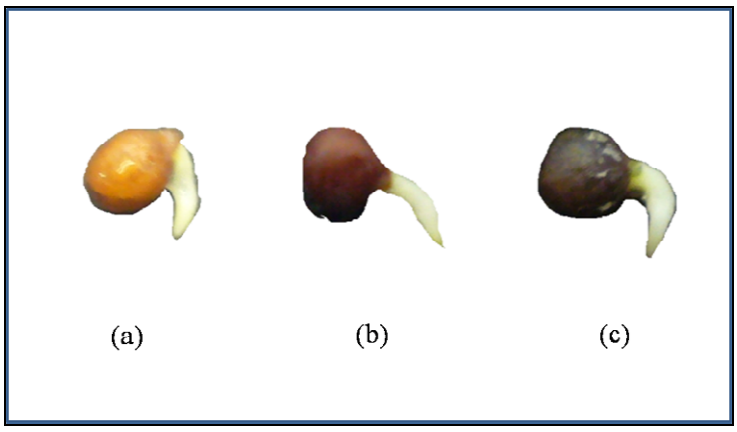


Figure 14 - Germinated seed of *B. insularis* for each chromatic category: orange (a), brown (b) and black (c).

NaCl stress and recovery on seed germination

Effect of temperature

Seed germination significantly decreased ($p < 0.05$) with increasing temperatures and salinity concentrations. At all tested temperatures, the higher germination percentages were detected in non-saline conditions (control, 0 mM NaCl) and at 100 mM (Figure 15 and Table 4). Under control conditions final germination ranged from 8.3 ± 10.4% (5°C) to 85.0 ± 13.2% (25/10°C), while at 100 mM, germination percentages ranged from 0% (5°C) to 53.3 ± 2.9% at 10°C (Figure 15 and Table 4). At 200 mM, germination occurred only at 15°C, with 1.7 ± 2.9% of germinated seeds. At NaCl concentrations higher than 200 mM, germination was totally inhibited.

At all temperatures, recovery response among temperatures significantly decreased ($p < 0.05$ at 10°C and 25°C and $p < 0.01$ at 5°C, 15°C and 25/10°C) with increasing NaCl concentrations to which seeds were exposed in the previous NaCl experimental phase. Significant differences ($p < 0.05$) were detected on RP among temperatures at the same NaCl concentration. At all salinities the highest RP was detected at 5°C (ranging from 30.0 ± 5.0 at 400 mM to 85.0 ± 5.0% at 100 mM), whereas the lowest at 25°C (ranging from 0% at 400 mM to 28.3 ± 14.4% at 200 mM). At 500 mM, only 1.7 ± 2.9% of seeds recovered, while no recovered seeds were observed at 600 mM.

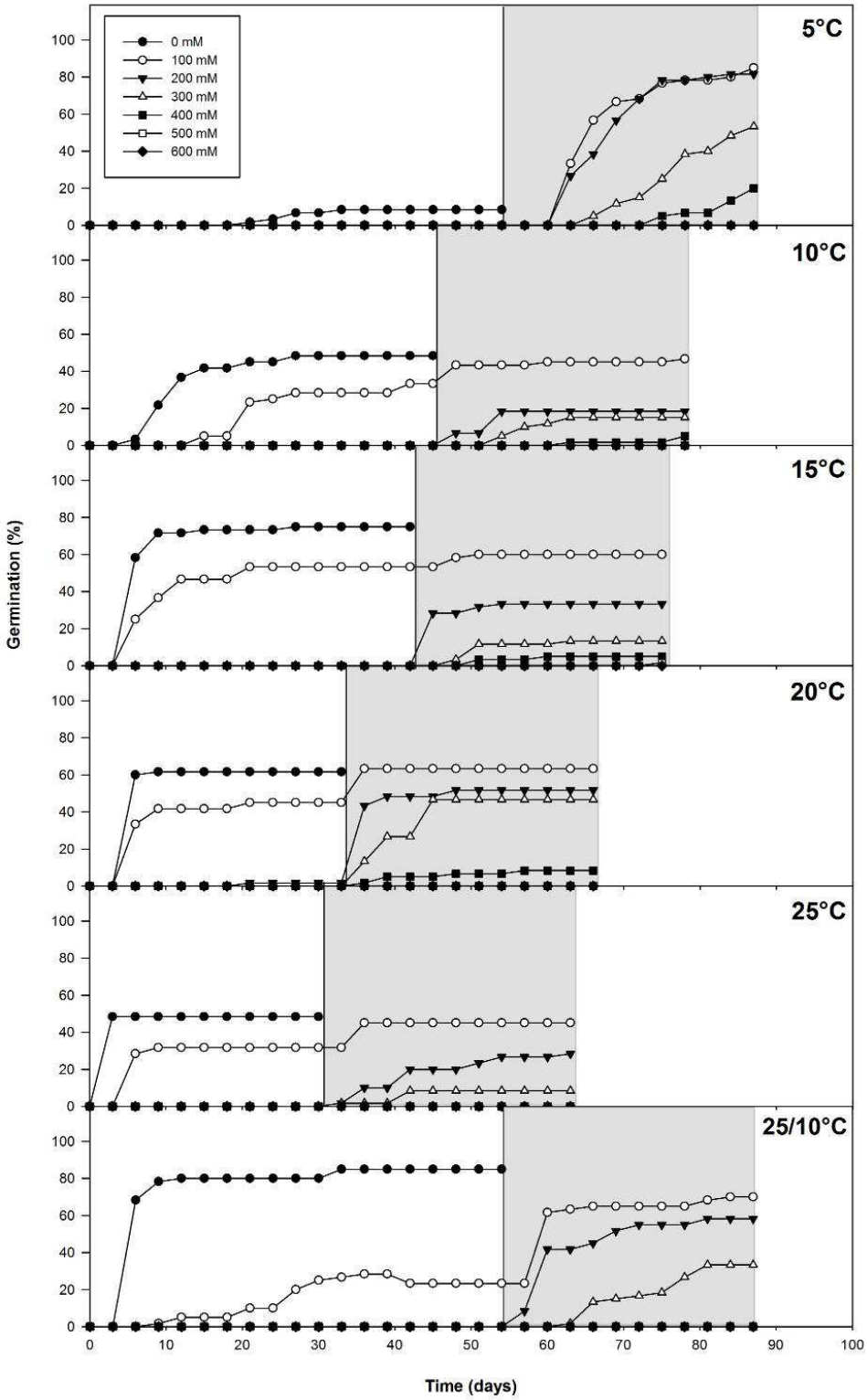


Figure 15 - Cumulative germination percentages at the tested constant temperatures (5-25°C) and at the alternating temperature regime (25/10°C), under different saline conditions (0-600 mM NaCl) and following transfer to distilled water (recovery, indicated by the shaded area in the graph) for *B. insularis*. Each point represents the mean (± 1 SD) of three replicates.

Table 4 - Germination and recovery (RP) percentages at each temperature regime, at different saline conditions (0-600 mM NaCl) for *B. insularis* (seed lot Br1, collected in 2011; see Table 2). Kruskal-Wallis tests were conducted to detect the effect of the same temperature on germination percentages and RP and that of the same salinity on germination percentages and RP; [p values were considered not significantly different ($p > 0.05$, ns), significantly ($p < 0.05$, *; $p < 0.01$, **), by Kruskal-Wallis test]. Data are the means (± 1SD) of three replicates. Capital letters in columns are related to the same salinity, while lower-case letters in rows to the same temperature. Values with different letters were used to indicate significant differences at $p < 0.05$ (Mann Whitney U-test).

Temperature (°C)	Percentage (%)	NaCl concentration (mM)							
		0	100	200	300	400	500	600	
5	Germination	8.3 ± 10.4^{A}	0^{A}	0	0	0	0	0	ns
	Recovery (RP)	-	85.0 ± 5.0^{aA}	81.7 ± 5.8^{aA}	66.7 ± 5.8^{bA}	30.0 ± 5.0^{cA}	0^{d}	0^{d}	**
10	Germination	45.0 ± 13.2^{aB}	33.3 ± 12.6^{aB}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	*
	Recovery (RP)	-	20.9 ± 7.6^{aBC}	18.3 ± 2.9^{aB}	15.0 ± 5.0^{abB}	5.0 ± 5.0^{bcBC}	0^{c}	0^{c}	*
15	Germination	75.0 ± 8.7^{aC}	53.3 ± 2.9^{bC}	0^{c}	0^{c}	0^{c}	0^{c}	0^{c}	**
	Recovery (RP)	-	14.4 ± 6.7^{abB}	33.3 ± 12.6^{bBC}	13.3 ± 5.8^{abB}	6.7 ± 2.9^{acB}	1.7 ± 2.9^{cd}	0^{d}	**
20	Germination	61.7 ± 5.8^{aB}	45.0 ± 5.0^{bBC}	1.7 ± 2.9^{c}	0^{c}	0^{c}	0^{c}	0^{c}	**
	Recovery (RP)	-	23.5 ± 12.1^{acBC}	49.1 ± 1.5^{bD}	46.7 ± 11.5^{bcAC}	8.3 ± 5.8^{adB}	0^{d}	0^{d}	**
25	Germination	48.3 ± 17.5^{aB}	31.7 ± 20.2^{aBC}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	**
	Recovery (RP)	-	16.7 ± 15.7^{aBC}	28.3 ± 14.4^{aBC}	8.3 ± 7.7^{abB}	0^{bC}	0^{b}	0^{b}	*
25/10	Germination	85.0 ± 13.2^{aC}	30.0 ± 13.2^{bB}	0^{c}	0^{c}	0^{c}	0^{c}	0^{c}	**
	Recovery (RP)	-	43.6 ± 24.0^{abC}	61.7 ± 20.2^{aACD}	40.0 ± 8.7^{bC}	1.7 ± 2.9^{cBC}	0^{c}	0^{c}	**
	Germination	*	*	ns	ns	ns	ns	ns	
	Recovery (RP)	-	*	*	*	*	ns	ns	

Seed mortality

Figure 16 shows the estimate of the relationship between NaCl concentration and seed mortality percentages at different temperatures. At temperatures between 5°C and 20°C and at the alternating temperature regime of 25/10°C, the regression lines showed that mortality significantly increased ($p < 0.05$ for 5°C and $p < 0.01$ for all other temperatures) with NaCl concentrations and temperatures (with r^2 ranging from 0.67 of 5°C to 0.85 of 20°C, while 0.84 for 25/10°C). At 25°C, significant differences ($p < 0.05$) were showed in the NaCl concentrations range from 0 mM to 400 mM ($p < 0.05$) and the linear regression equation showed highest values of r^2 (0.86), while in the range of salinity between 400 mM and 600 mM, regression was not significant ($p > 0.05$) (Figure 16). At 25°C the increase of seed mortality velocity was much greater than that detected at 5°C (with angular coefficient values of straight line of 0.23 and 0.14, for 25°C and 5°C, respectively; Figure 16), showing that seed mortality velocity increased with the increase in temperature.

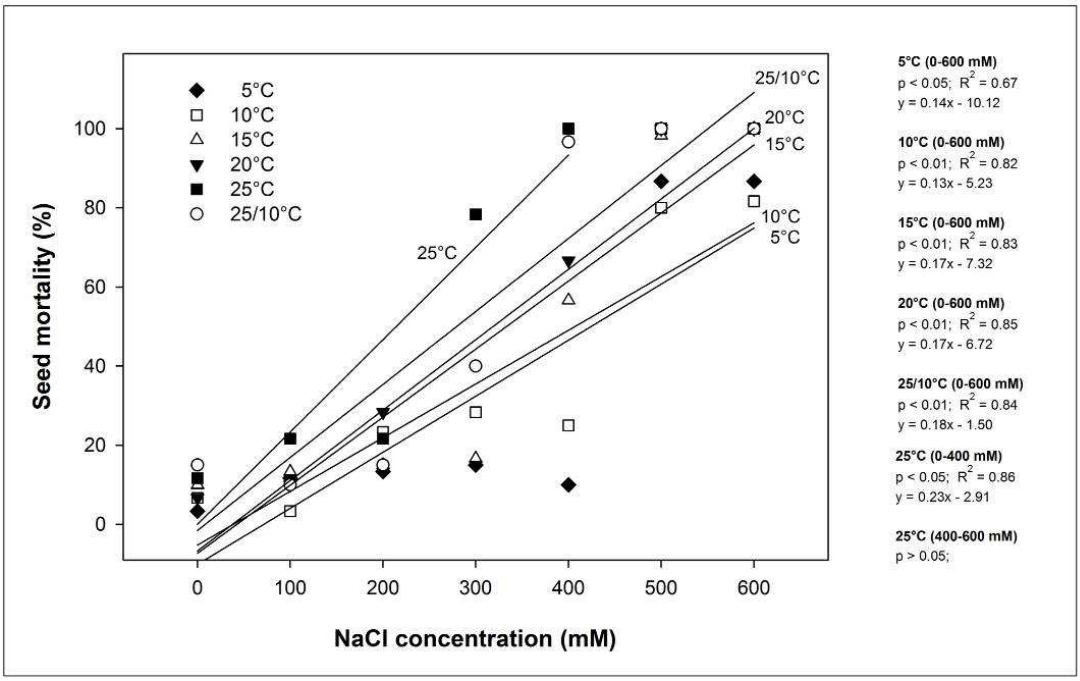


Figure 16 - Mortality of *B. insularis* **seeds at the tested NaCl concentrations (0-600 mM) and different temperatures (5-25°C; 25/10°C). Black lines indicate linear regressions for each temperature. Each symbol is the mean of three replicates.**

Inter-population variability

At 15°C, under control conditions (0 mM NaCl), no differences were detected on seed germination among populations, with percentages ranging from 68.3 ± 5.8% (Br2) to 80.0 ± 8.7% (Br3) (Table 5). At 200 mM germination percentages of the three populations significantly differed ($p < 0.05$) and the highest value was detected for Br3 (51.7 ± 7.7%), while no germination occurred for Br1 and only 10.0 ± 5.0% of germinated seeds were observed for Br2 (Table 5). At 400 mM and 600 mM, germination was totally inhibited for all populations (Table 5). For Br1, each salinity concentration inhibited germination. For Br2, final germination significantly decreased ($p < 0.05$) compared with control conditions and was inhibited at NaCl concentrations higher than 200 mM. For Br3, the trend was similar to that observed for Br2, but with higher germination percentages at 200 mM, while concentrations higher than 200 mM inhibited germination.

For RP, at 200 mM, recovery differed among populations, with the highest values detected for Br2 and Br3 (85.0 ± 3.9% and 62.6 ± 19.6%, respectively; Table 5). These values were statistically similar ($p > 0.05$) between themselves, but significantly different ($p < 0.05$) from Br1 (33.3 ± 12.6%; Table 5). At 400 mM, recovery response showed differences among populations and the highest RP values were detected for Br2 and Br3 (80.0 ± 5.0% and 88.3 ± 5.8%, respectively), while only 6.7 ± 2.9% of seeds recovered for Br1 (Table 5). At 600 mM, Br3 showed the highest RP (61.7 ± 7.7%) and values of all populations were significantly ($p < 0.05$) different from each others (10.0 ± 8.7% for Br2 and 0% for Br1; Table 5).

For Br1, recovery response significantly ($p < 0.05$) differed among all concentrations and was totally inhibited at 600 mM (see data cited above and Table 5). For Br2, differences were detected among RP, although percentages at 200 mM and 400 mM showed statistically similar ($p > 0.05$) values between themselves and RP at 600 mM significantly differed from all other. For Br3, no differences were detected and RP values without significant differences ($p > 0.05$) among NaCl concentrations (see Table 5).

At 25/10°C, at 0 mM, final germination was statistically similar ($p > 0.05$) among populations with values of ca. 90.0% for all populations. At 200 mM, no differences were detected among populations, although 1.7 ± 2.9% of seeds germinated for Br2 and 18.3 ± 5.8% for Br3, while none germinated seed was observed for Br1 (see Table 5). At 400 mM and 600 mM, germination was inhibited for all populations (see Table 5). For Br1 differences were detected although germinated seeds were observed only under control conditions (85.0 ± 13.2%). For Br2, final germination considerably decreased between control conditions and 200 mM (see Table 5), while for Br3 significant ($p < 0.05$) differences were detected among NaCl concentrations and the trend was the same observed for Br2 (see Table 7). For RP, at 200 mM, differences were detected among populations and the higher RP were detected both for Br2 and Br3 populations (ca. 94.0%; see Table 5) and these values were statistically ($p < 0.05$) different from RP of Br1 (61.7 ± 20.2%) (see Table 5). At 400 mM, recovery response showed differences among populations and RP of Br2 and Br3 were without significant differences ($p > 0.05$) between themselves (ca. 80.0%), but significantly ($p < 0.05$) different from that detected for Br1 (1.7 ± 2.9%). At 600 mM, recovery was totally inhibited both for Br1 than Br2 and this condition showed significant differences with RP detected for Br3 population (28.3 ± 17.6%). For Br1, differences were detected among NaCl concentrations and the higher values at 200 mM significantly ($p < 0.05$) differed from all other RP, while values detected at 400 mM and 600 mM (0%) were similar between themselves (see

Table 5). For Br2, recovery response showed differences among salinities and the higher values at 200 mM and 400 mM were statistically similar ($p > 0.05$) between themselves but significantly different ($p < 0.05$) from that detected at 600 mM, concentration for which no recovery occurred (see Table 5).

Table 5 - Inter-population variability of *B. insularis* in response to NaCl for germination and recovery percentages (RP) at 15°C and 25/10°C. Kruskal-Wallis tests were conducted to detect the effect of the same temperature on germination percentages and RP and that of the same salinity on germination percentages and RP; [p values were considered not significantly ($p > 0.05$, ns), significantly ($p < 0.05$, *; $p < 0.01$, **) different, by Kruskal-Wallis test]. Data are the means (± 1 SD) of three replicates. Capital letters in columns are related to the same salinity, while lower-case letters in rows to the same temperature. Values with different letters were used to indicate significant differences at $p < 0.05$ (Mann Whitney *U-test*). For each population, the code is the same of Table 1.

Temperature (°C)	Population	Percentage (%)	NaCl concentration (mM)				
			0	200	400	600	
15	*Br1*	Germination	75.0 ± 8.7^{a}	0^{bA}	0^{b}	0^{b}	**
		Recovery (RP)	-	33.3 ± 12.6^{aA}	6.7 ± 2.9^{bA}	0^{cA}	*
	Br2	Germination	68.3 ± 5.8^{a}	10.0 ± 5.0^{bB}	0^{c}	0^{c}	**
		Recovery (RP)	-	85.0 ± 3.9^{aB}	80.0 ± 5.0^{aB}	10.0 ± 8.7^{bB}	*
	Br3	Germination	80.0 ± 8.7^{a}	51.7 ± 7.7^{bC}	0^{c}	0^{c}	**
		Recovery (RP)	-	62.6 ± 19.6^{B}	88.3 ± 5.8^{B}	61.7 ± 7.7^{C}	ns
		Germination	ns	*	ns	ns	
		Recovery (RP)	-	*	**	**	
25/10	*Br1*	Germination	85.0 ± 13.2^{a}	0^{b}	0^{b}	0^{b}	*
		Recovery (RP)	-	61.7 ± 20.2^{aA}	1.7 ± 2.9^{bA}	0^{bA}	*
	Br2	Germination	91.7 ± 2.9^{a}	1.7 ± 2.9^{b}	0^{b}	0^{b}	*
		Recovery (RP)	-	93.1 ± 3.2^{aB}	86.7 ± 7.7^{aB}	0^{bA}	**
	Br3	Germination	91.7 ± 7.7^{a}	18.3 ± 5.8^{b}	0^{c}	0^{c}	*
		Recovery (RP)	-	96.1 ± 3.4^{aB}	73.3 ± 20.8^{aB}	28.3 ± 17.6^{B}	*
		Germination	ns	ns	ns	ns	
		Recovery (RP)	-	*	**	*	

Salt spray on seedling development

Seedling survival

For the coastal Br3, at the end (after eight week) of salt spray treatment, 100% of seedlings survived under control condition (no spray), as well as when nebulized one- and two days per week. When the frequency of applied salt spray was three days per week, 92.3% of seedling survived (see Figure 17).

For the inland Br4, survival was inversely correlated with frequency of nebulization: 92.3% of seedlings survived without salt spray nebulization (control), 46.1% when sprayed one day per week, only 15.4% with a frequency of two days per week and no one (0%) with a three days per week nebulization (Figure 17).

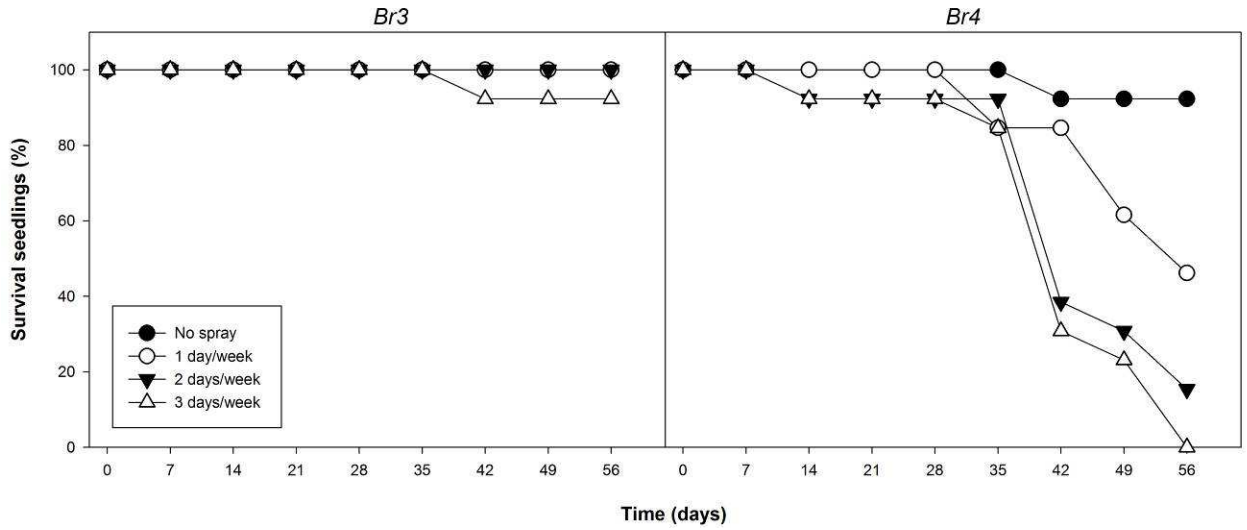


Figure 17 - Survival of *B. insularis* **seedlings from a coastal (Br3) and an inland population (Br4), for each treatment (no spray, one-day/week, two-days/week, three-days/week), during eight weeks of salt spray solution (600 mM NaCl) nebulization.**

Dry weight, epigeal and hypogeal length

For the coastal Br3, the one-way ANOVA showed a non significant ($p > 0.05$) effect of frequency of nebulization on seedlings mean dry weight (Fig. 18), with values of ca. 0.08 g for each seedling (see Figure 20A). For the inland population Br4, the one-way ANOVA showed the non significant ($p > 0.05$) effect of frequency among different nebulization treatments and values were of ca. 0.03 g for each seedling (see Figure 20A).

Figure 18 - Preparation of *B. insularis* **seedlings for dry mass measure (on the left) and dry mass measure with precision balance (four decimal places) (on the right).**

For population Br3, the one-way ANOVA showed a significant ($p < 0.05$) effect of frequency of salt spray nebulization on length of epigeal part of seedlings, in particular the increase of frequency caused a significant decrease of seedlings height (Fig. 19). The length of epigeal part (Figure 19) for three-days per week nebulized seedlings was significantly lower (104.6 ± 4.9 mm) than that of control condition or one-day per week nebulization (ca. 128.0 ± 6.0 mm, for both frequencies). Seedling heights with a frequency of two days per week was similar (116.5 ± 7.0 mm) to values detected for all frequencies (see Figure 20B). For population Br4, the one way ANOVA showed the non significant effect ($p > 0.05$) of salt spray frequency on seedlings height,

with lengths ranging from 76.5 ± 0.8 mm to 96.1 ± 5.4 mm (for two-days per week frequency and control conditions, respectively; Figure 20B).

For population Br3, the one-way ANOVA showed a significant ($p < 0.05$) effect of frequency of nebulization on hypogeal length of the seedlings. Root lengths of no sprayed and two- days per week sprayed seedlings were statistically similar ($p > 0.05$; ca. 22.5 ± 6.9 mm for both), while lengths of hypogeal parts of seedlings nebulized one way per week and three days per week were significantly different ($p < 0.05$; 25.7 ± 6.5 mm and 17.9 ± 4.9 mm, respectively; Figure 20C). For population Br4, the one-way ANOVA did not show significant differences ($p > 0.05$) and values of roots lengths were of ca. 19.0 mm for all frequencies (Figure 20C).

Figure 19 - Seedlings measurements with digital caliper.

During the salt spray experiment, conductivity values of substrate at the end of the experiment (eight weeks) significantly ($p < 0.05$) differed among frequencies of salt spray nebulization, ranging from 47.2 ± 7.8 mM to 272.4 ± 7.6 mM, for control conditions (no spray)

and three-days/week sprayed pots, respectively. pH values significantly ($p < 0.05$) differed among frequencies of salt spray treatment, ranging from 4.8 ± 0.1 to 4.6 ± 0.1, for no sprayed and three-days/week nebulized pots, respectively. No statistical correlation ($p > 0.05$) was found between these soil parameters and seedling growth data for both populations.

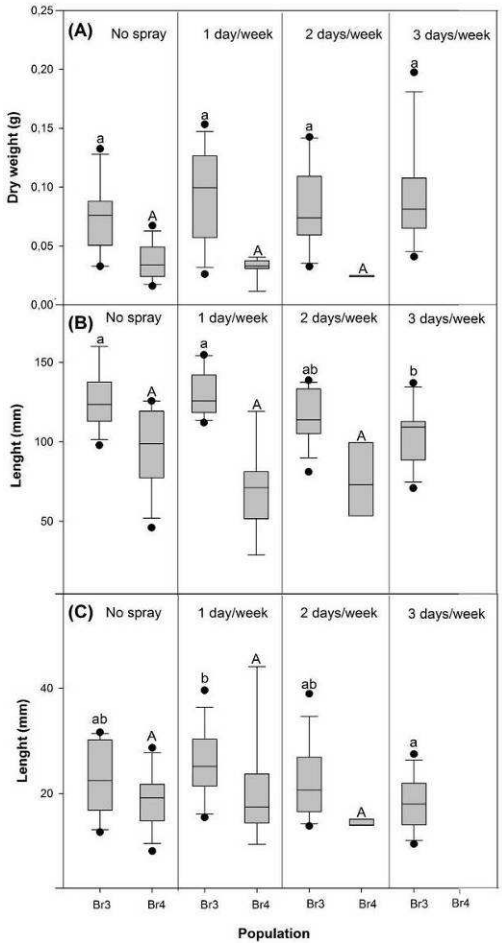


Figure 20 - Dry weight (A) and epigeal (B) and hypogeal lengths (C) of *B. insularis* seedlings from a coastal and an inland population, (Br3 and Br4, respectively), for each treatment (no spray, one-day/week, two-days/week, three-days/week), after eight weeks of salt spray solution (600 mM NaCl) nebulization. A one-way ANOVA was conducted, for each population, to detect the effect of treatment on seedlings growth. Different letters were used to indicate significant values at $p < 0.05$ (Fisher's LSD post hoc test). Lower-case letters were used for Br3, while capital letters for Br4. For each treatment, data are the mean of survived seedlings after eight weeks from the beginning of experiments.

Discussion

The morpho-colorimetric analysis, conducted on the three *B. insularis* populations, revealed high inter-population variability, with very low error percentages, showing an high differentiation among populations. This result, shows that also considerably near populations (only ca. 8 km between Br2 and Br3) have morphologically seed differentiation. This pattern is probably due of the presence of some ecologic barriers, which may obstacle inter-population gene flow. Phenotypic traits under genetic control often vary within and among populations of a species (Ramakrishnan *et al*., 2004). The extreme variability of *Brassica* populations is well known and this phenomenon has determined in some cases the description of several new *taxa* belonging to this genus, growing in short distances as, for example, in Sicily (Raimondo *et al*., 1991; Raimondo, 1997; Raimondo & Mazzola, 1997; Raimondo & Geraci, 2002; Scialabba *et al*., 2003; Raimondo *et al*., 2012).

B. insularis seeds achieved similar germination percentages both in the light and in the dark, therefore they are not photo-inhibited for germination, contrary to other coastal species, such as *Spinifex hirsutus* (Harty & McDonald, 1972), *Glaucium flavum* (Thanos *et al*., 1989), *Allium staticiforme*, *Brassica tournefortii*, *Cakile maritima*, *Otanthus maritimus* (Thanos *et al*., 1991), *Matthiola tricuspidata* (Thanos *et al*., 1994), *Crucianella maritima* (Del Vecchio *et al*., 2012). This germination pattern was confirmed by the seed mass of *B. insularis* (> ca. 3 mg) as species with seed < 0.1 mg in weight are largely light-requiring for germination and the incidence of light-dependence declines with increasing seed size (Grime *et al*., 1981; Pearson *et al*., 2002).

Low germination (≤ 60%) was observed at all tested conditions for fresh seeds of all populations sampled in 2010. The inter-population variability identified through morpho-colorimetric analysis was also observed in germination behavior. Br2 was the only population for which a preference of temperature (15°C) was detected, while, except for the high germination

percentages detected at 5°C for Br1, for this population and for Br3, none temperature was preferred respect to others.

The application of the dry after-ripening treatment had not a significant effect on *B. insularis* seed germination, although inter-population differences were detected. Therefore, *B. insularis* seeds do not need of a dry summer period which forego germination. The variation in germination behavior that occurs among different populations within the same species has been reported by several researchers (Pérez-García, 1993; Martin *et al*., 1995; Pérez-García *et al*., 1995; Beckstead *et al*., 1996; Andersson & Milberg, 1998; Meyer & Allen, 1999; Qaderi & Cavers, 2000a, b). These differences can arise from both environmental variations in light, moisture, temperature, and nutrients (Fenner, 1991; Gutterman, 1992; Perèz-Garcìa *et al*., 2003) and genetic factors (Meyer *et al*., 1989).

For Br1 seeds collected in 2010, heteromorphy was observed and morpho-colorimetric analysis identified three different seed testa colours (orange, brown and black). This phenomenon is largely documented for several species of the genus *Brassica*, but to our knowledge it has never been reported for *B. insularis*. Orange seeds showed a significant lower seed mass respect to both brown and black seeds. Therefore, it would be reasonable to assume that the different colours could be attributable to different seed maturation phases. Moisture content reduction in seeds is initiated during maturation of seeds (Mayer & Poljakoff-Mayber, 1989; Bewley & Black, 1995). When the seeds are subjected to higher temperatures, progressive removal of water occurs. During development, orthodox seeds (such as *B. insularis* seeds) pass through three distinct phases: embryogenesis, active biosynthesis of reserve material leading to a rapid increase in seed fresh and dry weight, and seed maturation when dry weight accumulation ceases and fresh weight declines markedly in dry dehiscent fruit types (Welbaum & Bradford, 1989; Daws *et al*., 2004). However, the three chromatic categories had the same water content and therefore it can be

assumed that they have also the same maturation stage. The maturation stage of seeds may influence germination behavior (Baskin & Baskin, 1998). Germination requirements and percentages of immature seeds may be different from those of mature seeds of the same species (Baskin & Baskin, 1998). Germination of the three chromatic *B. insularis* seed categories did not show particular differences among different colours, confirming moisture content measure results. Many different species of angiosperms show seed heteromorphy and differential germinative response of seeds with different form, size or testa colour within a species has been reported (Khan & Ungar, 1984; Imbert, 2002; Mira *et al*., 2011), expecially in Brassicaceae, Caryophyllaceae and Chenopodiaceae (Matilla *et al*., 2005). Mira *et al*. (2011) reported for *Silene diclinis* the production of three chromatic seed categories (red, grey and black) in the same fruit and demonstrate that seed water content did not show differences among colours as well as seed dormancy release and controlled seed ageing were not related to seed colour in that species. The heteromorphy phenomenon in *B. insularis* seeds seems to be more likely a physical seed testa property than an eco-physiologic differentiation. Seed coat colour in *Brassica* was found to vary from yellow to brown with intermediate shades (Liu *et al*., 2005). Various groups of researchers have studied the inheritance of seed colour in *Brassica* species (Sabharwal *et al*., 2004) and showed that seed heteromorphy in this genus is under genetic control (Mohammad *et al*., 1942; Vera *et al*., 1979; Rahman *et al*., 2001). In this study, seed heteromorphy was observed only in 2010 for Br1 seeds, which is clearly the population farthest and isolated from others, growing on a little island (Isola dei Cavoli - SE Sardinia). It may be argued that the geographical isolation of this population may have determined independent evolutionary divergence processes respect to the other Sardinian populations. Even so, more information on the chemical properties of seed testa and compared genetic analysis on plant individuals of “Isola dei Cavoli” population would be needed in future studies on this species, in order to address the reason for colour differences and

the occurrence of the heteromorphy phenomenon only in this population. However, this phenomenon is not constant as seeds collected in other years from the same population and stored at the Sardinian Germplasm Bank (BG-SAR) are heteromorphic. The occurrence of different seed colours production among years in function of plant growth temperature may be a reason of the non-constant occurrence of heteromorphy in Br1 seeds, probably related to inter-annual climatic aspects, as previously observed for *B. napus* by Van Deynze *et al*. (1993).

B. insularis seeds germinated with NaCl in the substrate up to 200 mM of concentration, although in salt substrate were observed lower germination percentages than under control conditions (0 mM NaCl). Many studies reports that percentages of germination decreased with increased salinity stress and highest germination occurs in absence of NaCl in the substrate (Khan & Ungar, 1984; El-Keblawy *et al*., 2010; Vallejo *et al*., 2010).

For *B. insularis*, recovery showed a good performance only at lowest temperature of 5°C, although at all temperatures recovery performances decreased progressively and inversely proportionally to concentration which seeds were exposed in the previous NaCl experimental phase. For example, at 400 mM recovery response reached the highest values at 5°C (ca. 30%), while at all other temperatures did not exceed 6.7% at this concentration. At NaCl concentrations higher than 400 mM, recovery was unsuccessful or minimal at all temperatures. At high temperatures, salinity exposure could result in a loss of viability and consequently, poor recovery response (Ungar, 1982). Salinity-temperature interactions may have significant eco-physiological implications in terms of time of germination under field conditions (Ungar, 1982). *B. insularis* seeds mortality in NaCl was highly influenced by temperature, in particular at 400 mM, the highest tested temperature of 25°C amplified the negative effect of salinity on seed viability (with mortality of 100%). At each tested temperature, seed mortality increased proportionally with NaCl concentration and the increase of temperature increased seed mortality velocity affecting

irreversibly seed viability, probably through ion toxicity. At 500 and 600 mM, *B. insularis* seeds did not tolerate the high concentrations to which were exposed and mortality percentages were not lower than 80%, independently from temperature, demonstrating high sensibility for seeds of this species to highest NaCl concentrations. The capability of a species to tolerate high salinities is reflected both on the maximum salt concentration at which seeds may germinate, both to tolerate high salinity concentrations and then to have recovery after this NaCl exposure (Ungar, 1982). Several studies reported that salt stress negatively affected seed germination, with consequent seed mortality, either osmotically (through reduced water absorption) or ionically (through the accumulation of Na^+ and Cl^-), causing an imbalance in nutrient uptake and toxicity effect (Li, 2008; Shokohifard *et al.*, 1989).

Intra-specific variability in germination patterns has been reported for several species and investigated in various studies (Bischoff *et al*., 2006; Kremer *et al*., 2009; Bischoff & Müller-Schärer, 2010). Differences in salt stress response were showed among populations of *Panicum turgidum* (El-Keblawy *et al*., 2010) and *Spartina patens* (Hester *et al*., 1996), but not in *Crucianella maritima* (Del Vecchio *et al.*, 2012). Different levels of salt stress tolerance of *B. insularis* seeds in saline conditions and variability in recovery response were detected among populations. Moreover, germination percentages under control condition (0 mM NaCl) for seeds from the three tested populations collected in 2010 were significantly lower than those detected in 2011 and tested under the same conditions from the same populations. Variations in seed germination among different years has been studied through several researches (Urbanska & Schütz, 1986; Chambers, 1989; Gutterman, 1994b; Kigel, 1995; Beckstead *et al*., 1996). The variability of germination characteristics could be interpreted as one of the most important survival strategies for species growing under unpredictable environmental conditions (Gutterman, 1994a; Kigel, 1995) and will reduce the risk of seedlings being subjected to poor growing

conditions due to the establishment of intense competition hierarchies (Giménez-Benavides *et al*., 2005).

Salt spray experiment highlighted an high inter-population variability for *B. insularis* seedlings in marine aerosol tolerance. Seedlings of the inland (with a distance from the sea > 6.0 km) population Br4 showed high sensibility to salt spray, resulting in a drastic decrease of seedling survival with the increase of the frequency of salt spray nebulization and life span less than the eight weeks. On the contrary, not less than 90% of Br3 seedlings survived also when nebulized with the highest NaCl solution. For both populations, the frequency of nebulization did not influence seedling biomass. For the coastal population the increase in salt spray nebulization frequency caused a reduction in seedling growth, while for the inland population the effect did not influence their growth, but caused seedling death for the highest nebulization frequency. The ability to tolerate and possibly adapt to airborne saltwater sprays may be critical to the maintenance of coastal plant populations (Maun, 1994; Greipsson & Davy, 1996). In coastal communities, the distribution of species can sometimes be tied to their tolerance of salt spray (Sykes & Wilson, 1988; Wilson & Sykes, 1999). This suggests that adaptation to salt spray within species might be detected within subpopulations that are closest to shore, analogous to the way that intra-specific variation in the tolerance of soil salinity has been documented (Rozema *et al*., 1985; Hester *et al*., 1996; Greipsson *et al*., 1997). Given that salt spray deposition levels can vary greatly within a population, depending not only on the proximity to the shore, but also on wind intensity and direction, topography, and the timing of rainfall episodes (Boyce, 1954; Barbour, 1978; Cheplick & Demetri, 1999), plasticity of growth and reproduction may be a viable buffer against the selective elimination of suboptimal genotypes (Sultan, 1987; Rice & Mack, 1991). In our case, the highest tolerance to marine salt spray, detected for the *B. insularis* coastal population, may be due to environmental induced adaptation to this adverse factor, widely present

on coastal cliffs, while the highest sensibility of the inland population reflects the environmental conditions of the population, where the impact of salt spray episodes, if present, is negligible.

In conclusion, our results highlighted the absence of dormancy for *B. insularis* seeds, as reported for other Brassicaceae (Finch Savage & Leubner-Metzger, 2006). However, considering the high inter-population and inter- annual variability detected in this study, this pattern should be considered cautiously. Light, temperature and dry after-ripening did not affect final germination percentages whereas it was totally inhibited when seeds were exposed to high salt concentrations. *B. insularis* seeds showed the capability to germinate up to concentration of 200 mM NaCl, salt affected seed viability and recovery response decreased, proportionally with increase in salinity and temperature. Seed heteromorphy was observed for the first time in this species and only in an isolated population, and this phenomenon may be due to independent evolutionary divergence processes of “Isola dei Cavoli “ population. *B. insularis* seed germination behavior differs from that of other “typical” Mediterranean plants, for which germination at low temperatures is a widely extended trait (Thanos *et al*., 1995), demonstrating that germination of this species may occur in a wide time window during the year. Further studies are need on this species to clarify the causes of seed heteromorphy and to investigate inter-population variability through a higher number of considered populations.

Appendix 1 - List of 36 morpho-colourimetric features measured on seeds, excluding the 78 Elliptic Fourier Descriptors (EFDs) calculated according to Hâruta (2011).

	Feature	Description
A	Area	Seed area (mm^2)
P	Perimeter	Seed perimeter (mm)
P_{conv}	Convex Perimeter	Convex perimeter of the seed (mm)
P_{Crof}	Crofton Perimeter	Crofton perimeter of the seed (mm)
P_{conv}/P_{Crof}	Perimeter ratio	Ratio between P_{conv} and P_{Crof}
D_{max}	Max diameter	Maximum diameter of the seed (mm)
D_{min}	Min diameter	Minimum diameter of the seed (mm)
D_{min}/D_{max}	Feret ratio	Ratio between D_{min} and D_{max}
Sf	Shape Factor	Seed shape descriptor = $(4 \cdot \pi \cdot A)/ P^2$ (normalized value)
Rf	Roundness Factor	Seed roundness descriptor = $(4 \cdot A)/(\pi \cdot D_{max}^2)$ (normalized value)
Ecd	Eq. circular diameter	Diameter of a circle with equivalent area (mm)
EA_{max}	Maximum ellipse axis	Maximum axis of an ellipse with equivalent area (mm)
EA_{min}	Minimum ellipse axis	Minimum axis of an ellipse with equivalent area (mm)
F	Fiberlength	Seed length along the fiber axis
C	Curl degree	Ratio between D_{max} and F
$Conv$	Convessity degree	Ratio between P_{Crof} and P
Sol	Solidity degree	Ratio between A and convex area
Com	Compactness degree	Seed compactness descriptor = $[\sqrt{(4/\pi)}\, A]/ D_{max}$
R_{mean}	Mean red channel	Red channel mean value of seed pixels (grey levels)
R_{sd}	Red std. deviation	Red channel standard deviation of seed pixels
G_{mean}	Mean green channel	Green channel mean value of seed pixels (grey levels)
G_{sd}	Green std. deviation	Green channel standard deviation of seed pixels
B_{mean}	Mean blue channel	Blue channel mean value of seed pixels (grey levels)
B_{sd}	Blue std. deviation	Blue channel standard deviation of seed pixels
H_{mean}	Mean hue channel	Hue channel mean value of seed pixels (grey levels)
H_{sd}	Hue std. deviation	Hue channel standard deviation of seed pixels
L_{mean}	Mean lightness channel	Lightness channel mean value of seed pixels (grey levels)
L_{sd}	Lightness std. deviation	Lightness channel standard deviation of seed pixels
S_{mean}	Mean saturation channel	Saturation channel mean value of seed pixels (grey levels)
S_{sd}	Saturation std. deviation	Saturation channel standard deviation of seed pixels
D_{mean}	Mean density	Density channel mean value of seed pixels (grey levels)
D_{sd}	Density std. deviation	Density channel standard deviation of seed pixels
S	Skewness	Asymmetry degree of intensity values distribution (grey levels)
K	Kurtosis	Peakness degree of intensity values distribution (densitometric units)
H	Energy	Measure of the increasing intensity power (densitometric units)
E	Entropy	Dispersion power (bit)

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Chapter IV - Inter- and intra-specific variability in seed germination requirements and salt stress tolerance of three *Lavatera* L. (sect. *Glandulosae* R. Fern.) species

Abstract

Genus *Lavatera* comprises perennial and annual species growing in different habitat typologies. In this study we investigated seed germination ecology of *L. agrigentina*, *L. triloba* ssp. *pallescens* and *L. triloba* ssp. *triloba*, all of them belonging to the *Lavatera triloba* aggregate (sectio *Glandulosae*). For each species, the effect of mechanical scarification, light and constant (5-25°C) and alternating (25/10°C) temperatures on germination were evaluated, as well as the effect of a dry after-ripening period (90 days at 25°C). The salt stress effect (0-600 mM NaCl) and its recovery on seed germination and seedlings salt spray tolerance were also evaluated for the two coastal *taxa* (*L. pallescens* and *L. triloba*). A combinational dormancy (PY+PD) for *L. agrigentina* and physical (PY) for the two other species were detected. For all the *taxa*, highest germination was reached at low temperatures (e.g. 10°C and 15°C), and light did not affect final germination percentages. Dry after-ripening highly promoted germination only in *L. agrigentina*. *L. pallescens* seeds germinated up to concentration of 600 mM NaCl, those of *L. triloba* up to 200 mM.Salt affected seed viability and recovery response decreased with increases of salinity. Differences were detected in salt spray tolerance between the two *taxa*, with higher sensibility to this abiotic factor for *L. triloba*.

Keywords: dry after-ripening, *Lavatera triloba* aggregate, Mediterranean flora, NaCl, recovery, salt spray, scarification.

Introduction

Successful germination is crucial in the life cycle of terrestrial angiosperms and dormancy is an innate seed property that defines the environmental conditions in which the seed is able to germinate (Baskin & Baskin, 1998). Seed dormancy is defined as an intrinsic block to the completion of germination of a viable seed under favorable conditions for germination such as temperature, humidity, light, etc. (Finch-Savage & Leubner-Metzger, 2006). Seed dormancy associated with the seed embryo and caused by a physiological inhibiting mechanism, which prevents embryo growth and seed germination until chemical changes occur, is called physiological dormancy (PD; Baskin and baskin, 1998). A water-impermeable seed coat can be cause of a physical dormancy (PY), which develops during maturation drying of the seed (Van Staden *et al*., 1989) and prevention of water uptake causes the seed to remain dormant until some factors render the covering layers permeable to water (Baskin *et al*., 2000), while combinational dormancy can be observed when physical dormancy is associated to physiological dormancy (PY + PD; Baskin & Baskin, 1998, 2004; Finch-Savage & Leubner-Metzger, 2006).

Several environmental factors, including light, moisture, temperature, soil composition and distance from the sea, can determine differences in germination behavior and cause specific adaptations also in phylogenetically related species (e.g. of the same genus; Ellison, 2001). Differences or similarities among closely related *taxa* in seed dormancy and germination preferences may explain different environmental adaptations and changes (Pérez-Garcìa *et al*., 2006). Several studies highlighted the presence of intra-specific variation (inter-population

variability) in germination and dormancy (Baskin & Baskin, 1998; Andersson & Milberg, 1998; Keller & Kollman, 1999). The inter-population variability in germination can be due to environmental differences or to genetic variations (Fenner, 1991; Gutterman, 1992; Kigel, 1995) while inter-population variability in seed dormancy can serve as an adaptive strategy in unpredictable environments (Cohen, 1968; Cruz *et al*., 2003).

Seed drying under warm temperatures (dry after-ripening) is a natural mechanism that controls dormancy in dry climates (Finch-Savage *et al*., 2007). A period of usually several months of dry storage at room temperature of freshly harvested, mature seeds is a common method used in laboratory to mimic this mechanism and release seed dormancy (Bewley, 1997). Coastal plants are exposed to frequent fluctuations of salinity levels in relation to seasons, also depending on their distance from the sea (Weber and D'Antonio, 1999). Salt stress affects plant growth at various stages of development including germination and establishment, vegetative growth and reproduction (Ungar, 1995, Katembe *et al*., 1998). An increase in salinity can induce a reduction in the percentage of germinating seeds as well as a delay in the initiation of the germination process (Ungar, 1995). It can also causes a complete inhibition of the germination process at NaCl concentrations beyond the tolerance limits of the species (Pujol *et al*., 2000). When salinity stress is reduced, partial to complete germination recovery has been observed for several species (Khan, 2003). The ability to tolerate and possibly adapt to airborne saltwater spray may be critical to the maintenance of coastal plant populations (Boyce, 1954; Cheplick & White, 2002). The total concentration of salt deposited on coastal plants as salt spray is influenced by the physical factors of impact deposition (Maun, 2009).

In the Mediterranean area, several species of Malvaceae occur both in coastal and inland habitats; in particular the genus *Lavatera* comprises perennial and annual species growing in different habitats, such as coastal cliffs, open habitats, endorheic lagoons, ditches, etc. and on

various substrates (limestones, chalkyes, clays, saline sediments; Fernandes, 1968; Bacchetta *et al*., 2011). The *Lavatera triloba* aggregate is a monophyletic group of perennial herbs or sub-shrubs endemic to the Western Mediterranean region (Escobar Garcìa *et al*., 2009). The three *taxa* object of the present study [*Lavatera agrigentina* Tineo, *Lavatera triloba* L. subsp. *pallescens* (Moris) Nyman and *Lavatera triloba* L. subsp. *triloba*] belong to section *Glandulosae* R. Fern. of the genus *Lavatera* (Escobar Garcìa *et al*., 2010). Seeds of *Lavatera* species are reported to have orthodox seeds (Hong *et al*., 1998; Royal Botanic Gardens Kew, 2008) and Martin (1946) described for this genus an axile folded embryo with a firm-fleshy endosperm. Finch-Savage and Leubner-Metzger (2006) reported seeds of Malvaceae as non dormant (ND), physically dormant (PY) or with a combinational dormancy (PY + PD). The only data available on seed germination of the three investigated species in this study are on *Lavatera triloba* ssp. *triloba* and are reported in the Seed Information Database (SID), for which high germination percentages (ca. 85%) are reported for incubation at 15°C and 20°C, in the light (8 h of irradiance per day) after seed chipping with scalpel (Royal Botanic Gardens of Kew, 2008). However, no factorial germination experiments were carried out on seeds of these species to determine the key factors in stimulating germination, their response to salinity and recovery and none information is available about the effects of sea salt spray on seedlings.

The aims of this study were: (1) to characterize seed dormancy and germination requirements of the three investigated species, (2) to evaluate the effects of NaCl and recovery on their seed germination and (3) to evaluate the effects of salt spray on seedlings development.

Materials and Methods

Study species

L. agrigentina Tineo is a caespitous nanophanerophyte, with a plant height of 30-200 cm (Pignatti, 1982) and displays a dimorphic upper leaf surface indumentum of sparse fasciculate hairs and abundant single glands, resulting in fetid, viscid and not hispid plant (Escobar Garcìa *et al*., 2010). Leaf shape is orbicular to oblong, subentire to 3-lobed, while leaf margin is dentate and undulate. Upper leaves are progressively triangular, while basal leaves are larger up to 7 x 7 cm. Inflorescence is a cylindrical and loose ear, with pale yellow or white flower colour and nerves not darker than the lamina. Petals are (15)20-25 mm and clearly longer than calyx (Fig. 1a). Flowering is basal-staggered and it occurs from April to May. Fruit is a schizocarp with 15-23 mericarps fused together and fruiting occurs from late May to late July (Escobar Garcìa *et al*., 2010). It occurs in open habitats on clayey-chalky sediments at 200-750 m a.s.l. (Fig. 3a). This *taxon* is distributed in Southern Sicily and historically recorded also in Calabria (south Italy; Pignatti, 1982; Giardina *et al*., 2007; Bacchetta *et al*., 2011).

L. triloba subsp. *pallescens* (hereafter *L. pallescens*) is a caespitous nanophanerophyte, with a plant height of (50)70-150 cm and displays a dimorphic upper leaf surface indumentum of numerous fasciculate hairs and sparse single glandular hairs (Escobar Garcìa *et al*., 2010). Plants are not hispid. Leaves are of a pale green colour, white-yellowish when senescent, usually up to 10 x 10 cm and they have three to five lobes (sometimes seven) and an undulate margin (Fig. 1b). Upper leaves are gradually smaller and distinctly 3-lobed, while basal leaf are larger than 10 x 10 cm (Escobar Garcìa *et al*., 2010). Inflorescence is a cylindrical and loose ear, with 30-70(200) flowers. Flowers are disposed in axillary clusters and petals are longer than 20 mm, clearly going over of calyx, with a variable colour from white to pale pink, sometimes with yellowish shades.

Flowering is basal staggered and it occurs from late April to late June. Fruit is a schizocarp with 10-20 mericarps fused together and fruiting occurs from late May to late July (Fenu *et al*., 2010).

This *taxon* occurs on limestone cliffs with an high exposition to wind and marine aerosol, from 20 to 48 m of share (Fenu *et al*., 2010; Fig. 3b). It is a narrow endemic, growing in only one population in southwestern Sardinia.

L. triloba subsp. *triloba* (hereafter *L. triloba*) is a caespitous nanophanerophyte, with a plant height of 30-200 cm, strongly glandular, often viscid and with fetid smell. It displays a trimorphic adaxial leaf surface indumentum, with numerous fasciculate and long-radiated single glandular hairs. Plants are hispid. Leaves are with an undulate or crenate margin and orbicular to oblong shape, subentire to profoundly 3-5 lobed. Basal leaves are larger up to 10 x 10 cm. Inflorescence is glomerular in groups of 3-7 subsessil flowers. Petals are of a deep purple colour, only occasionally white and with darker petal nerves, and of dimensions (15)20-30 mm, clearly longer than the calyx (Escobar Garcìa *et al*., 2010) (Fig. 1c). Flowering occurs from May to late June. Fruit is a schizocarp with 12-16 mericarps fused together and fruiting occurs from late June to late August (Pignatti, 1982). This *taxon* occurs in open habitats on clayey saline sediments, often subruderal, and can be locally abundant growing around endorheic lagoons. It can be rarely in primary habitats (open scrubland on limestone bedrock; Escobar Garcìa *et al*., 2010; Fig. 3c). It is distributed on the Iberian Peninsula and in south Sardinia.

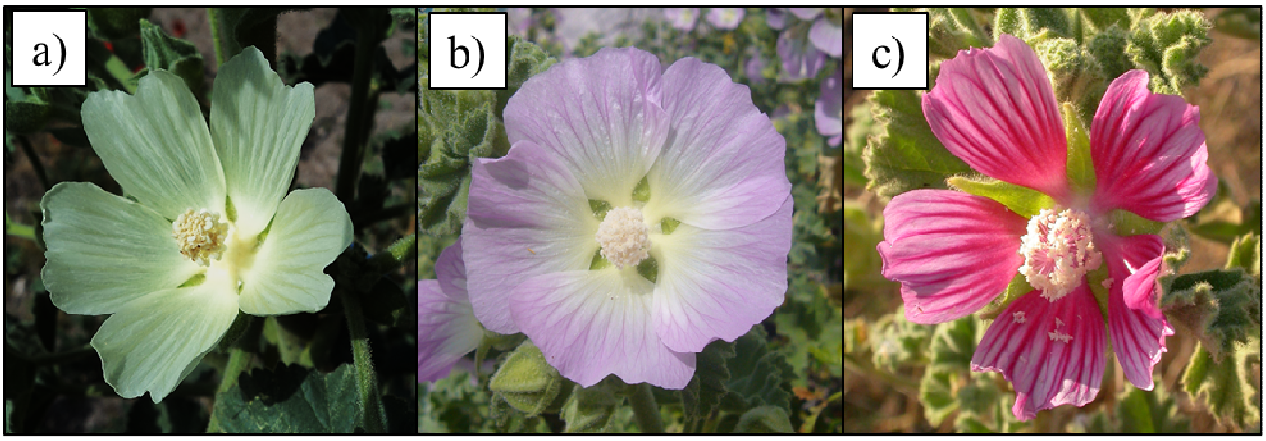

Figure 1 - Flower of *L. agrigentina* **(a),** *L. pallescens* **(b) and** *L. triloba* **(c).**

Seed lot details

Mericarps (hereafter seeds) of the three *taxa* were collected in their natural populations (Table 1 and Fig. 2) at the time of natural dispersal (Table 1). Seeds were separated from the rest of the fruit using an Agriculex CB-2 Column Seed Cleaner and selected by hand. Mean seed mass (± 1SD) for each seed lot was calculated by weighing 10 replicates of 20 seeds each (Table 2).

Table 2 - Population data.

Species	Locality	Code	Coordinates	Substrate	Mean altitude (m a.s.l)	Slope (°)	Aspect
L. agrigentina	Agìra (EN)	*La1*	37°33’ N 14°32’ E	Clays	232	40	S-SW
L. agrigentina	Ássoro (EN)	*La2*	37°37’ N 14°25’ E	Chalky clays	417-719	0	-
L. pallescens	Buggerru (CI)	*Lp1*	39°24’ N 08°24’ E	Calcareous cliffs and coastal limestone screes	7-30	20-80	W-NW
L. triloba	Elmas (CA)	*Lt1*	38°16’N 08°01’E	Clayey saline sediments	0	0	-
L. triloba	Pula (CA)	*Lt2*	38°59’ N 08°59’ E	Clayey saline sediments	2	0-5	-
L. triloba	Domus de Maria (CA)	*Lt3*	38°54’ N 08°52’ E	Clayey saline sediments	0	0	-
L. triloba	Assemini (CA)	*Lt4*	39°16' N 08° 59' E	Clayey saline sediments	1	0-5	-

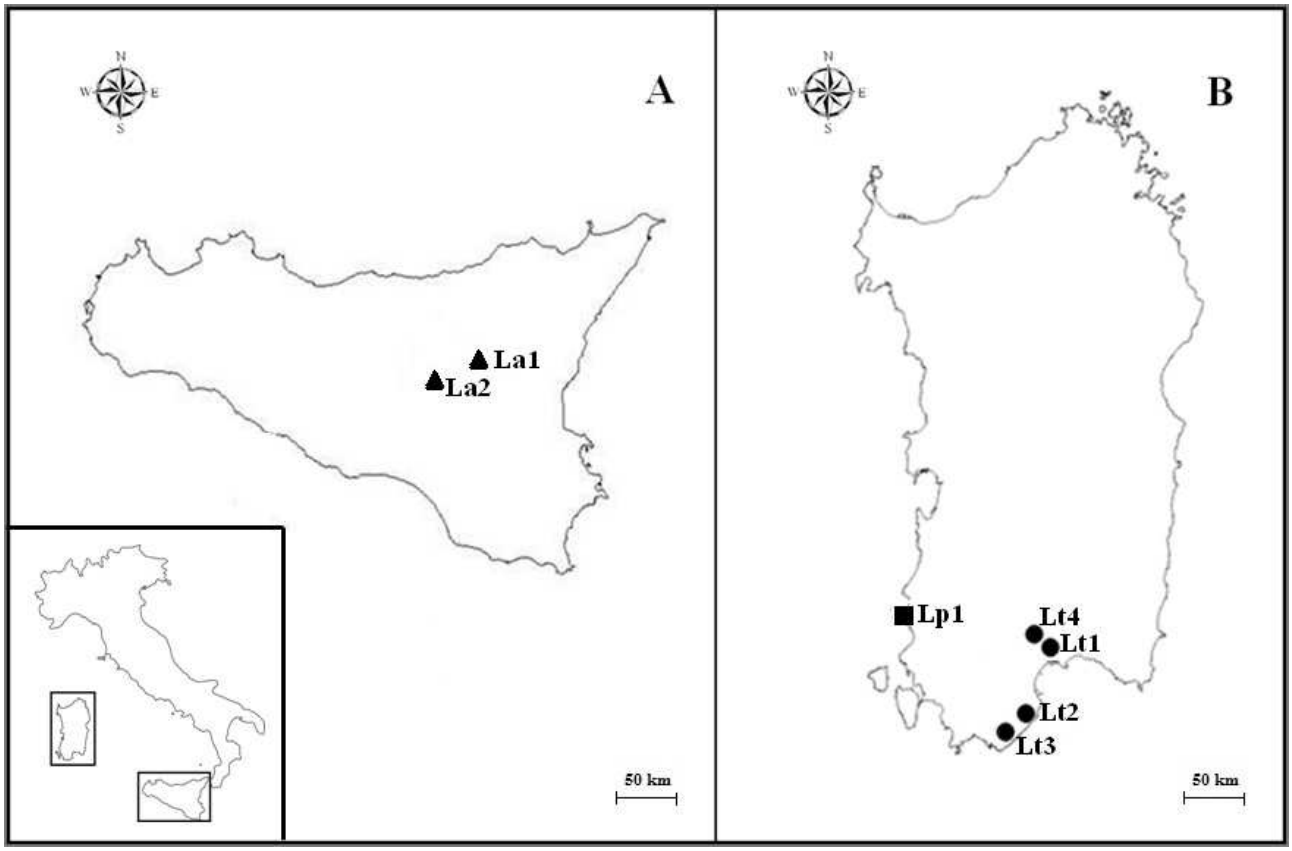

Figure 2 - Seed provenances of the three *Lavatera* species investigated in this study, in Sicily (A) and Sardinia (B). Symbols indicate: ▲ = *L. agrigentina*, ■ = *L. pallescens* and ● = *L. triloba*. See Table 1 for the explanation of the populations codes.

Figure 3 - Natural environment of *L. agrigentina* (a; La2), *L. pallescens* (b; Lp1) and *L. triloba* (c; Lt3). See Table 1 for the explanation of the population codes.

Table 2 - Seed lot details. In the column "Experimental trials": Imb = Imbibition tests; L = Light; T = Temperature; DAR = Dry after-ripening; NaCl = Salinity tests; Spray = Salt spray experiments).

Code	Data of collection	Mean seed mass (mg ± SD)	Experimental trials
La1	07/07/2010	8.69 ± 0.10	T
La2	07/07/2010	8.66 ± 0.01	Imb, L, T, DAR, NaCl
Lp1	22/07/2010	3.53 ± 0.07	Imb, L, T, DAR, Spray
Lp1	14/07/2011	3.50 ± 0.10	NaCl
Lt1	19/07/2010	6.35 ± 0.02	T
Lt2	24/07/2010	5.66 ± 0.03	T
Lt2	14/07/2011	5.87 ± 0.22	NaCl
Lt3	24/07/2010	6.65 ± 0.02	Imb, L, T, DAR, Spray
Lt3	01/08/2011	6.41 ± 0.28	NaCl
Lt4	14/07/2011	6.72 ± 0.29	NaCl

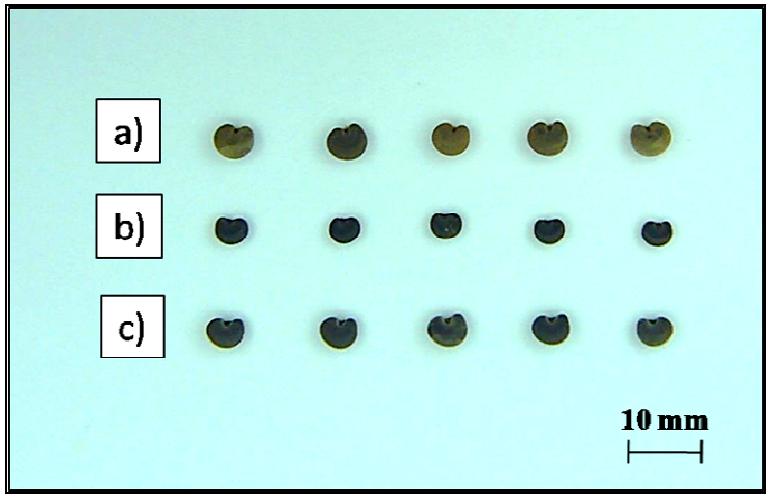

Figure 4 - Seeds of *L. agrigentina* **(a),** *L. pallescens* **(b) and** *L. triloba* **(c).**

Imbibition tests

In order to detect the presence of water impermeable teguments and therefore of a physical component of seed dormancy, three replicates with scarified and three with intact seeds of 50 seeds each, from one seed lot for each *taxon* (see table 2), were soaked in distilled water in six 40 ml glass jars (Fig. 5a) and incubated in a growth chamber (SANYO MLR-351) at the constant temperature of 20°C, in the light (12 h of irradiance per day). Seeds of each replicate were blotted dry and weighed every hour for the first 12 hours, and every 24 hours for a total of 120 hours (Fig. 5b).

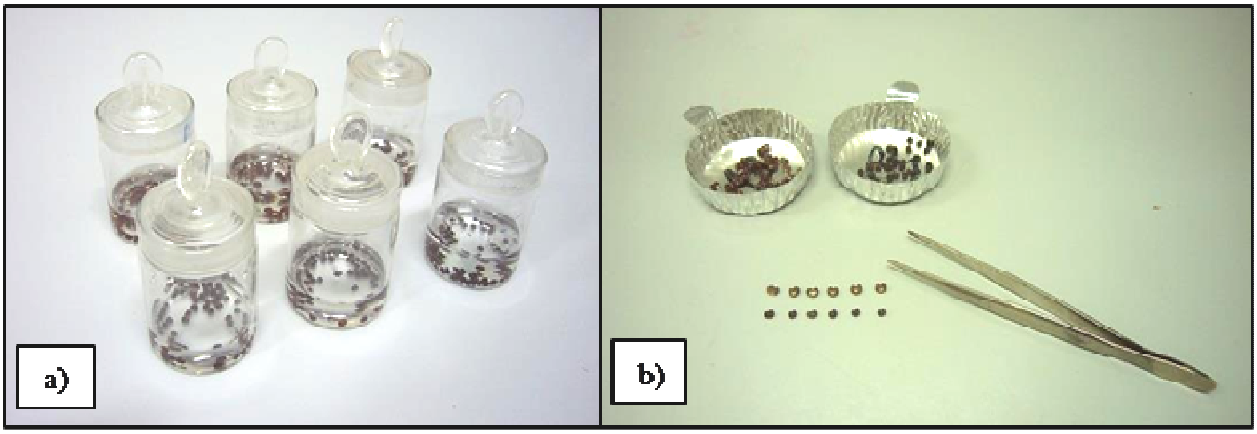

Figure 5 - Imbibition tests: glass jars with scarified and intact seeds (a) and blotted dry seeds before weighing.

Germination tests

Effect of light

A preliminary test was carried out in order to evaluate the effect of light on seed germination for seeds from one seed lot for each *taxon* (see Table 2). Manually scarified seeds (with a scalpel) were sown in 2010 on 1% water agar substrate, which provided a solid, non-sterile medium for germination, in plastic Petri dishes of 90 mm diameter. Three replicates of 20 seeds each were incubated in the light (12 h of irradiance per day) and in the dark, in growth chambers (SANYO

MLR-351) at 15°C. This temperature was chosen on the basis of the best conditions reported for seed germination of *L. triloba* (Royal Botanic Gardens Kew, 2008). Darkness was achieved by wrapping dishes in two aluminum foils (Fig. 6).

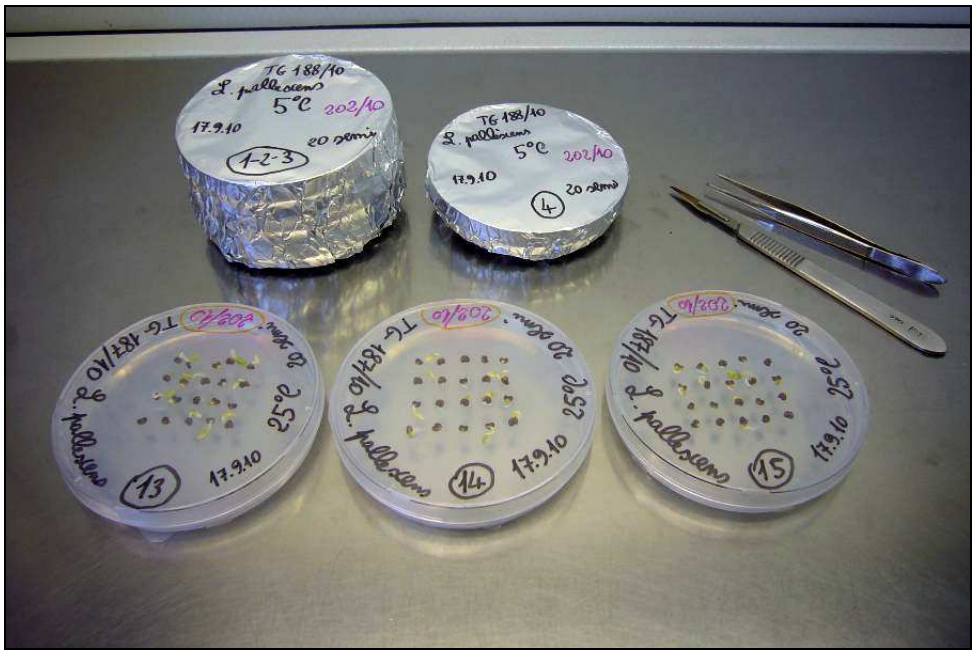

Figure 6 - Germination tests on *L. pallescens* seeds in the light (below) and in the dark (above).

The criterion for germination was visible radical protrusion. Seeds incubated in the light were scored daily and germinated seeds discarded, while seeds incubated in the dark were scored only at the end of the test to avoid any exposure to irradiance (Baskin *et al*., 2006). When no additional germination occurred in the light for two consecutive weeks, test were stopped both in the light and in the dark and the viability of any remaining seeds was checked.

Effect of temperature

In order to evaluate the effect of temperature, germination tests were conducted on seeds of each seed lot (see table 2). Three replicates of 20 seeds each were incubated in a range of constant

temperatures (5, 10, 15, 20 and 25°C) and at an alternating temperature regime (25/10°C) in the light (12 h of irradiance per day) in growth chambers. In the alternating temperature regime, the higher temperature period coincided with the light period (Baskin *et al.*, 2006).

Effect of dry after-ripening on seed germination

A sub-lot of freshly collected seeds from one seed lot of each *taxon* (see table 2), was placed in 2010 in a dry room (15°C and 15% R.H.). The advancement of drying, was monitored by measuring the activity water (aw) by the hygrometer Hygropalm Aw1 (Rotronic), equipped with the AW-DIO probe. When seeds reached aw = 0.180, they were closed in a sealed transparent polyethylene envelopes, together with two microbags containing silica gel (0.5g each) within a hermetic 2000 ml glass jar (mod. Fido, Bormioli Rocco S.p.a), with granular brown silica gel (diameter 2-5 mm), to maintain low level of humidity. The jar was then incubated at 25°C in a growth chamber and after three months, seeds were sown in Petri dishes in the light (12 h of irradiance per day) to the above specified germination conditions.

Effect of NaCl on seed germination and recovery

To evaluate the effect of salt stress on seed germination, three replicates of 20 seeds each, from one seed lot for *L. pallescens* and *L. triloba* (see Table 2), were sown in 2011 in 1% water agar substrate, with different NaCl concentrations (0, 100, 200, 300, 400, 500, 600 mM) and incubated in a range of constant temperatures (10, 15, 20 °C), in the light. *L. agrigentina* seeds were not tested due to the habitat of the species (see Table 1), with its populations being far (> 50 km) of the coastline. To evaluate the inter-population variability in response to NaCl for *L. triloba*, germination in four NaCl concentrations (0, 200, 400, 600 mM) at 15°C, was tested for seed lots Lt2 and Lt4 (see Table 2). After two consecutive weeks without additional germination under control conditions (NaCl 0 mM), non-germinated seeds were washed with distilled water and then

sown in new Petri dishes containing 1% water agar substrate for additional 30 days (recovery phase) at the same incubation temperatures.

Effect of salt spray on seedling development

To evaluate the effect of salt on the vegetative growth, a solution of NaCl was applied by spraying on early seedlings, from one seed lot for *L. pallescens* and one for *L. triloba* in 2011 (see Table 2). Seeds were sown on 1% water agar substrate in plastic Petri dishes of 90 mm diameter. In order to obtain the number of seedlings required for the start of the experiments, five replicates of 100 seeds each were incubated in the light at 15°C and 20°C, for *L. pallescens* and *L. triloba*, respectively, in a growth chamber. One week after seed germination, seedlings were sown in polyethylene pots (70 x 70 x 90 mm) in number of four for each pot, but only one seedling per pot was kept for the experiment. Before the use, all pots were disinfected by immersion in a solution of NaClO (860 mM) per two hours and then washed in distilled water. Pots were filled by a substrate, constituted by turf (55%), perlite (35%) and coconut fiber (10%), sterilized at 80°C per five hours in an oven. Four replicates of 13 seedlings each per condition, were inserted in a phytotron (8 m^3) at the alternating regime 20/10°C, with 12 h of irradiance per day (the higher temperature period coincided with the light period) (Fig. 7). Conductivity (conductometer microCM200, Crison) and pH (pH-meter GLP 21, Crison) values of the substrate were measured at the end of experiments. Humidity values inside the phytotron were monitored for all the duration of the experiments by a humid bulb hygrometer and they ranged from 73% (during the light period) to 91% (in the dark period). For eight weeks, four replicates were sprayed with a 600 mM NaCl solution (to mimic sea water) at a distance of 200 mm, with different frequencies (1 day/week, 2 days/week and 3 days/week; Cheplick & Demetri, 1999), while other four replicates did not get any spraying (control). The temperature of the salt spray solution was 15°C and all

epigeal parts of each seedling were equally exposed to the solution. Weekly the number of dead seedlings was annotated. After eight weeks, at the end of the experiment, the length of epigeal and hypogeal parts for each survived seedling was measured by a digital caliper (Fig. 17) and the dry weight calculated by drying in oven at 103°C per 17 hours.

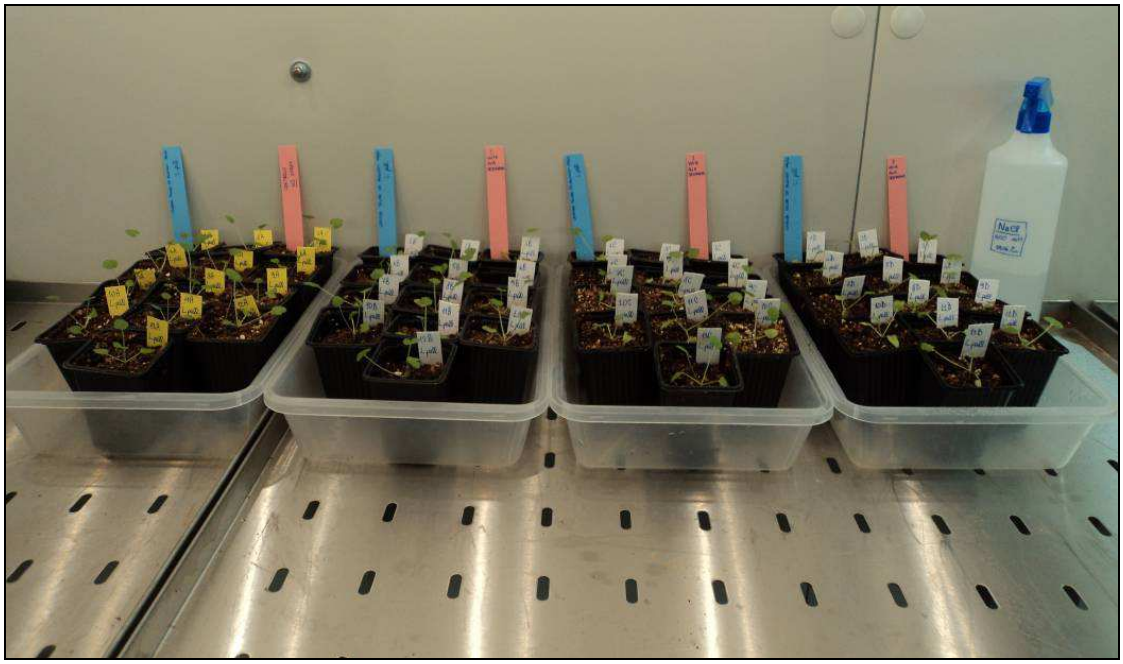

Figure 7 - Replicates of *L. pallescens* **seedlings for salt spray experiments.**

Data analysis

Percentage water uptake was calculated following Hidayati *et al*. (2001) in relation to seed mass at to:

$$\%Ws = [(Wi - Wd) / Wd] \times 100,$$

where Ws = increase in mass of seed, Wi = mass of seed after a given interval of imbibition, and Wd = seed mass at to.

Final germination percentage was calculated as the average of the three replicates (± 1SD) on the basis of filled seeds. The rate of germination was estimated by using a modified Timson's index (TI) of germination velocity:

$$TI = \sum G/t ,$$

where *G* is the percentage of seed germination at two-days intervals and *t* is the total germination period (Khan & Ungar, 1984). Using this index, higher the value, more rapid is germination.

For NaCl experiments, the recovery percentages (RP) according to the following equation (Pujol *et al*., 2000):

$$RP = \{[(a - b)/(c - b)] \times 100\},$$

where *a* is the total number of seeds germinated in salt solutions plus those that recovered to germination in the fresh water, *b* is the total number of seeds germinated in saline solutions, and *c* is the total number of seeds. For salt spray experiments, a value of dry weight (mean ± SD) was calculated by weighing all survived seedlings for each treatment after eight weeks from the beginning of experiments. Arcsin-transformed germination percentages were analysed by one- and two-way ANOVA and a Fisher's LSD *post hoc* test was used to determine significant differences ($p < 0.05$) among means. Log_{10}-trasformed TI were calculated, both for fresh and DAR seeds, only for seeds germinated in the light and analysed by two-way ANOVA (followed by a Fisher's LSD *post hoc* test). For salt stress experiments, germination, RP and mortality percentages, as well as conductivity and pH values, were analysed by a non-parametric Kruskal-Wallis test followed by a Mann-Whitney *U-test*. For salt spray experiments, seedlings dry mass and seedling lengths of epigeal and hypogeal parts were analysed by Kruskal-Wallis test, (followed by a Mann-Whitney *U-test*). Linear regressions to correlate soil parameters (conductivity and pH) with seedling growth

were realized using the software Sigmaplot 11.0, while all the statistical analyses were carried out using the software Statistica 8.0 for Windows.

Results

Imbibition tests

While intact seeds of all the three species did not increase in their seeds mass more than 15% even after 120 h, scarified seeds of all three species reached, in the first hour, seed mass increases of 23.0 ± 0.4%, 69.1 ± 2.1% and 54.6 ± 4.5%, for *L. agrigentina*, *L. pallescens* and *L. triloba*, respectively (Fig. 8). *L. agrigentina* and *L. triloba* scarified seeds reached their maximum increase in mass after 90 h with percentages of water uptake of 111.9 ± 1.8 and 109.2 ± 6.0%, respectively, while those of *L. pallescens* after 120 h (112.6 ± 2.1%). Moreover, germination occurred during the test for all three species, for scarified seeds, starting after 9 h for *L. pallescens* (Fig. 9), 48 h for *L. agrigentina* and 120 h for *L. triloba*.

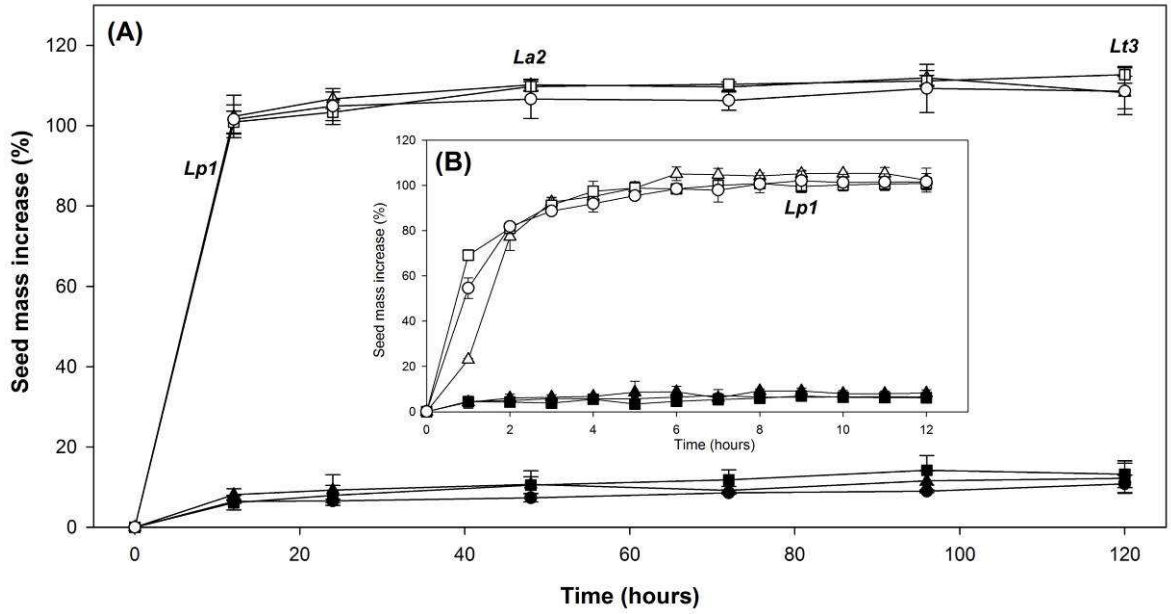

Figure 8 - Cumulative curves of imbibition for the three *Lavatera* species, (A) total duration of 120 h, (B) detail of the first 12 h; Symbols indicate: ▲ = *L. agrigentina*, ■ = *L. pallescens* and ● = *L. triloba*. Symbols in black indicate intact seeds, in white scarified seeds. See Table 1 for the explanation of the population codes. The position of the codes highlights the hour when seeds of the different species started germinating during the imbibition test.

Figure 9 - Scarified (above) and intact (below) *Lavatera pallescens* seeds after 9 hours of imbibition.

Germination tests

Effect of light

For all the three *taxa*, the one-way ANOVA showed a non significant ($p > 0.05$) effect of light on germination (*L. agrigentina*: 63.0 ± 7.6% and 60.0 ± 10.0%; *L. pallescens*: 88.0 ± 12.0% and 78.3 ± 7.6%; *L. triloba*: 80.0 ± 10.0% and 93.3 ± 5.8%, for light- and dark-incubated seeds of each *taxon*, respectively).

Effect of temperature

L. agrigentina

The two-way ANOVA showed a highly significant effect for temperature (T: $p < 0.001$), population (P: $p < 0.05$) as well as for their interaction (T x P: $p < 0.05$). For La1, the higher germination percentages were detected at low temperatures, between 5°C and 15°C (ca. 55% for the three temperatures; see Table 3). Germination decreased at 20°C (35.0 ± 17.3%) and the lowest percentages were detected at the highest constant temperature of 25°C (5.0 ± 5.0%) and at 25/10°C (6.7 ± 5.8%) and these values significantly ($p < 0.05$) differed from that of all other temperatures. For La2, the highest germination percentages were detected between 10°C and 20°C (ca. 65% for all the three tested temperatures; see Table 3), while lowest values were observed at 5°C (31.7 ± 12.6%) and 25°C (15.0 ± 5.0%) and they did not differ significantly ($p > 0.05$) between themselves. At 25/10°C, germination percentages (ca. 50%) were not statistically ($p > 0.05$) different from that at 5°C (see Table 3). Significant ($p < 0.05$) differences among germination percentages of the two populations were observed at 20°C and 25/10°C, with the higher values at both temperatures, detected for La2.

The two-way ANOVA conducted for TI showed an highly significant effect ($p < 0.001$) for temperature (T), population (P) and their interaction (T x P). For La1, the most rapid germination was detected at 10°C and 15°C (TI of ca. 2.3). TI values decreased at 5°C and 20°C, with values

of ca. 1.3. The lowest values were detected at the highest constant temperature of 25°C and at the alternating regime of 25/10°C (TI of ca. 1.5) and these values differed significantly ($p < 0.05$) from TI at all other temperatures (see Table 3). For La2, the most rapid germination was detected at 15°C (TI: 7.9 ± 0.9) and this value significantly ($p < 0.05$) differed from all others. TI values decreased at 10°C and 20°C (ca. 2.7 for both temperatures) and at 25°C and 25/10°C (ca. 1.5 for both). The lowest TI were detected at 5°C (TI: 0.8 ± 0.3) and these value were significantly ($p < 0.05$) different from all others. TI differed significantly ($p < 0.05$) between the two populations only at all temperatures highest than 10°C (see Table 3) and germination velocity at these temperatures was higher (ca. > 50% more) for La2 than La1.

L. pallescens

The one way ANOVA showed a significant effect of temperature (T) on germination ($p < 0.05$) and on germination velocity ($p < 0.001$). The highest germination percentages were detected at 5°C, 10°C and 15°C (ca. 90%) and these values were significantly ($p < 0.05$) different from those at higher temperatures. The lowest value was detected at 20°C (67.0 ± 7.7%) and it was not statistically different ($p > 0.05$) only from 25°C (73.0 ± 7.7%). At the alternating temperature regime of 25/10°C, germination percentage (81.7 ± 2.9%) was not significantly ($p > 0.05$) different from that detected at 10°C, 15°C and 25°C (with values ranging from ca. 75% at 25°C to ca. 92% at 10°C; see Table 3).

Germination velocity showed the highest value at 10°C (TI: 6.5 ± 0.5), which significantly differed ($p < 0.05$) from all other TI values. There was not significant difference ($p > 0.05$) between TI at 5°C and 15°C (ca. 4.5 for both temperatures). TI values significantly decreased ($p < 0.05$) at temperatures highest than 15°C (with values of ca. 3.0 at 20°C, 25°C and 25/10°C) (see Table 3).

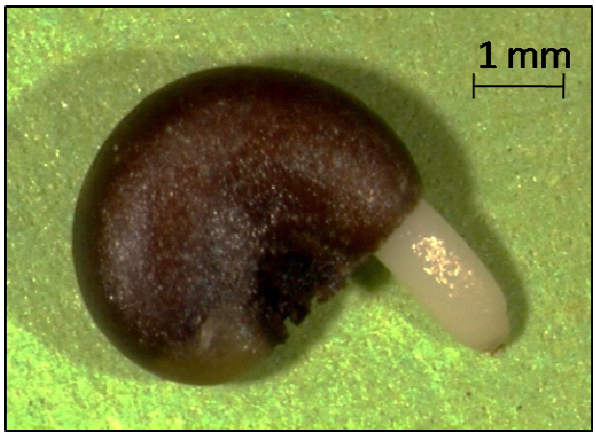

Figure 10 - Particular of a germinated seed of *L. pallescens*

L. triloba

The two-way ANOVA showed a highly significant effect ($p < 0.001$) on germination both for temperature (T) and population (P), but not for their interaction (T x P; $p > 0.05$). For Lt1, the highest values were detected at temperatures between 10°C and 20°C and at 25/10°C (ca. 40%; see Table 3), while lowest values were at 5°C and 25°C (ca. 22%, for both temperatures) and they were significantly different ($p < 0.05$) only from values at 20°C. For Lt2, high germination percentages were detected at all temperatures higher than 5°C (ranging from 65.0 ± 15.0% at 25°C to 76.6 ± 15.5% at 15°C) and significantly differed ($p < 0.05$) from the lower values detected at 5°C (46.6 ± 20.2%), with the exception of 25°C, which were not statistically different ($p > 0.05$) (see Table 3). For Lt3, the highest germination percentages were detected at temperatures between 10°C and 20°C (with values ranging from 80.0 ± 10.0% at 15°C to 87.0 ± 7.7% at 20°C), while germination decreased at 5°C (58.3 ± 14.4%) and at 25°C and 25/10°C (ca. 65% for both). Lt1 showed at all temperatures significantly ($p < 0.05$) lower germination percentages respect to Lt2 and Lt3 populations, which did not differ significantly ($p > 0.05$) between themselves.

For Lt1 the highest TI were detected in the range 15-25°C (TI of ca. 5.0) and they were not statistically different ($p > 0.05$) from that at 25/10°C (ca. 4.0). The lowest germination velocity was at 5°C (with TI of ca. 0.6) and differed significantly ($p < 0.05$) from all other values (see Table 3). For Lt2, the highest TI was detected at 20°C (ca. 9.0) and was significantly different ($p < 0.05$) from all other TI values, whereas the lowest germination velocity, was detected at 5°C (with TI of 0.7 ± 0.3; see Table 3). For Lt3, the most rapid germination was at 15 and 20°C (TI: 6.7 ± 0.8 and 8.7 ± 0.8, respectively), and these values were significantly different ($p < 0.05$) from all other TI. The lowest TI were detected at 5°C and 25/10°C, with values of ca. 0.9, for both temperatures. Lt2 and Lt3 showed a statistically similar ($p > 0.05$) trend in germination velocity, in particular at 5°C, 15°C and 20°C. TI values were significantly different ($p < 0.05$) for each population at 10°C and 25/10°C, while, at 25°C, did not show significant differences ($p > 0.05$) between Lt1 and Lt3.

Table 3 - Germination percentages and Timson's index (TI) in the light (12/12 h) at each temperature regime for different populations of the three *Lavatera* species. A two-way ANOVA was conducted among germination percentages and TI for populations of the same species in order to detect the effect of temperature (T), population (P) and their interaction (T x P), while a one-way ANOVA was conducted for the only one *L. pallescens* population to evaluate the effect of temperature (T) on germination and Timson'index. A Fisher's LSD *post hoc* test was conducted to identify significant differences at $p < 0.05$. Data are the mean (± 1 SD) of three replicates. Capital letters in columns are related to the same temperature, while lower-case letters in rows to the same population. For each population, the code is the same of Table 1.

Species	Code	Parameter	Temperature (°C)					
			5	10	15	20	25	25/10
L. agrigentina	***La1***	**Germination (%)**	50.0 ± 5.0^{abA}	63.3 ± 15.3^{bA}	55.0 ± 17.3^{abA}	35.0 ± 17.3^{aA}	5.0 ± 5.0^{cA}	6.7 ± 5.8^{cA}
		TI	1.3 ± 0.1^{acA}	2.4 ± 0.6^{bA}	2.3 ± 0.7^{bcA}	1.3 ± 0.7^{aA}	0.3 ± 0.3^{dA}	0.2 ± 0.2^{dA}
	La2	**Germination (%)**	31.7 ± 12.6^{adA}	71.7 ± 2.9^{bA}	63.3 ± 7.7^{bcA}	65.0 ± 13.2^{bcB}	15.0 ± 5.0^{dA}	48.3 ± 20.2^{acB}
		TI	0.8 ± 0.3^{aA}	2.7 ± 0.1^{bA}	7.9 ± 0.9^{cB}	2.7 ± 0.5^{bB}	1.5 ± 0.5^{dB}	1.6 ± 0.7^{dB}
L. pallescens	***Lp1***	**Germination (%)**	96.7 ± 2.9^{a}	91.7 ± 7.7^{ab}	88.0 ± 11.5^{ab}	67.0 ± 7.7^{c}	73.0 ± 7.7^{cd}	81.7 ± 2.9^{bd}
		TI	4.4 ± 0.1^{a}	6.5 ± 0.5^{b}	4.9 ± 0.6^{a}	3.0 ± 0.3^{c}	2.8 ± 0.3^{c}	2.9 ± 0.1^{c}
L. triloba	***Lt1***	**Germination (%)**	21.7 ± 10.4^{aA}	35.0 ± 5.0^{abA}	40.0 ± 13.2^{abA}	45.0 ± 13.2^{bA}	21.7 ± 10.4^{aA}	31.7 ± 2.9^{abA}
		TI	0.6 ± 0.5^{aA}	0.8 ± 0.1^{bA}	4.0 ± 1.3^{cA}	5.6 ± 1.6^{cA}	5.4 ± 2.6^{cA}	3.9 ± 0.4^{cA}
	Lt2	**Germination (%)**	46.6 ± 20.2^{aB}	75.0 ± 5.0^{bB}	76.6 ± 15.5^{bB}	73.3 ± 11.5^{bB}	65.0 ± 15.0^{abB}	75.0 ± 8.7^{bB}
		TI	0.7 ± 0.3^{aAB}	1.9 ± 0.1^{bB}	5.5 ± 0.9^{cAB}	9.2 ± 1.4^{dB}	1.4 ± 0.3^{bB}	2.1 ± 0.2^{bB}
	Lt3	**Germination (%)**	58.3 ± 14.4^{aB}	85.0 ± 5.0^{bdB}	80.0 ± 10.0^{bcdB}	87.0 ± 7.7^{bB}	68.0 ± 10.0^{adB}	60.0 ± 8.7^{acB}
		TI	0.8 ± 0.2^{aB}	3.5 ± 0.2^{bC}	6.7 ± 0.8^{cB}	8.7 ± 0.8^{cB}	3.4 ± 0.5^{bA}	1.0 ± 0.1^{aC}

Effect of dry after-ripening

L. agrigentina

A significant effect ($p < 0.001$) of pretreatment (P) and temperature (T), and of their interaction (P x T; $p < 0.05$) was detected among germination percentages (see Table 4). For DAR seeds, 100% of germination was reached in at least one of the three replicates, at 5°C, 10°C and 15°C (96.7 ± 2.9%, 96.7 ± 3.3, 100 ± 0 %, respectively). The lowest values were detected at 25°C (66.7

± 7.6%) and were significantly ($p < 0.05$) different from that of other all temperatures. Germination percentages at 20°C and 25/10°C (ca. 87%, for both) were not statistically different ($p > 0.05$) with the values detected at 10°C. Values of DAR seeds were significantly higher ($p < 0.05$) respect to that of fresh seeds, with the highest increases at 5°C and 25°C (with 70% and 50% of increase in final germination percentages, respectively) (see Figure 3).

The two way ANOVA for TI showed an highly significant effect ($p < 0.001$) of pretreatment (P), temperature (T), as well as their interaction (P x T). For DAR seeds, the higher values of germination velocity were detected at 15°C (TI of ca. 13) and significantly differed ($p < 0.05$) from all other TI. The lower values were at 5°C and 25/10°C (TI of ca. 1.5) and showed statistical differences ($p < 0.05$) from all other TI, but not between themselves ($p > 0.05$). Germination velocity values at 10°C, 20°C and 25°C did not differ significantly ($p > 0.05$) among themselves. TI values were statistically similar ($p > 0.05$) between fresh and DAR seeds only at 20°C and 25/10°C ($p > 0.05$), while at all other temperatures were detected significant increases ($p < 0.05$) in germination velocity, in particular at 10°C and 15°C (with increases in germination velocity of ca. 65% and ca. 35%, respectively) (see Figure 3).

L. pallescens

Pretreatment (P) did not influence significantly ($p > 0.05$) seed germination, while temperature (T) and their interaction (P x T) had a significant effect ($p < 0.001$ and $p < 0.05$, respectively; Table 4). For DAR seeds, the higher germination values were observed at temperatures below 20°C (ca. 95% for all temperatures), while percentages at 25°C and 25/10°C were significantly ($p< 0.05$) lower (ca 70%). Values of DAR seeds were significantly ($p < 0.05$) higher respect to that of fresh seeds only at 20°C, with an increase of ca. 35% in final germination percentages (Figure 11).

The two-way ANOVA for TI showed a significant effect of pretreatment (P; $p < 0.05$), temperature (T; $p < 0.001$) as well as their interaction (P x T; $p < 0.05$). For DAR seeds, the higher

germination velocity values were detected at 10°C, with TI of ca. 6.5 and they were significantly ($p < 0.05$) different from all the others. The lowest TI were at 25/10°C (ca. 3.0) and differed significantly ($p < 0.05$) from those detected at all other tested temperatures. Significant ($p < 0.05$) increases of germination velocity for DAR seeds respect to fresh seeds were detected only at the high temperatures of 20°C and 25°C (TI of ca. 4.0) with increases of ca. 30% (Figure 11).

L. triloba

Pretreatment (P) did not influenced significantly ($p > 0.05$) seed germination, while temperature (T) was highly significant ($p < 0.001$), but not their interaction (P x T; $p > 0.05$) (see Table 4). For DAR seeds, the higher germination percentages were detected at 20°C (90.0 ± 5.0%) and did not differ significantly ($p > 0.05$) from that at 10°C and 15°C (ca. 85% for both). The lower percentages were observed at 5°C and 25/10°C (ca. 55%, for both) and did not show significant differences ($p > 0.05$) from that detected at 25°C (72.0 ± 2.9%) (see Figure 11). Germination percentages of DAR seeds were statistically similar ($p > 0.05$) from that detected for fresh seeds at all tested temperatures.

The two-way ANOVA for TI showed the non significant effect of pretreatment (P; $p > 0.05$), but highly significant ($p < 0.001$) both for temperature (T) and their interaction (P x T). For DAR seeds, the highest germination velocity was detected at 20°C (TI: 7.5 ± 0.4) and significantly ($p < 0.05$) differed from that at all other temperatures. The lower TI values were at 5°C and 25/10°C (ca. 1.4 for both) and resulted statistically different ($p < 0.05$) from all other. Germination velocity at 10°C, 15°C and 25°C (TI of 2.5 ± 0.3; 6.0 ± 0.3 and 3.2 ± 0.1, respectively) were significantly different ($p < 0.05$) among themselves, and from values detected at all other temperatures. Significant increases ($p < 0.05$; ca. 45%) of germination velocity for DAR seeds respect to fresh seeds were detected only at 5°C and 25/10°C, while a significant decrease ($p < 0.05$; ca. 25%) were at 10°C (see Figure 11).

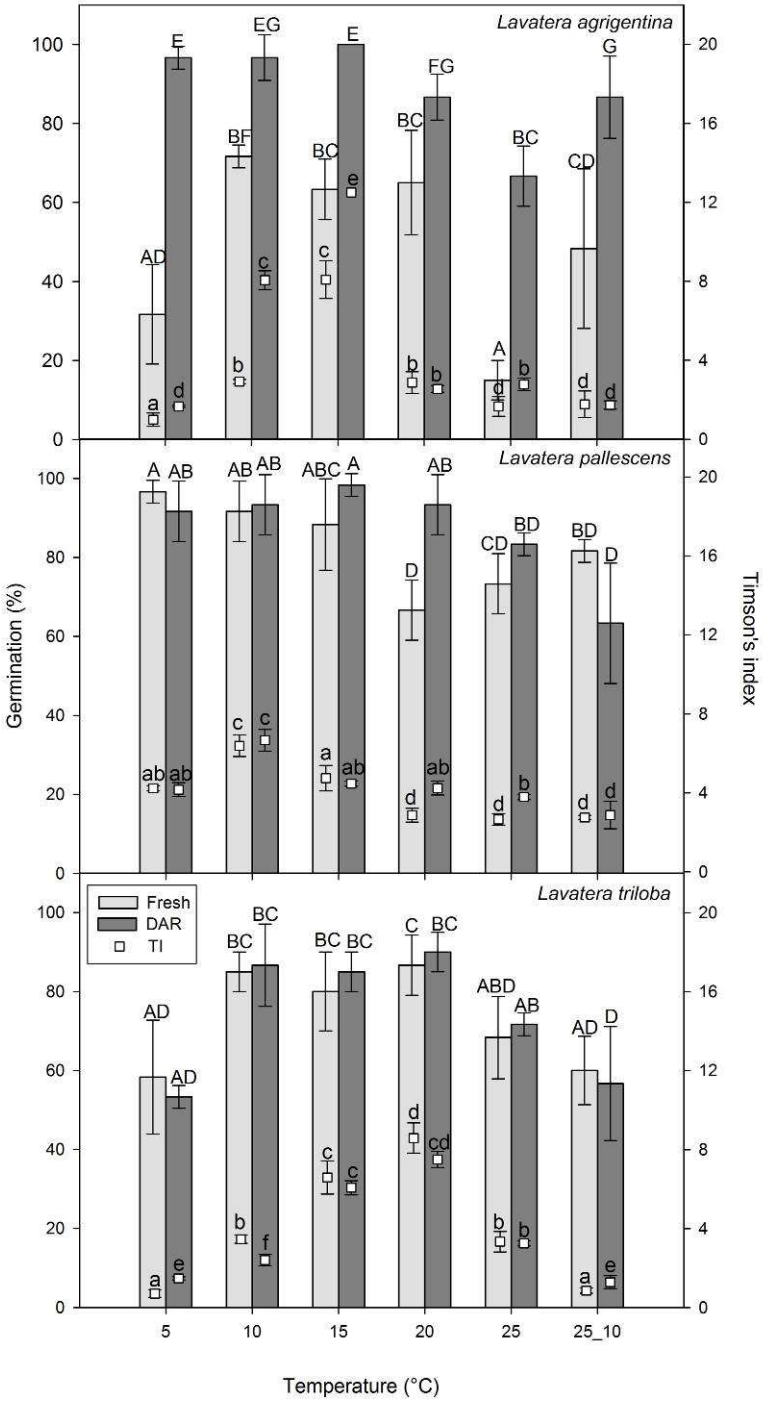

Figure 11 - Final germination percentage and Timson's index (TI) at each temperature regime for fresh and dry after-ripened (DAR) seeds in the light for *L. agrigentina*, *L. pallescens* **and** *L. triloba*. **The same letters for bars (capital letters) and squares (lower-case letters) are not significantly different at $p < 0.05$ (two-way ANOVA followed by Fisher's LSD** *post hoc* **test). Data are the means (± 1 SD) of three replicates, for each treatment. See Table 2 for the explanation of the tested population for each** *taxon*.

Table 4 - Effect of the dry after-ripening pretreatment (P), temperature (T) and their interaction (P x T) on germination percentages for the three *Lavatera* species; [p values were considered not significantly (p > 0.05, ns), significantly (p < 0.05, *) and highly significant (p < 0.001, *) different, by two-way ANOVA].**

Species	Factor	SS	DF	MS	F	p
Lavatera agrigentina	Pretreatment (P)	9758,2	1	9758,2	171,053	***
	Temperature (T)	4247,6	5	849,5	14,891	***
	P x T	1338,9	5	267,8	4,694	*
	Error	1369,2	24	57,0		
Lavatera pallescens	Pretreatment (P)	218,4	1	218,4	2,889	ns
	Temperature (T)	2361,9	5	472,4	6,248	***
	P x T	1219,2	5	243,8	3,225	*
	Error	1814,5	24	75,6		
Lavatera triloba	Pretreatment (P)	11,1	1	11,1	0,144	ns
	Temperature (T)	5666,7	5	1133,3	14,703	***
	P x T	172,2	5	34,4	0,447	ns
	Error	1850,0	24	77,1		

NaCl and recovery on seed germination

Effect of temperature

L. pallescens

Seed germination decreased ($p < 0.05$) with an increase in temperature and salinity. At all tested temperatures, the higher germination percentages were detected in non-saline control (0 mM NaCl) and at 100 mM conditions and significantly differed ($p < 0.05$) from those at all other conditions (Fig. 12 and Table 5A). Under control condition final germination ranged from 83.3 ± 15.3% (20°C) to 96.7 ± 5.8% (15°C), while at 100 mM, germination percentages ranged from 63.3 ± 10.4% (20°C) to 90.0 ± 5.0% at 10°C (see Table 5A and Figure 12). At 200 mM, germination percentages decreased significantly ($p < 0.05$) respect to lower salinities, but were significantly ($p < 0.05$) higher of those detected at higher NaCl concentrations. Final germination decreased with the increase of temperature, ranging from 76.7 ± 5.8% at 15°C to 13.3 ± 14.4% at 20°C, and these differences were statistically significant ($p < 0.05$). At salinities higher than 200 mM,

independently from temperature, a significant decrease ($p < 0.05$) of germination percentages was observed with the increase of NaCl concentration. At 200 and 400 mM, the differences among the low temperatures and the highest temperature of 20°C were significant ($p < 0.05$; Table 5A).

For each tested temperature, no differences were detected among RP at different concentrations. At 100 mM, RP did not show differences ($p > 0.05$) at 15°C and 20°C, and at 10°C was not possible to test for salt recoverying, because the few ungerminated seeds were not viable after the previous NaCl experiment phase. Recovery percentages significantly decreased ($p < 0.05$) with the increase of temperature at 200 mM (with RP ranging from ca. 100% at 15°C to ca. 5% at 20°C), 400 mM (with RP ranging from ca. 70% at 10°C to 2% at 20°C) and 600 mM (from ca. 30% to 8% from 10°C to 15°C). At 500 mM and 600 mM, at 20°C it was not possible to test for salt recoverying, due the total seed mortality at these concentrations in the previous NaCl experiment phase (Table 5A).

L. triloba

Final germination decreased with the increase of salinity concentration ($p < 0.05$), but not with temperature ($p > 0.05$). The highest germination percentages were detected under control condition (ca. 80% for all temperatures) and decreased significantly ($p < 0.05$) at 100 mM, with the exception of 15°C, for which non-saline control and 100 mM did not show significant ($p > 0.05$) differences (ca. 75% for both concentrations). Germination was totally inhibited at salinities higher than 100 mM, at all tested temperatures. No differences were detected ($p > 0.05$) in the recovery response among temperatures at the same NaCl concentration, although RP showed significantly higher values ($p < 0.05$) at lower salinities respect to higher concentrations at all temperatures (see Table 5B). No recovered seeds were observed at 20°C at 500 mM.

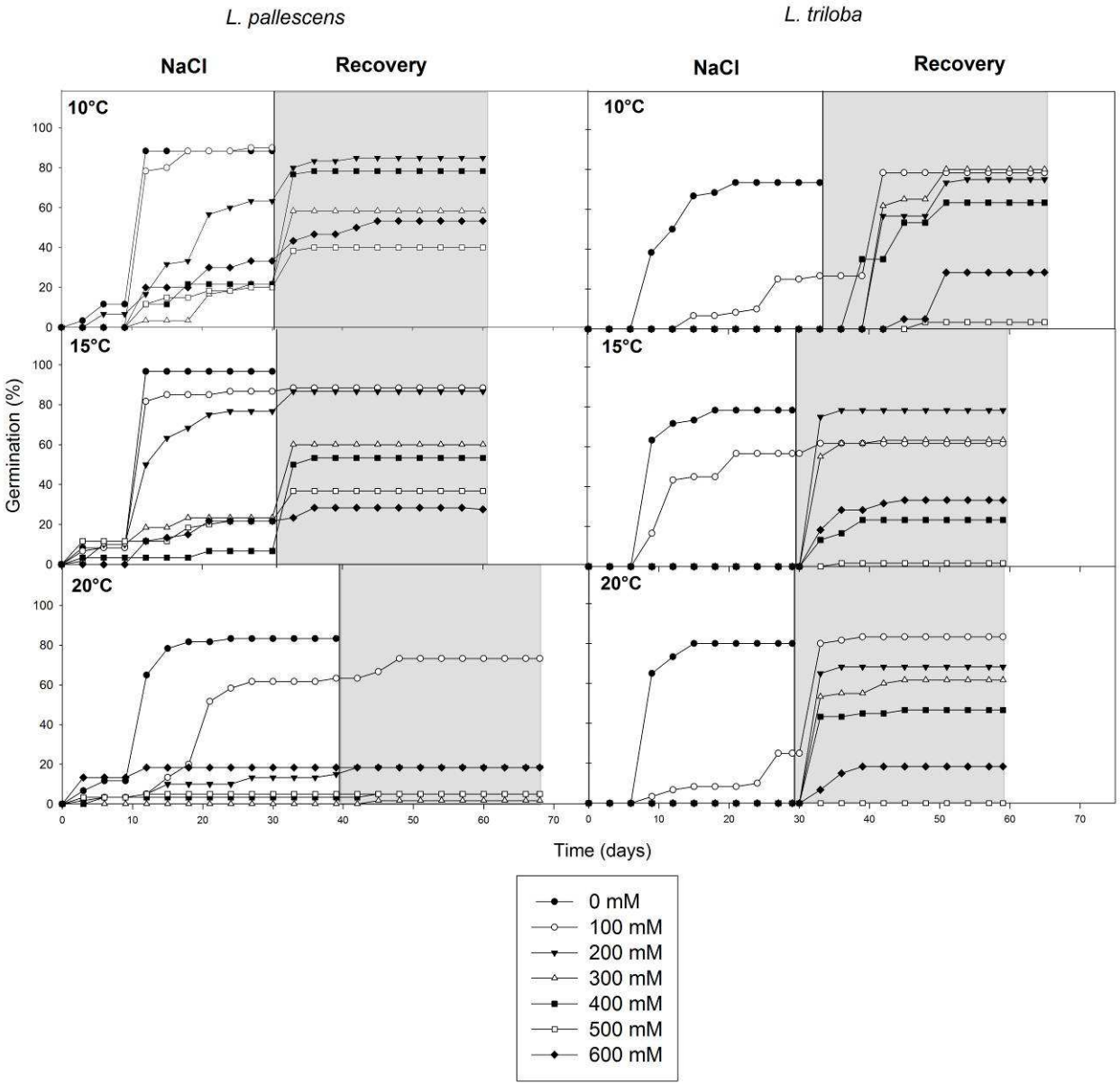

Figure 12 - Cumulative germination percentages at the tested temperatures (10°C, 15°C, 20°C), under different saline concentrations (0-600 mM NaCl) and following transfer to distilled water (recovery, indicated by the shaded area in the graph) for *L. pallescens* (on the left) and *L. triloba* (on the right) . Each point represents the mean (± 1 SD) of three replicates.

Table 5 - Germination and recovery (RP) percentages at each temperature regime, at different saline concentrations (0-600 mM NaCl) for *L. pallescens* (A) and *L. triloba* (B). Kruskal-Wallis tests were conducted to detect the effect of the same temperature on germination percentages and RP and that of the same salinity on germination percentages and RP; [p values were considered not significant (p > 0.05, ns) and significant (p < 0.05, *; p < 0.01, **) by Kruskal-Wallis test]. Data are the means (± 1 SD) of three replicates. Capital letters in columns are related to the same salinity, while lower-case letters in rows to the same temperature. Values with different letters were used to indicate significant differences at p < 0.05 (Mann Whitney *U-test*). – were used to indicate the lack of recovery experiment.

(A) *L. pallescens*

Temperature (°C)	(%)	NaCl concentration (mM)							
		0	100	200	300	400	500	600	
10	Germination	88.3 ± 2.9^{a}	90.0 ± 5.0^{a}	65.0 ± 26.0^{bB}	30.0 ± 5.0^{bd}	21.7 ± 2.9^{cdA}	20.0 ± 13.2^{cd}	33.3 ± 7.6^{bd}	**
	Recovery (RP)	-	-	84.6 ± 21.7^{A}	49.1 ± 22.7	72.8 ± 18.9^{A}	23.8 ± 21.2^{A}	31.6 ± 23.7^{A}	ns
15	Germination	96.7 ± 5.8^{a}	86.7 ± 11.6^{ab}	76.7 ± 5.8^{bB}	23.3 ± 20.2^{cd}	6.7 ± 5.8^{dB}	21.7 ± 7.7^{c}	21.7 ± 7.7^{c}	**
	Recovery (RP)	-	25.0 ± 0	100 ± 0^{A}	45.4 ± 15.8	50.4 ± 10.5^{A}	18.4 ± 11.1^{A}	8.1 ± 7.0^{B}	ns
20	Germination	83.3 ± 15.3^{a}	63.3 ± 10.4^{a}	13.3 ± 14.4^{bA}	0^{c}	3.3 ± 5.8^{bcB}	5.0 ± 5.0^{bc}	18.3 ± 7.7^{b}	**
	Recovery (RP)	-	36.1 ± 19.7	5.3 ± 0^{B}	2.5 ± 3.5	1.8 ± 3.2^{B}	-	-	ns
	Germination	ns	ns	*	ns	*	ns	ns	
	Recovery (RP)	-	ns	*	ns	*	ns	*	

(B) *L. triloba*

Temperature (°C)	Percentage (%)	NaCl concentration (mM)							
		0	100	200	300	400	500	600	
10	Germination	73.3 ± 14.4^{a}	26.7 ± 20.9^{b}	0^{c}	0^{c}	0^{c}	0^{c}	0^{c}	**
	Recovery (RP)	-	68.3 ± 22.7^{abc}	73.3 ± 10.4^{bc}	80.0 ± 8.7^{bc}	63.3 ± 16.1^{abc}	3.3 ± 2.9^{d}	30.0 ± 18.0^{a}	*
15	Germination	80.0 ± 13.2^{a}	68.3 ± 5.8^{a}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	**
	Recovery (RP)	-	35.2 ± 19.4^{ac}	78.3 ± 7.6^{b}	63.3 ± 7.6^{bc}	23.3 ± 17.5^{ad}	1.7 ± 2.9^{d}	33.3 ± 11.5^{a}	*
20	Germination	80.0 ± 10.0^{a}	25.0 ± 21.8^{b}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	**
	Recovery (RP)	-	76.2 ± 9.2^{a}	68.3 ± 19.0^{ab}	63.3 ± 10.4^{ac}	46.7 ± 15.3^{bcd}	0^{e}	18.3 ± 10.4^{d}	*
	Germination	ns	ns	ns	ns	ns	sn	ns	
	Recovery (RP)	-	ns	ns	ns	ns	ns	ns	

Mortality

For both species, figure 13 shows the estimate of the relationship between NaCl concentration and seed mortality percentages at different temperatures. For *L. pallescens*, at 10°C and 15°C, the regression lines showed that mortality increased with NaCl concentrations and temperatures (with r^2 = 0.70 and 0.76, for 10°C and 15°C, respectively) and for both temperatures, percentages differed significantly ($p < 0.05$) among salinities. At 20°C, significant differences were showed in the NaCl concentrations range from 0 mM to 300 mM ($p < 0.05$) and the linear regression equation showed highest values of r^2 (0.92), while in the range of salinity between 300 mM and 600 mM, regression was not significant (Fig. 13A). At 20°C the increase of seed mortality velocity was much greater than that detected at 10°C (with angular coefficient values of straight line of 0.30 and 0.08, for 20°C and 10°C, respectively; Fig. 13A), showing that seed mortality velocity increased with the increase in temperature. For *L. triloba*, at all the three tested temperatures, final mortality was significantly ($p < 0.05$) different among tested salinities and increased with NaCl concentration, with r^2 values of linear regression equations of 0.58, 0.72, 0.86, for 10°C, 15°C and 20°C, respectively (Fig. 13B). Seed mortality velocity increased sligthly with temperature (with angular coefficient values of straight line of 0.10, 0.12 and 0.15, for 10, 15, 20°C).

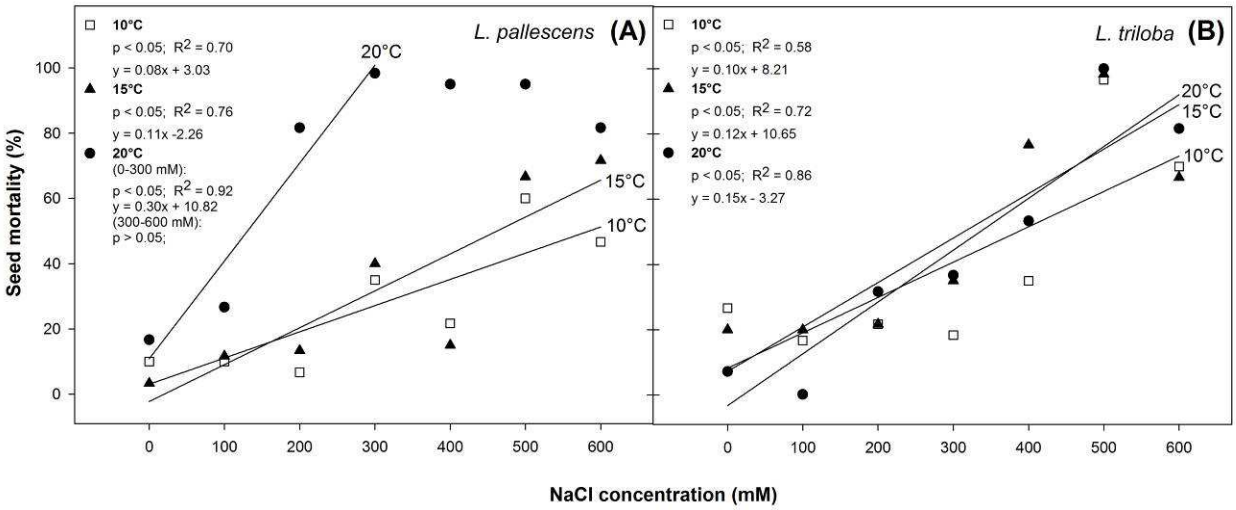

Figure 13 - Mortality of seeds of *L. pallescens* **(A) and** *L. triloba* **(B) at the tested NaCl concentrations (0-600 mM) and different temperatures (10, 15, 20°C). Black lines indicate linear regressions for each temperature of each species. Each symbol is the mean of three replicates.**

Inter-population variabiliy

Seed germination significantly decreased ($p < 0.05$) with the increase in salinity for all populations and the highest germination percentages were detected under control condition, ranging from 98.3 ± 2.8% for Lt2 to 80.0 ± 13.2% for Lt3. At 200 mM, germination values for Lt2 and Lt4 (6.7 ± 11.5% and 16.7 ± 20.8%, respectively) showed significant differences ($p < 0.05$) with non-saline control, while, for Lt3, were not observed germinated seeds (see Table 6). Final germination was totally inhibited at NaCl concentrations higher than 200 mM for all populations.

Recovery response showed significant decreases ($p < 0.05$) with the increase of salinity, to which seeds were exposed, among RP of all populations, although, for Lt3, no differences ($p > 0.05$) were detected, with percentages ranging from ca. 23% at 400 mM to ca. 80% at 200 mM (see Table 6). RP ranged from ca.17% at 600 mM to ca. 100% at 200 mM for Lt2 and from ca. 4% at 400 mM to ca. 75% at 200 mM for Lt4. For all three populations, a decrease of RP was detected from lower salinities (200 mM) to higher NaCl concentrations (600 mM). No significant differences ($p > 0.05$) were detected among populations at the same salinity, with the exception of

400 mM, for which RP values ranged from 93.3 ± 2.9% for Lt2 to 3.3 ± 2.9% for Lt4 ($p < 0.05$) (see Table 6).

Table 6 - Inter-population variability of *L. triloba* in response to NaCl for germination and recovery percentages (RP) at 15°C. Kruskal-Wallis tests were conducted to detect the effect of the same population on germination percentages and RP and that of the same salinity on germination percentages and RP; [p values were considered not significant ($p > 0.05$, ns) and significantly different ($p < 0.05$, *) by Kruskal-Wallis test]. Data are the means (± 1 SD) of three replicates. Capital letters in columns are related to the same salinity, while lower-case letters in rows to the same temperature. Values with different letters were used to indicate significant differences at $p < 0.05$ (Mann Whitney *U-test*). For the explanation of population code, see Table 1.

Code	Percentage (%)	NaCl concentration (mM)				
		0	200	400	600	
Lt2	**Germination**	98.3 ± 2.8^{a}	6.7 ± 11.5^{b}	0^{b}	0^{b}	*
	Recovery (RP)	-	98.3 ± 2.9^{a}	93.3 ± 2.9^{aA}	16.7 ± 7.7^{b}	*
Lt3	**Germination**	80.0 ± 13.2^{a}	0^{b}	0^{b}	0^{b}	*
	Recovery (RP)	-	78.3 ± 7.6	23.3 ± 17.5^{B}	33.3 ± 11.5	ns
Lt4	**Germination**	81.7 ± 2.9^{a}	16.7 ± 20.8^{b}	0^{b}	0^{b}	*
	Recovery (RP)	-	75.2 ± 12.0^{a}	3.3 ± 2.9^{bB}	15.0 ± 5.0^{b}	*
	Germination	ns	ns	ns	ns	
	Recovery (RP)	-	ns	*	ns	

Salt spray

Seedling survival

For *L. pallescens*, 100% of seedlings under control condition (no spray) and nebulized two days per week, 92.3% of those nebulized one day per week and 76.9%, when the frequency was three days per week, survived at the end of salt spray treatment (Figure 14A and Fig. 15). For *L. triloba*, seedling survival drastically decreased with the increase of salt spray nebulization. Under no spray

condition, 100% of seedlings survived, while 92.3% for one day per week nebulized seedlings. When salt spray nebulization was of two days per week only 30.8% of seedlings survived and none for the three days per week treatment (Figure 14B and Fig. 15).

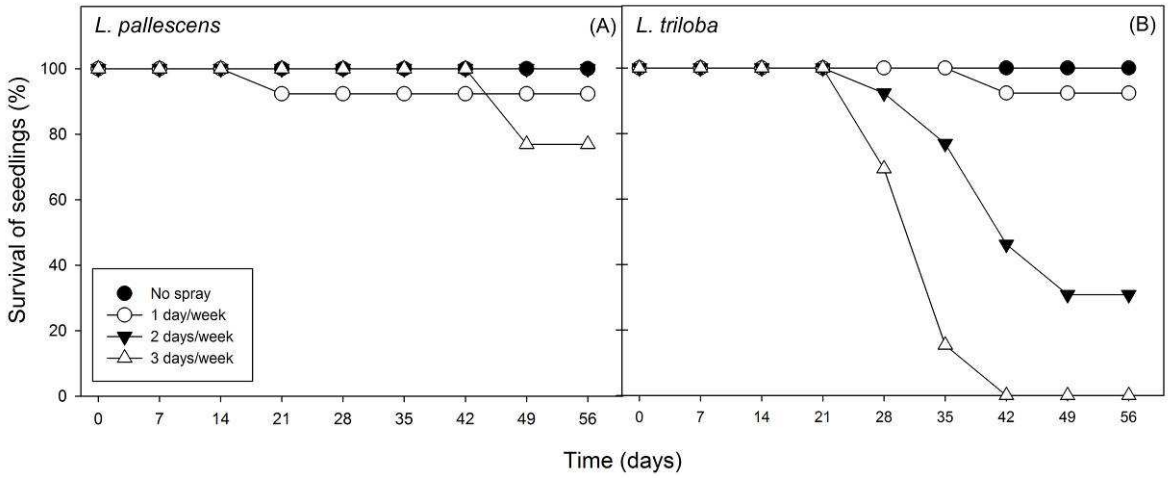

Figure 14 - Survival of seedlings of *L. pallescens* **(A) and** *L. triloba* **(B), for each treatment (no spray, one day/week, two days/week, three days/week), during eight weeks of salt spray solution (600 mM NaCl) nebulization.**

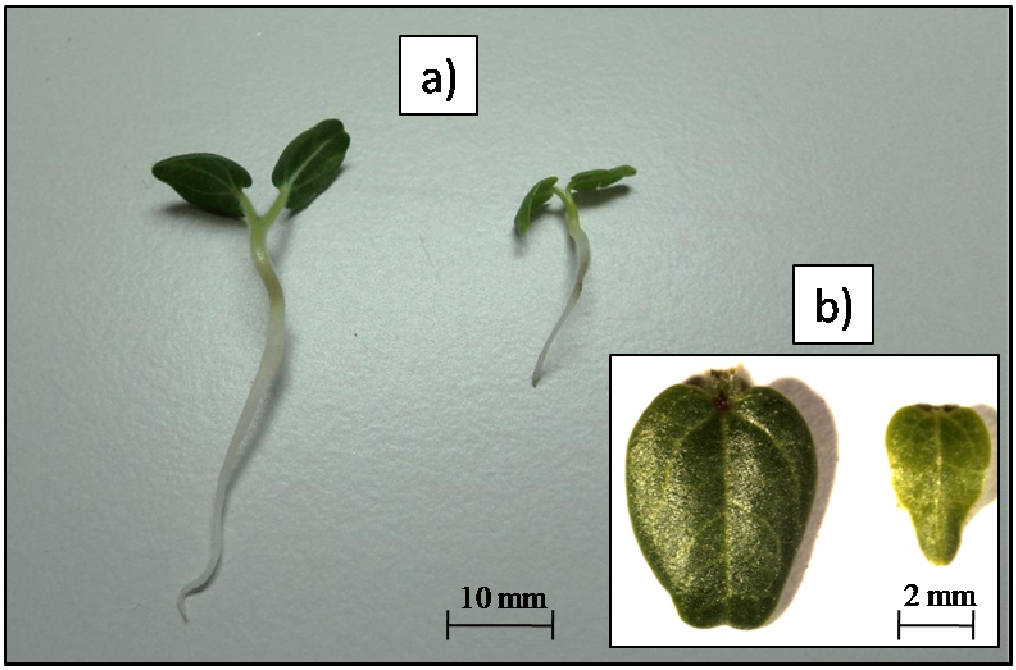

Figure 15 - Seedlings (a) and particular of cotyledons (b) of *L. triloba* (on the left) and *L. pallescens* (on the right).

Seedling dry weight, epigeal and hypogeal parts

No significant differences ($p > 0.05$) were detected on mean dry weight of *L. pallescens* seedlings among salt spray frequencies, with values of ca. 0.02 g for each seedling, independently on the treatment (Figure 16A). On the contrary, the frequency of salt spray nebulization had a significant effect ($p < 0.05$) on *L. triloba* seedlings. Mean dry weight under control condition (ca. 0.04 g for each seedling) resulted statistically higher ($p < 0.05$) than for one- and two-days nebulized seedlings values (ca. 0.02 g for each seedling for both treatments; Figure 16A). For *L. triloba*, the data for the frequency of three-days/week are missing due to none seedling survived until the octave week of salt spray treatment (Figure 14B and 16A).

The length of epigean part of *L. pallescens* seedlings significantly decreased ($p < 0.05$) with the increase of salt spray nebulization. Higher values were detected for seedlings under control condition and one-day per week treatment (ca. 50.0 mm, for both conditions) than from that of other treatments (ca. 37.0 mm, both for two- and three-days per week spraying; Figure 16B). Also for *L. triloba*, nebulization frequency significantly ($p < 0.05$) affected seedling mean length of epigeal part. The higher values were detected under no spray condition (ca. 70.0 mm for each seedling), and significantly ($p < 0.05$) than from those measured in the two-days per week treatment (ca. 58.0 mm for each seedling; Figure 16B). For the same reasons above citied, the data of *L. triloba* seedlings for the three-days/week frequency are missing.

The increase of frequency of nebulization significantly ($p < 0.05$) affected also the length of hypogeal parts of *L. pallescens* seedlings. Higher values were detected under no spray condition (ca. 52.0 mm) than from all treatments (see Figure 16C). The lowest root lengths were measured for the three-days per week treatment, with values of ca. 25.0 mm for each seedling and did not show significant ($p > 0.05$) differences only with values of two-days/week sprayed seedlings (ca. 30.0 mm). Also for *L. triloba* nebulization frequency had a significant ($p < 0.05$) effect on root

length of seedlings. The higher values, detected under control condition (ca. 65.0 mm) significantly ($p < 0.05$) differed from one- and two-days per week sprayed seedlings which root length values of ca. 29.0 mm (Figure 16C). For the same reasons above citied, the data about *L. triloba* seedlings for the three-days/week frequency are missing.

During the salt spray experiment, conductivity values of substrate at the end of the experiment (eight weeks) significantly differed ($p < 0.05$) among frequencies of salt spray nebulization, ranging from 47.2 ± 7.8 mM to 272.4 ± 7.6 mM, for control conditions (no spray) and three-days/week sprayed pots, respectively. pH values significantly differed ($p < 0.05$) among frequencies of salt spray treatment, ranging from 4.8 ± 0.1 to 4.6 ± 0.1, for no sprayed and three-days/week nebulized pots, respectively. No statistical correlation ($p > 0.05$) was found between these soil parameters and seedling growth data for both species, except for of the root lengths of *L. triloba* which significantly decreased ($p < 0.05$) as soil conductivity increased.

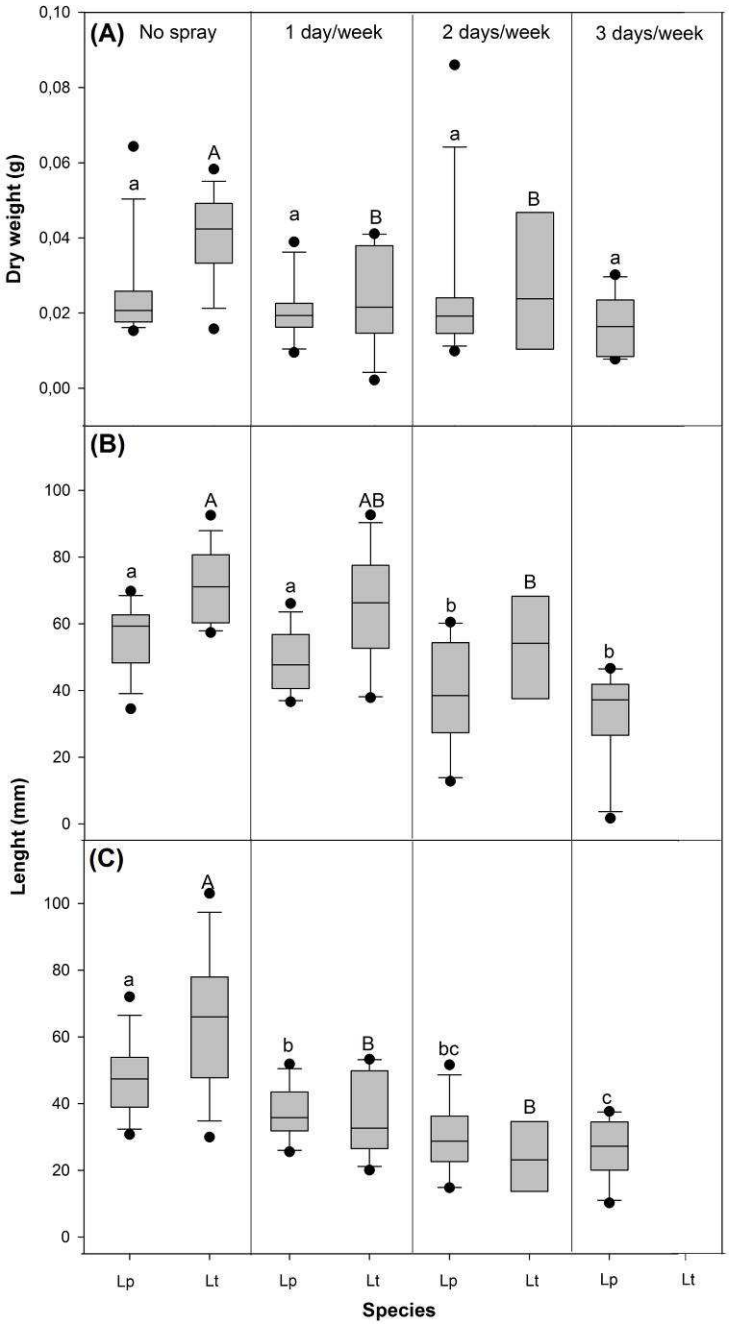

Figure 16 - Dry weight (A), lenght of epigean (B) and hypogean part (C) of seedlings of *L. pallescens* **(Lp) and** *L. triloba* **(Lt) for each treatment (no spray, one-day/week, two-days/week, three-days/week), after eight weeks of salt spray solution (600 mM NaCl) nebulization. Kruskal-Wallis test was conducted, for each species, to detect the effect of treatment on seedlings growth. Letters (lower-case for** *L. pallescens* **and capital for** *L. triloba*) **were used to indicate values different at p < 0.05 (Mann Whitney *U-test*). For each treatment, data are the mean of survived seedlings after eight weeks from the beginning of experiments.**

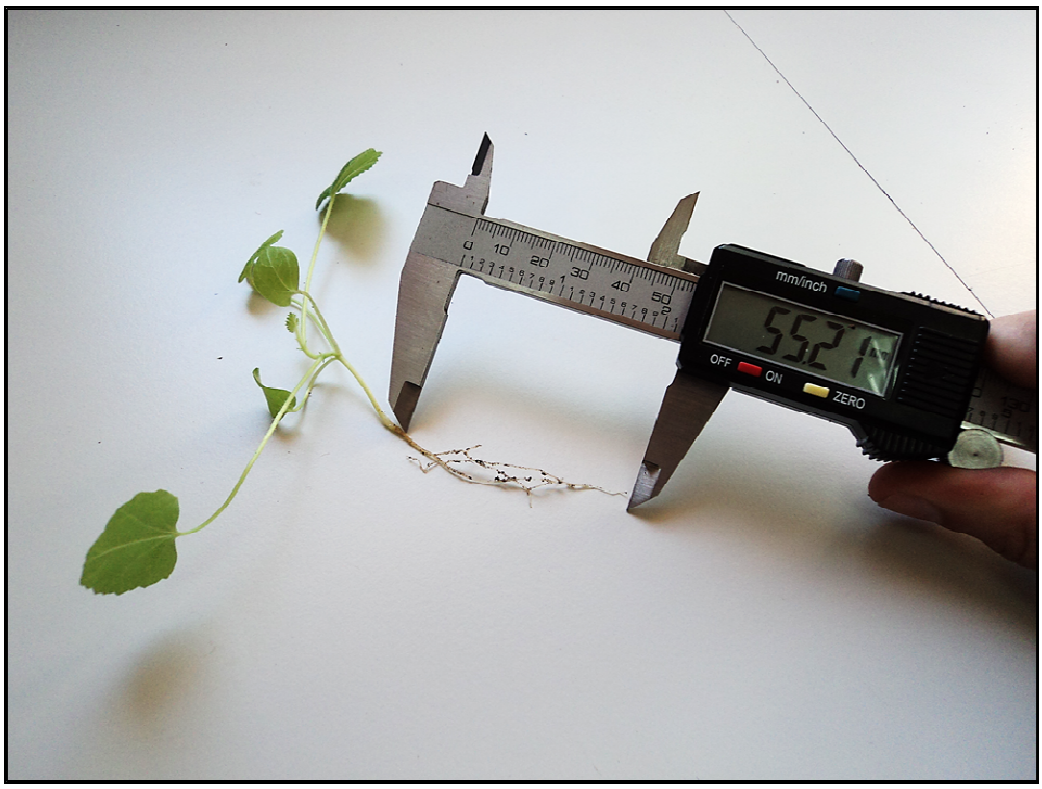

Figure 17 - Measurement of hypogeal part in *L. triloba* seedling.

Discussion

Mechanical scarification highly improved water uptake in all the three species of this study, highlighting the need of this pretreatment and the presence of a physical component of dormancy, (PY) as reported for several *Lavatera* species (Royal Botanic Gardens of Kew, 2008). In nature, various biotic and abiotic factors can produce seed scarification, including extreme temperatures (e.g. fire or chilling), changes in the chemical environment (e.g. seed ingestion by frugivores and passage through the digestive tract) and mechanical abrasion with rocks (Vilela & Ravetta, 2001). Several studies on various species showed enhancement of germination by mechanical scarification (Baskin & Baskin, 1998; Argaw *et al*., 1999; Sy *et al*., 2001) that could be attributed

to the increase in water uptake and also reducing the mechanical resistance to the protrusion of the radicle. For the species investigates in this study, the most reasonable and probable scarification modality, considering the natural environment of each species, may be abrasion with rocks (in particular for *L. pallescens*) and ingestion by birds.

Thanos *et al.* (1989, 1995) found that germination of several Mediterranean species is photo-inhibited, highlighting a surface avoiding mechanism, which enables seeds to avoid germinating under the harsh conditions of the soil surface. However, seeds of all the three *Lavatera* species, which achieved high germination percentages both in the light and in the dark, did not show this kind of surface-avoiding mechanism, therefore resulting not photo-inhibited for germination. Grime *et al*. (1981), in a survey study of 271 species, found that species with seeds that weigh less than 0.1 mg were largely light-requiring, and that the incidence of light-dependence declined with increasing seed size. The indifference to irradiance of *L. agrigentina* seeds, with a mass of ca. 8 mg, confirmed this observation, as well as for *L. pallescens* (ca. 3 mg) and for *L. triloba* (ca. 5 mg).

For all the three *Lavatera* species of this study the higher germination rate and percentages detected in the range 10-20°C (*L. agrigentina* and *L. triloba*) and 5-15°C (*L. pallescens*) and the significant decrease of germination at the highest temperature (25°C) are in accordance with Thanos *et al*. (1989; 1995), for which germination at low temperatures is a widely extended trait in many Mediterranean species. Germination in a period from autumn to spring (when water availability, soil moisture and rainfalls are high, and temperatures are not prohibitive for germination and consequent seedlings establishment) ensures ecological success in an unpredictable climate such as that Mediterranean (Thanos *et al*., 1995; Kadis & Georghiou, 2010).

Inter-population variability in seed germination was detected for both *L. agrigentina* and *L. triloba*, also between considerably near populations (e.g. only ca. 9 km between La1 and La2), but

germination behavior may vary greatly within a single species from one population to another, from year to year and among individuals (Urbanska & Schütz, 1986), in function of environmental factors (e.g. light, moisture, temperature, nutrients, substrate, altitude) (Fenner, 1991; Gutterman, 1992) during seed maturation (Meyer *et al*., 1989).

For *L. agrigentina* the dry after-ripening pretreatment highly promoted germination at all temperatures highlighting a physiological componenet of seed dormancy (PD). In particular the pretreatment widened the germination range both at low and high temperatures (type 3 of nondeep PD; Baskin and Baskin, 1998, 2004). Therefore this species exhibits combinational dormancy (PY+PD). Dry after-ripening pretreatment was able to break physiological dormancy and scarification also permitted germination. Combinational dormancy is evident in seeds with water-impermeable coats (as in PY) combined with physiological embryo dormancy (Baskin & Baskin, 2004) such as that of *Sicyos angulatus* (Qu *et al*., 2011), *Geranium robertianum* (Vandelook & Van Assche, 2010) and genus *Trifolium* (Fenner & Thompson, 2005). The application of the dry after-ripening treatment had not a significant effect on *L. pallescens* and *L. triloba* seed germination, showing as seeds of these two species do not need of a dry summer period which forego germination, and exhibit only a physical dormancy.

L. pallescens seeds germinated with NaCl in the substrate at all tested concentrations (until 600 mM), although in salt substrate were observed lower germination percentages, in comparison with that higher under control conditions (0 mM NaCl) and temperature influenced germination under salt stress. Many studies report that percentages of germination decreased with increased salinity stress and highest germination occurs in absence of NaCl in the substrate (Khan & Ungar, 1984, El-Keblawy *et al*., 2010; Vallejo *et al*., 2010). Recovery response was influenced by temperature but not by NaCl concentration. The highest tested temperature (20°C) interfered with seeds recovery and amplified the deleterious effect of salinity in their capability to recover from

saline conditions causing total mortality at the highest salinities (500 and 600 mM). Similar effects were previously detected by Guma *et al*. (2010) for *Salsola vermiculata*. It is well known that the limit of tolerance to salt vary among different also phylogenetically close, species (Ungar, 1995). For *L. triloba*, differently to *L. pallescens*, temperature did not influence germination, high recovery response capability was observed at all tested temperatures and decreased in function of salt concentration to which seeds were exposed in the previous NaCl experimental phase. Seed mortality of both species increased with salinity, although *L. pallescens* showed higher sensibility in salinity-temperature interaction respect to *L. triloba*. Several studies reported that salt stress negatively affected seed germination, with consequent seed mortality, either osmotically through reduced water absorption or ionical, through the accumulation of Na^+ and Cl^-, causing an imbalance in nutrient uptake and toxicity effect (Baskin & Baskin, 1998; Ungar, 1995; Vallejo *et al*., 2010). Salinity-temperature interactions may have significant eco-physiological implications in terms of time of germination under field conditions (Ungar, 1995). The different NaCl tolerance and recovery response behavior for the two *Lavatera* species confirm as asserted by Khan and Ungar (1984), that is that tolerance and recovery from salinity and temperature stress are species specific as well as that seeds of some species did not recover or showed little recovery response when subjected to high salinity and temperature stress (Khan & Gul, 2006).

For *L. triloba*, different behavior in germination in NaCl and recovery capability were detected among populations (Table 6), confirming (1) the ability of *L. triloba* seeds to germinate at low NaCl concentrations in the substrate, (2) the occurrence of inhibition of germination when seeds were exposed to highest concentrations (> 200 mM), (3) the capability of seeds to have high recovery of germination when subjected to low NaCl concentrations and (4) the deleterious effect of salinity increase on seed viability. Intra-specific variability in germination patterns has been reported for several species (Bischoff *et al*., 2006; Kremer *et al*., 2009; Bischoff & Müller-

Schärer, 2010). Differences in salt stress response were showed among populations of *Panicum turgidum* (El-Keblawy *et al*., 2010) and *Spartina patens* (Hester *et al*., 1996), but not in *Crucianella maritima* (Del Vecchio *et al*., 2012). According Gutterman (1994) and Kigel (1995) the variability of germination characteristics could be interpreted as one of the most important survival strategies for species growing under unpredictable environmental conditions.

L. pallescens seedlings showed both an high capability to tolerate salt spray with a high life span (≥ 80%) also at the highest frequency of nebulization. The habitat where this species grows (coastal limestone cliffs), due to its morphology and high wind exposition, receives significant quantities of marine aerosol, so this species is constantly exposed to this environmental abiotic factor. It may be reasonable to assume that this species has evolved adaptations to tolerate this factor, ineluctable in its habitat. Therefore, the frequency of nebulization did not influence seedling biomass, while increase in salt spray nebulization caused a reduction in seedling growth. Probably, in total absence of this environmental factor in natural habitat, seedlings would grow with a faster rate. The increase of salt spray nebulization on seedling development of *L. triloba* showed as seedling life span was highly influenced by frequency with an inverse proportionality and only small quantities of salt spray did not interfere with seedlings survival. Biomass production as well as seedlings lengths (both epigeal than hypogeal) were negatively affected by salt spray, demonstrating as for *L. triloba,* the optimal condition would be absence of marine aerosol. The results obtained in salt spray experiments highlighted the different sensibility to this factor between the two *Lavatera* subspecies. In fact, the higher distance from seashore (ca. 1 km) of *L. triloba* shows as distance and/or interposed vegetation may determine a lower impact of salt spray for this species, respect to *L. pallescens*, directly exposed to this factor (< 50 m). Therefore, *L. triloba* appears adapted to its habitat (Table 1), with germination at low salt concentrations and high capability to recover from saline conditions, when salt concentration level is reduced by

rainfalls. *L. pallescens* instead, growing in an habitat where spindrifts by marine waves and salt spray tend to accumulate, into rocks fissures, small quantities of marine water and consequent NaCl, is a species adapted to germinate even to salt concentrations equal to seawater. The ability to tolerate and possibly adapt to airborne saltwater sprays may be critical to the maintenance of coastal plant populations (Maun, 1994; Greipsson & Davy, 1996). In coastal communities, the distribution of species can sometimes be tied to their tolerance of salt spray (Sykes & Wilson, 1988; Wilson & Sykes, 1999).

In conclusion, our results highlighted the presence of a combinational dormancy in *L. agrigentina* and of physical dormancy in both *L. pallescens* and *L. triloba*, as reported for other Malvaceae (Finch Savage & Leubner-Metzger, 2006). For the three *taxa*, light, temperature and dry after-ripening did not affect final germination percentages, while this pretreatment highly promoted germination in *L. agrigentina*. Seed germination behavior was in accordance with that of other "typical" Mediterranean plants, for which germination at low temperatures is a widely extended trait. Our results confirmed that L. pallescens and L. triloba could be distinguished not only for morphological and molecular characters, but also for their different seed ecology and response to the same abiotic factors. These findings are coherent with a field germination in a period between autumn and spring, when temperatures and salinity concentrations in the soil are low and moisture in the soil is high, representing an advantageous ecological adaptation towards the unpredictable Mediterranean rainfall pattern (Thanos *et al*., 1989, 1991).

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Chapter V - Light, temperature and salt stress responses on seed germination of *Halopeplis amplexicaulis* (Vahl) Ces., Pass. & Gibelli

Abstract

H. amplexicaulis is a halophytic species widespread throughout the Mediterranean area. Light and temperature requirements for germination were evaluated by incubation at 12 h of irradiance per day and darkness at constant (5-25°C) and alternating (25/10°C) temperatures regimes. Salt stress effect (0-600 mM NaCl) and its recovery on seed germination as well as seedling salt spray tolerance were also investigated. Light did not affect final germination of seeds, with highest germination percentages (ca. 90%) detected at 25/10°C. Seeds germinated up to 600 mM NaCl, salt did not affect seed viability and recovery response did not decrease with increasing salinities. High seedling sensibility to highest salt spray frequency was detected for this species. Soil analysis in the field revealed considerable variations in salt soil concentrations between seasons and depths. Our results highlighted that this halophytic species has its optimum for germination in autumn when, under a Mediterranean climate, water availability is highest and soil salinity levels are minimal.

Keywords: *Amaranthaceae*, conductivity, NaCl, recovery, salt marshes, salt spray.

Introduction

Salt marshes are among the most valuable ecosystems (Costanza *et al*., 1997; UNEP, 2006) and are characterized by fine sediments and halophytic vegetation, often with a significant component of annuals plant species (Watkinson & Davy, 1985). However, this typology of habitats is exposed to increasing threats due to the development of human activities such as tourism and pollution (Gedan *et al*., 2009) and the human-driven alterations can induce drastic vegetation changes (Roman *et al*., 1984; Leagdsgaard, 2006; Álvarez-Rogel *et al*., 2007).

Salt marsh species have to endure strong physiological stress due to soil salinity and drought (Chapman, 1974; Chen *et al*., 2002), especially in the Mediterranean area (Álvarez-Rogel *et al*., 2000). Such species show morphological and physiological adaptations allowing the avoidance of damaging and lethal consequences of these environmental factors (Hellings & Gallagher, 1992; Justin & Armstrong, 1987; Baumberger *et al*., 2012). The existence of spatial-temporal gradients of soil salinity and moisture has traditionally been considered one of the most important physical factors in the plant zonation of salt marshes (Chapman, 1974). These soil-plant relationships are particularly interesting under Mediterranean climate, where the areas farthest from the coast are not always those with the lowest soil salt concentrations (Callaway *et al*., 1990; Pennings & Callaway, 1992). Alternating periods of rainfall, during which salts are leached towards the deepest soil horizons, and periods of drought when they are brought to the surface horizons, bring about an important variation in salinity, both in regard to the quantity and type of salt (Chapman, 1974; Alvarez-Rogel *et al*., 1997; 2000).

The most critical stages in the life cycle of halophytes are seed germination and seedling establishment (Ungar, 1982). Germination in the field is controlled by several environmental factors, in particular water availability, light, temperature and salinity, and their interaction are very important (Baskin & Baskin, 1998). Halophytic species vary in their tolerance to salinity

during seed germination (Khan *et al*., 2002; Ungar, 1995) and they can recover the capability to germinate after exposure to salt stress that inhibits germination (Woodell, 1985; Khan, 2003). Salt spray is an abiotic factor of great importance for coastal species, influencing seedlings establishment and growth (Maun, 2009).

Halopeplis amplexicaulis (Vahl) Ces., Pass. & Gibelli is a salt marsh annual species belonging to the Amaranthaceae. The Seed Information Database (Royal Botanic Gardens Kew, 2008) reports high germination percentages (100%) at the alternating regime of 25/10°C, in the light (8 h of irradiance per day), without pre-treatments. Albert *et al*. (2002) investigated the effect of pre-treatments (manual scarification and dry heat) and of constant (15°C, 20°C, 25°C) and alternating temperatures (15/25°C), with 16 hours of irradiance per day, on seed germination of two populations of this species from NE Spain. These authors showed that all pre-treatments enhanced final germination, a positive effect of the tested alternating temperature regime respect to constant temperatures and the absence of inter-population variability in seed germination. Tremblin and Binet (1982) studied the effect of light and darkness on seed germination of *H. amplexicaulis* and germination in NaCl (from 50 mM to 600 mM). In this study the following findings were found: (1) the highest germination percentages were at 20°C and that germination was totally inhibited at 35°C; (2) darkness slowed germination, but after ten days of incubation, final germination was the same for light and dark conditions; (3) high germination percentages were detected up to 300 mM NaCl and germination occurred up to 500 mM. Tremblin (1982) reported that in Algeria germination in the field started in January, flowering and fruiting occurred in June and August, while seeds ripened in September. Tremblin and Binet (1984) tested the effect of two conditions (28°C with 55% of relative humidity and 25°C with 80% of r.u.) in presence of different salinities (up to 200 mM) and the effect of NaCl + Na_2SO_4 (50 mM + 50 mM), showing that sulphates affected plant growth of *H. amplexicaulis*. Moreover, these authors measured water

content, biomass and N content in plant from field and from laboratory culture (66 days at 100 mM NaCl), observing no differences in chemical compositions. Tremblin and Ferard (1994) measured growth and roots/shoots ions accumulation (Na^+, Cl^-, K^+, $SO4^{--}$) growing at different NaCl concentrations (from 10 mM to 500 mM) and showed that growth (dry mass production) was optimum when seedlings were exposed to 200 mM and 300 mM NaCl. De Martis *et al*. (1988) tested germination in NaCl (18‰, 36‰ and 72‰ NaCl, ca. 300 mM, 600 mM and 1200 mM NaCl, respectively) at 20°C in light (12/12 h) of *H. amplexicaulis* seeds, collected in the same Sardinian station of this study; these authors observed high germination percentages (ca. 76%) under control condition (0 mM), ca. 79% at 18‰ NaCl, ca. 20% at 36 ‰ NaCl and ca. 2% at 72‰ NaCl and showed a salt induced dormancy at higher NaCl concentrations. The same authors argued that the favourable period for seedling emergence could be starting from February, while months of July, August and December would unfavourable for seedling establishment of this species in Sardinia. No research investigated the recovery response of *H. amplexicaulis* seeds after exposition to NaCl and no data is available on the effects of salt spray on seedling growth and establishment of this species.

Seeds of *H. amplexicaulis* are reported to be orthodox (Royal Botanic Gardens Kew, 2008) and Martin (1946) described for Chenopodiaceae (now included in the Amaranthaceae; Peruzzi, 2010) a peripheral embryo with firm to flinty and glass-like to whitish endosperm. Finch-Savage and Leubner-Metzger (2006) reported seeds of Chenopodiaceae as physiologically dormant (PD) or non dormant (ND).

The aims of this study were to: (1) characterize seed germination of this species, by identifying its germination requirements in terms of light and temperature and evaluating the effect of an alternating temperature regime; (2) evaluate the effects of NaCl and recovery on its seed germination; and (3) evaluate the effects of salt spray on seedlings development.

Materials and Methods

Study species

Halopeplis amplexicaulis (Vahl) Ces., Pass. & Gibelli is a scapous therophyte, with an erect glaucous stem, 5-20 cm high (Pignatti, 1982; De Martis, 2011; Figure 1). Leaves are distant, with lamina of 2 mm, spikes of 5-15 mm, and bracts ovate-orbicular (Tutin *et al*., 1993). Seeds are 0.5-0.8 mm, with cylindrical papillae (Aguilella *et al*., 2009; Figure 1).

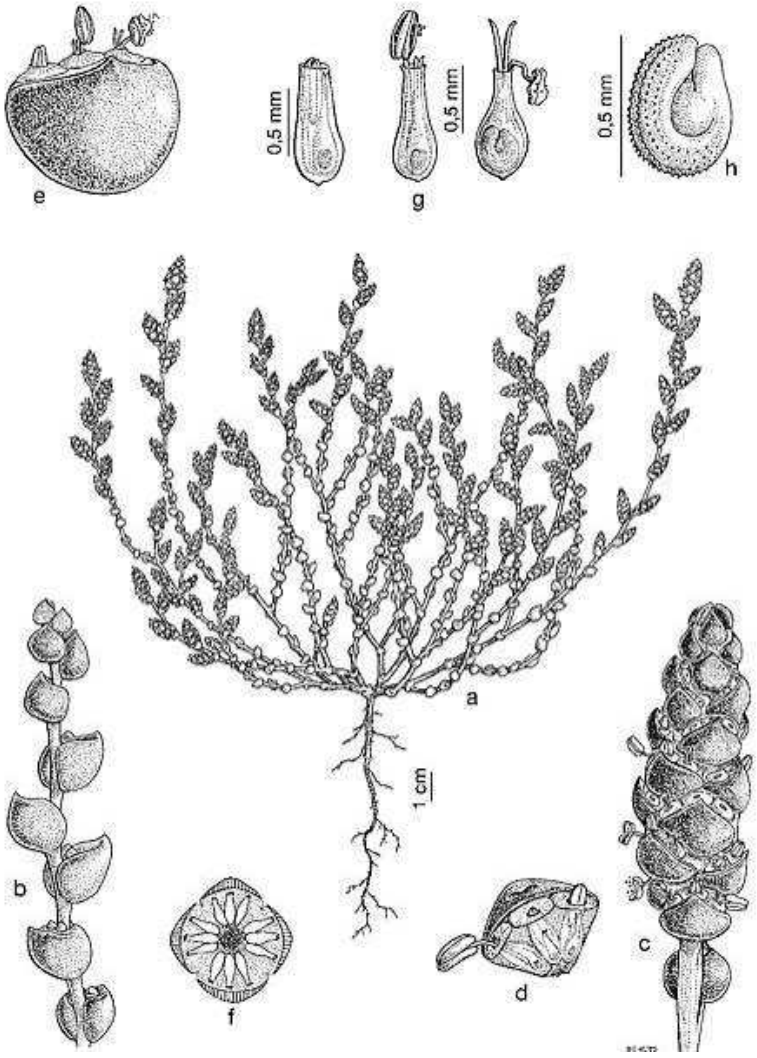

Figure 1 - *Halopeplis amplexicaulis***: a) general habit; b) branch with flowers; c) flowers in anthesis; d) internal part of a bractea and flowers; e) external part of a bractea and flowers; f) cross section of inflorescence with radial disposition of the flowers; g) different phases of flower maturation; h) seed. (from Castroviejo, 1990).**

Flowering occurs in June and fruiting in August. This species, typical of salt marshes and coastal saline swamps (Yaprak, 2006), is widespread throughout Mediterranean countries (Portugal, Spain, Sardinia, Sicily, Italy, Bulgaria, Asiatic Turkey, Cyprus, Lebanon, Syria, Israel, Jordan, Egypt, Libya, Tunisia, Algeria and Morocco; Greuter *et al.*, 1984; Blanche & Molero, 1987; Tremblin, 2000). In Italy, this species is inserted in IUCN Lists as vulnerable "VU" at national and regional (Sardinia) levels (Conti *et al*., 1992; Conti *et al*., 1997; Scoppola & Spampinato, 2005).

Seed lot details

Achenes of *H. amplexicaulis* (hereafter seeds) were collected at the time of natural dispersal (August 2011) in the "Saline di Molentargius"(South-Sardinia; Figure 2). This site is characterized by a set of solar salterns and in the past this human-made environment was exploited to obtain halite (NaCl) for human consumption and industrial purposes, through a process based on the evaporation of brines using the sun and the wind as the energy sources. *H. amplexicaulis* plants grow on clay substrate (at 1 m a.s.l) on the banks of these artificial evaporation basins. This population is the only known station for the island (De Martis *et al*., 1988). Seeds were cleaned with tweezers and separated from other plant residues with immersion in water. A seed mass of 0.12 ± 0.04 mg (mean ± SD) was calculated by weighing ten replicates of 50 seeds each.

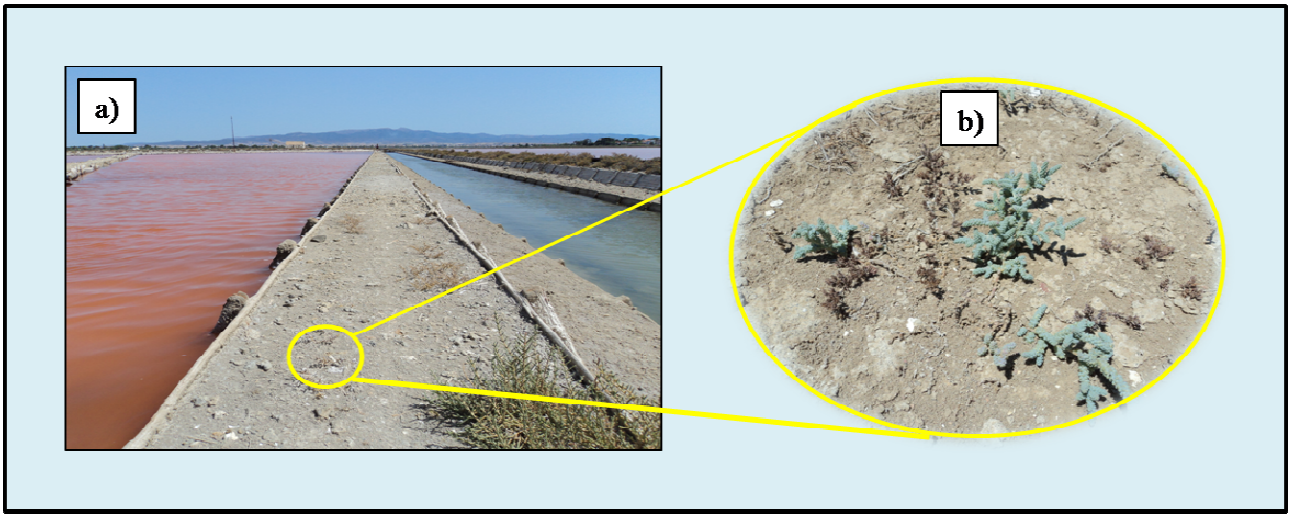

Figure 2 - "Saline di Molentargius" (a) and particular of *H. amplexicaulis* in its habitat (b).

Soil analysis

Soil samplings were conducted in the period of highest drought (August 2012; summer) and of highest rainfalls (December 2012, winter) to evaluate salt concentrations through seasons. For each season, three samplings was carried out random, in the area where *H. amplexicaulis* grows, at three different depths (0-5 cm; 5-10 cm and 10-15 cm; Figure 3a). Conductivity measure were conducted through conductometer microCM200 (Crison) at the Sardinian Germplasm Bank (BG-SAR), for each depth and investigated season (Figure 3b).

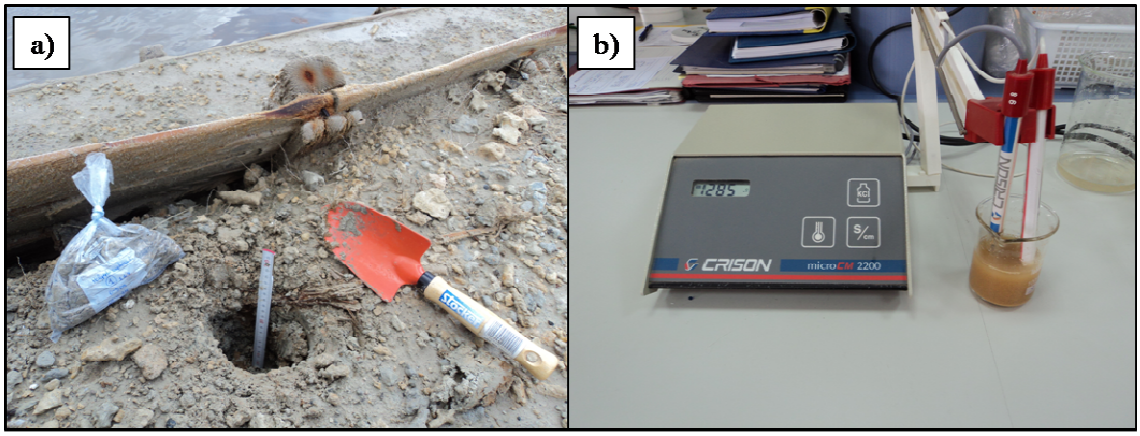

Figure 3 - Soil sampling at the "Saline di Molentargius" (a) and conductivity measure (b).

Germination tests

Effect of light and temperature

Germination tests were conducted in 2011 on 1% water agar substrate, which provided a solid, non-sterile medium for germination, in plastic Petri dishes of 60 mm diameter. Three replicates of 20 seeds each were incubated in growth chambers, in a range of constant temperatures (5, 10, 15, 20 and 25°C) and at an alternating temperature regime (25/10°C) in the light (12 h of irradiance per day) and in the dark, in growth chambers (SANYO MLR-351). In the alternating temperature regime, the higher temperature period coincided with the light period (Baskin *et al.*, 2006). Darkness was achieved by wrapping dishes in two aluminum foils. The criterion for germination was visible radical protrusion. Seeds incubated in the light were scored daily and germinated seeds discarded, while seeds incubated in the dark were scored only at the end of the test to avoid any exposure to irradiance (Baskin *et al*., 2006). When no additional germination occurred for two consecutive weeks, the viability of any remaining seeds was checked. The low number of replicates and of seeds per replicate used in all experiments were due to a limited seed availability, resulting from this species being endangered and rare and were chosen in order to allow testing a wide range of germination conditions.

Effect of NaCl on seed germination and recovery

To evaluate the effect of salt stress on seed germination, three replicates of 20 seeds each, were sown in 1% water agar substrate, with different NaCl concentrations (0, 100, 200, 300, 400, 500, 600 mM) and incubated in an alternating temperature regime (25/10°C), in the light (12/12 h). After two consecutive weeks without additional germination under control conditions (NaCl 0 mM), non-germinated seeds were washed with distilled water and then sown in new Petri dishes

containing 1% water agar substrate for additional 30 days (recovery phase) at the same incubation temperatures.

Effect of salt spray on seedling development

To evaluate the effect of salt on the seedling development, a solution of NaCl (600 mM) was applied by spraying on early seedlings of *H. amplexicaulis*. Seeds were sown on 1% water agar substrate in plastic Petri dishes of 60 mm diameter. In order to obtain the number of seedlings required for the start of the experiments, five replicates of 100 seeds each were incubated in the light at 20°C. One week after seed germination, seedlings were sown in polyethylene pots (70 x 70 x 90 mm) in number of four for each, but only one seedling per pot was kept for the experiment (Figure 4). Before the use, all pots were disinfected by immersion in a solution of NaClO (860 mM) per two hours. Pots were filled by a substrate, constituted by turf (55%), perlite (35%) and coconut fiber (10%), sterilized at 80°C per five hours in an oven. Four replicates of 13 seedlings each per condition, were inserted in a phytotron (8 m^3) at the alternating regime 20/10°C, with 12 h of irradiance per day (the higher temperature period coincided with the light period). Conductivity (conductometer microCM200, Crison) and pH (pH-meter GLP 21, Crison) values of the substrate were measured at the end of experiments. Humidity values inside the phytotron were monitored for all the duration of the experiments by a humid bulb hygrometer and they ranged from 73% (during the light period) to 91% (in the dark period).

For eight weeks, 13 replicates for each treatment were sprayed with a 600 mM NaCl solution (to mimic sea water) at a distance of 200 mm, with different frequencies (1 day/week, 2 days/week and 3 days/week; Cheplick & Demetri, 1999), while other 13 replicates did not get any spraying (control, no spray). The temperature of the salt spray solution was 15°C and all epigeal parts of each seedling were equally exposed to the solution. Weekly the number of dead seedlings was

annotated. After eight weeks, at the end of the experiments, the length of epigeal and hypogeal parts for each survived seedling was measured by a digital caliper and the dry mass calculated by drying in oven at 103°C per 17 hours.

Figure 4 - *H. amplexicaulis* **seedling in the pot for salt spray experiment.**

Data analysis

Conductivity soil values were calculated as the average of three replicates (± 1 standard deviation) for each depth and for each seasonal sampling. Final germination percentage was calculated as the average of the three replicates (± 1 SD) on the basis of filled seeds. For NaCl experiments, the recovery percentages (RP) according to the following equation (Khan & Ungar, 1984):

$$RP = \{[(a-b)/(c-b)] \times 100\},$$

where *a* is the total number of seeds germinated in salt solutions plus those that recovered to germination in the fresh water, *b* is the total number of seeds germinated in saline solutions, and *c* is the total number of seeds. For salt spray experiments, the dry mass (mean ± SD) was calculated by weighing all survived seedlings of each treatment after eight weeks from the beginning of experiment. Germination percentages and RP were analysed by a non-parametric Kruskal-Wallis test, followed by a Mann-Whitney *U-test*. Soil conductivity measure were analysed by two-way ANOVA followed by a Fisher LSD *post hoc* test when $p < 0.05$. All the analyses were carried out using the software Statistica 8.0 for Windows.

Results

Germination tests

Effect of light and temperature

Germination percentages did not show significant ($p > 0.05$) differences between light- and dark-incubated seeds at all tested constant temperatures. The higher germination percentages were detected in the alternating temperature regime of 25/10°C, with germination in the light of 88.3 ± 5.8 % which did not statistically ($p > 0.05$) differ from that detected in the dark (55.0 ± 17.3 % ; see Figure 5). Among the constant temperatures, germination was always lower than 45% both in the light and in the darkness. Values at 5°C, 10°C and 25°C were not significantly ($p > 0.05$) different among themselves and between light- and dark-incubated seeds, with the exception of 15°C and 20°C in the light (13.3 ± 7.6 % and 41.7 ± 37.5 %, respectively; see Figure 5).

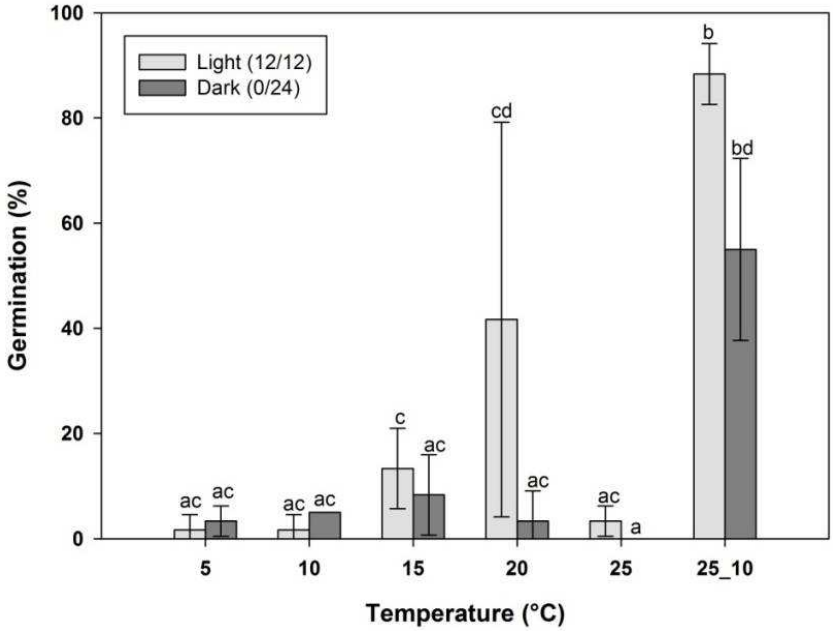

Figure 5 - Germination percentages in the light (12/12 h) and in the dark (0/24 h) at constant (5-25°C) and alternating temperature regime (25/10°C).Values with different letters were significantly different at $p < 0.05$ (by Mann Whitney *U-test*).

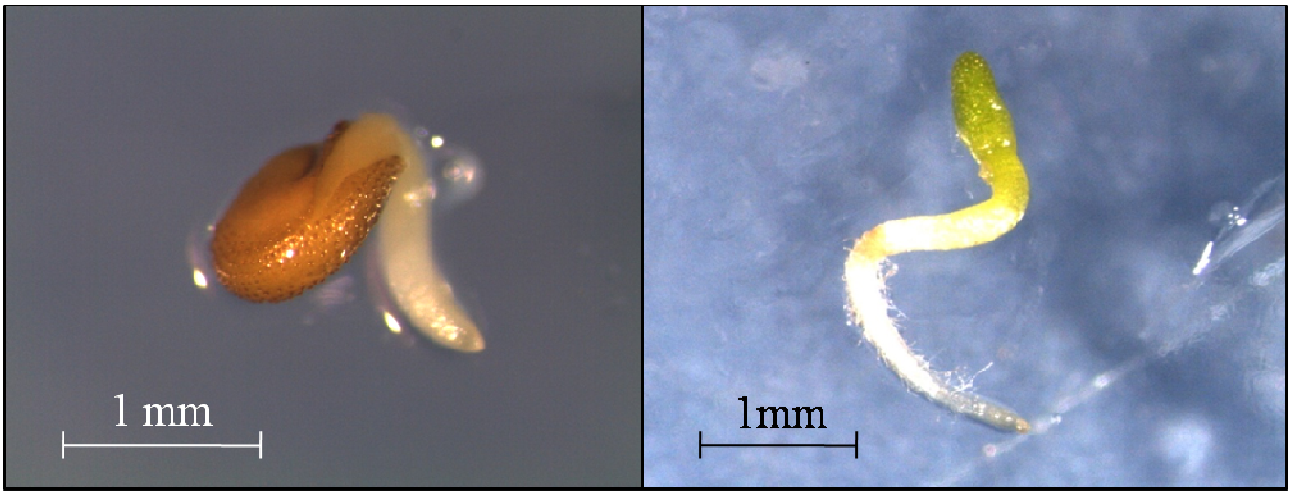

Figure 6 - Germinated seed (on the left) and seedling at 72 hours after germination (on the right) of *H. amplexicaulis*.

Effect of NaCl on seed germination and recovery

Seed germination decreased ($p < 0.05$) with increasing salinities. The higher germination percentage was detected in the non-saline control (0 mM) and was not significantly different ($p > 0.05$) from those detected at 100 mM and 200 mM NaCl (ranging from 81.7 ± 7.6% at 100 mM to 95.0 ± 0% at 200 mM; Figure 7 and Table 2). Values at 300 mM (ca. 70%) did not statistically differ ($p > 0.05$) only with that at 100 mM. Germination percentages at the salinity concentrations ≥ 400mM were not significantly different ($p > 0.05$) among themselves (ranging from 3.3 ± 5.8% at 600 mM to 21.7 ± 20.2% at 400 mM), but were significantly lower ($p < 0.05$) than those at low salinity concentrations (see Table 2). Recovery response was not statistically different ($p > 0.05$) among NaCl concentrations at which seeds were exposed (Figure 7) and RP ranged from 33.3 ± 57.7% at 200 mM to 96.3 ± 6.4% at 500 mM (see Table 2).

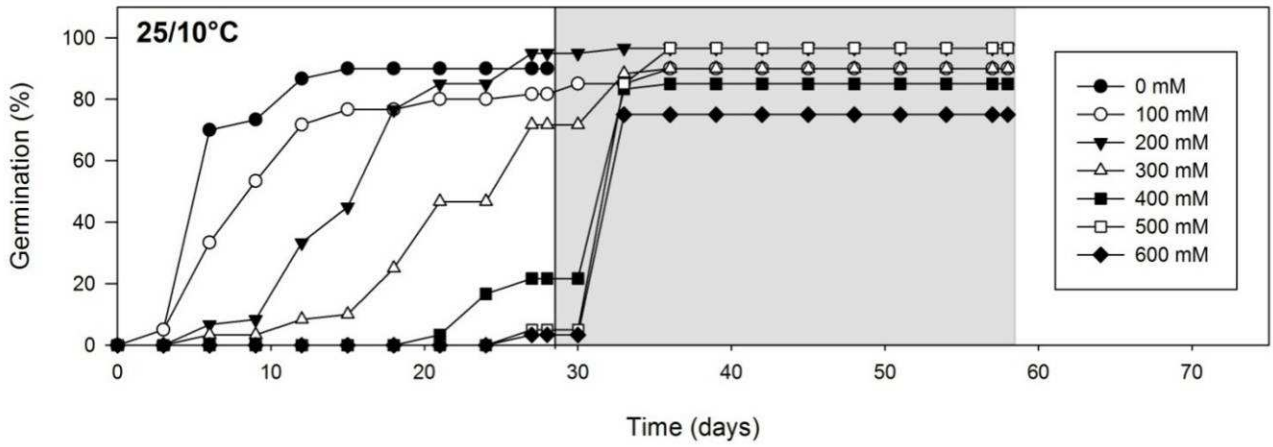

Figure 7 - Cumulative germination percentages at the alternating temperature regime (25/10°C), under different saline concentrations (0-600 mM NaCl) and following transfer to distilled water (recovery, indicated by the shaded area in the graph) for *H. amplexicaulis.* **Each point represents the mean (± 1 SD) of three replicates.**

Table 2 - Germination and recovery (RP) percentages at the alternating temperature regime (25/10°C), at different saline conditions (0-600 mM NaCl) for *H. amplexicaulis.* **Kruskal-Wallis tests were conducted to detect the effect of temperature on germination percentages and RP; ns = p > 0.05, and ** = p < 0.01. Data are the means (± 1 SD) of three replicates. Values with different letters differed at p < 0.05, by Mann Whitney *U-test*.**

Temperature (°C)	Percentage (%)	NaCl concentration (mM)							
		0	100	200	300	400	500	600	
25/10	Germination	90.0 ± 0[a]	81.7 ± 7.6[ab]	95.0 ± 0[a]	68.3 ± 16.1[b]	21.7 ± 20.2[d]	5.0 ± 5.0[d]	3.3 ± 5.8[d]	**
	Recovery(RP)	-	56.7 ± 40.4	33.3 ± 57.7	68.3 ± 7.6	79.4 ± 28.7	96.3 ± 6.4	74.2 ± 27.7	ns

Soil analysis

The two-way ANOVA showed a significant ($p < 0.01$) effect of season (Se), but not ($p > 0.05$) of depth (Dp), as well as of their interaction (Dp x Se) on soil conductivity values (Table 3). Significant differences ($p > 0.05$) were detected between conductivity values during summer (August) and winter (December) at the depth of 0-5 cm, while no differences ($p > 0.05$) between seasons were detected at 5-10 cm and 10-15 cm (Figure 8).

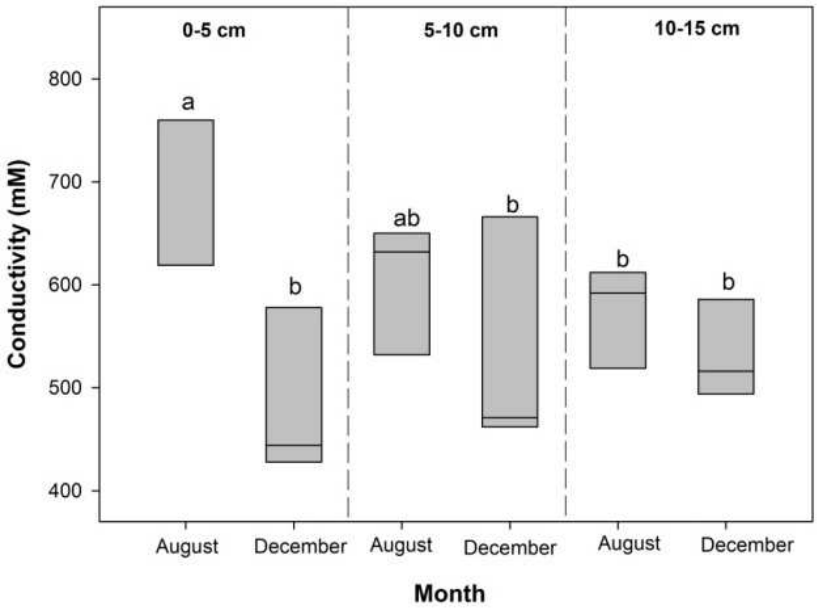

Figure 8 - Conductivity values between seasons at different depths. A two-way ANOVA was conducted among soil conductivity values in order to detect the effect of depth (Dp), season (Se) and their interactions (Dp x Se). A Fisher's LSD *post hoc* **test was conducted to identify significant differences at $p < 0.05$, for the same depth among seasons and among depths in each season. Data are the mean (± 1 SD) of three replicates.**

Salt spray on seedling development

Seedling survival

Seedling survival decreased with increasing salt spray nebulizations. At the end (week eight) of salt spray treatment, 92.3% of seedlings survived under control condition (no spray), while the survival of one-day/week nebulized seedlings was 69.2%. When the frequency of applied salt spray increased at two- and three-days/week, seedling survival was 53.8% and 30.7%, respectively (Figure 9).

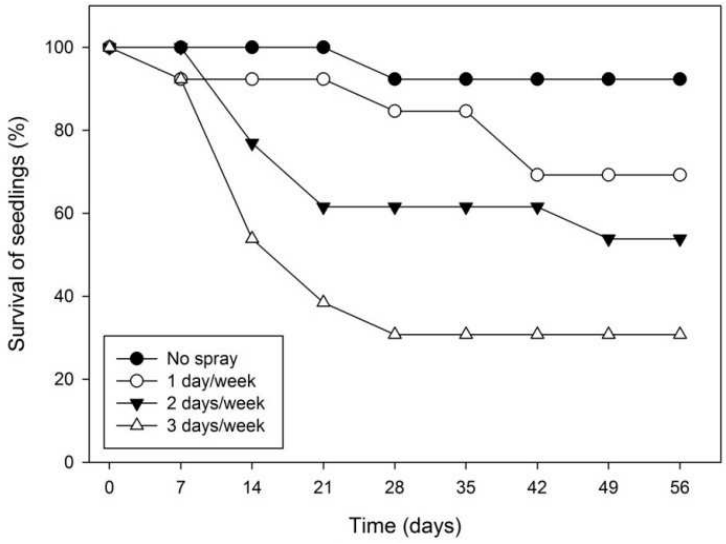

Figure 9 - Survival of *H. amplexicaulis* seedlings, for each treatment (no spray, one day/week, two days/week, three days/week), during eight weeks of salt spray solution (600 mM NaCl) nebulization.

Seedling dry mass and length of epigeal and hypogeal parts

Significant ($p < 0.05$) differences were detected among different frequencies of salt spray nebulization. Mean dry mass for no sprayed and one-day/week nebulized seedlings were not statistically ($p > 0.05$) different and, for both, values were of ca. 32 mg for each seedling (see Figure 10A). Values for two-days/week and three-days/week sprayed seedlings were of ca. 28 and 17 mg, respectively and were significantly ($p < 0.05$) lower (Figure 10A).

Length of epigeal part of seedlings significantly ($p < 0.05$) decreased with the increase in the frequency of salt spray nebulization (Figure 10B). The higher values were detected under no spray condition (ca. 9 mm) and significantly ($p < 0.05$) differed from all other values. Values of one- and two-days/week sprayed seedlings were statistically ($p > 0.05$) similar between themselves (ca. 7.1 mm, for both frequencies), but not ($p < 0.05$) with values detected for all treatments. Lengths of three/days week nebulized seedlings, with values of ca. 4.0 mm, for each seedling, were significantly ($p < 0.05$) different from all others.

The increase of frequency of nebulization significantly ($p < 0.05$) affected length of hypogeal parts of seedlings. The highest value was detected under control (no spray) conditions, with root lengths of ca. 5.8 mm and were statistically ($p > 0.05$) similar with that of one-day/week nebulized seedlings. The lowest value was measured for three-days/week sprayed seedlings (ca. 2.1 mm) and did not show significant ($p > 0.05$) differences with the length measured for the seedlings in the two-days/week treatment (ca. 2.8 mm), which were statistically ($p > 0.05$) similar also with root lengths of one-day/week sprayed seedlings (Figure 10C).

During the salt spray experiment, conductivity values of substrate at the end of the experiment (eight weeks) significantly ($p < 0.05$) differed among frequencies of salt spray nebulization, ranging from 47.2 ± 7.8 mM to 272.4 ± 7.6 mM, for control conditions (no spray) and three-days/week sprayed pots, respectively; pH values significantly ($p < 0.05$) differed among frequencies of salt spray treatment, ranging from 4.8 ± 0.1 to 4.6 ± 0.1, for no sprayed and three-days/week nebulized pots, respectively. No statistical correlation ($p > 0.05$) was found between these soil parameters and seedling growth data, except for the epigeal lengths which significantly ($p < 0.05$) decreased as soil conductivity increased.

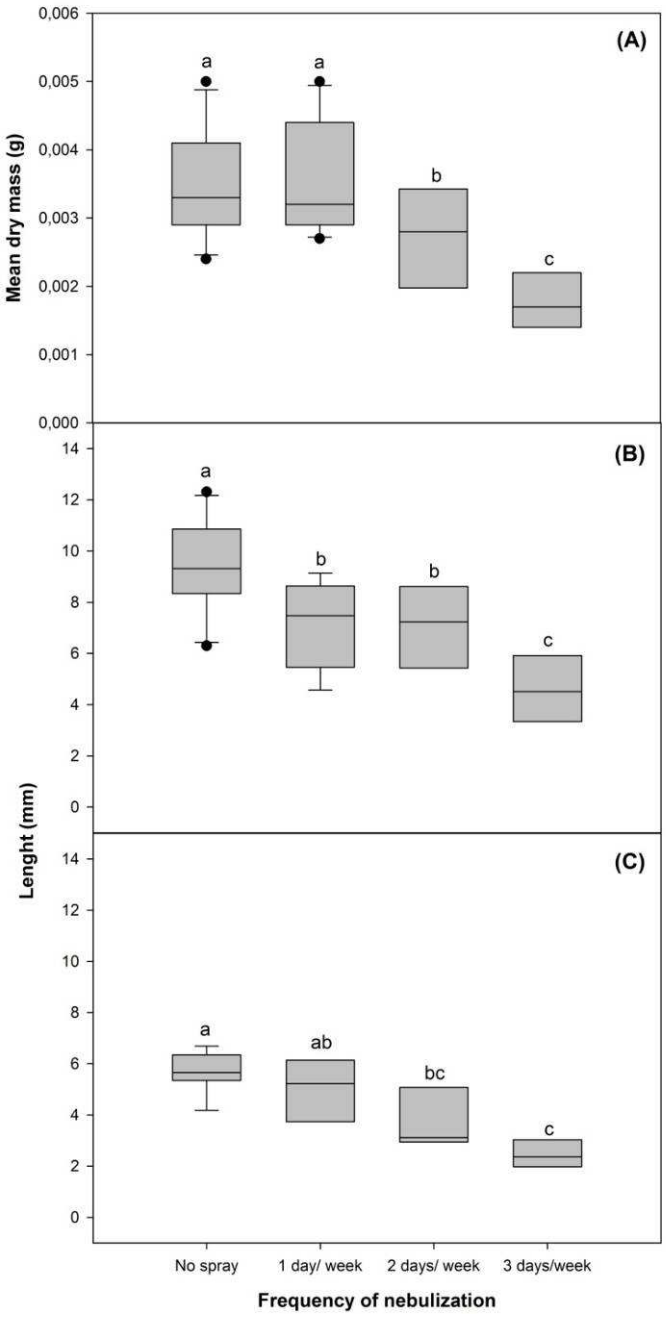

Figure 10 - Dry mass (A), epigeal (B) and hypogeal (C) lengths of *H. amplexicaulis* **seedlings, for each treatment (no spray, one day/week, two days/week, three days/week), after eight weeks of salt spray solution (600 mM NaCl) nebulization. A one-way ANOVA was conducted (for each graph) to detect the effect of treatment on seedlings growth. Values with different letters are significantly different at p < 0.05 (***post hoc* **Fisher's LSD test). For each treatment, data are the mean of survived seedlings after eight weeks from the beginning of experiments.**

Discussion

H. amplexicaulis seeds showed comparable, although generally low, final germination percentages in the light and in the dark at all tested temperatures, therefore they were not photo-inhibited for germination, unlike seeds of several other Mediterranean coastal species (Thanos *et al*., 1989; 1991; 1994). This germination pattern was also confirmed by the seed mass of *H. amplexicaulis* (ca. 0.12 mg) as species with seeds > 0.1 mg in weight are largely light-requiring for germination, and the incidence of light-dependence declines with increasing seed size, allowing seed germination on the soil surface (Grime *et al*., 1981).

H. amplexicaulis seeds germinated with highest percentages (ca. 90%) at the alternating temperature regime of 25/10°C, while germination at constant temperatures was sensibly lower, confirming the findings of Albert *et al.* (2002) and Royal Botanic Gardens Kew (2008). Probert (1992) suggested that responding to alternating temperatures represents an adaptation of small-seeded species which ensure that germination occurs only close to the soil surface. The stimulation of seed germination by alternating temperatures is extremely common and diurnal fluctuations in temperature may initiate or accelerate germination in certain plants and the effectiveness of the stimulus varies according to the amplitude of fluctuation (Grime & Thompson, 1976) and the presence or absence of light (Toole & Borthwick, 1971; Danielson & Toole, 1976). Steinbauer and Grigsby (1957) found that out of 85 species selected from 15 families more than 80% showed higher germination at alternating temperatures compared to constant temperatures. Thompson *et al*. (1977) argued that requirements for diurnal fluctuations in temperature are characteristic of species from particular types of habitat and provides mechanisms which cause seeds to germinate at times and in places favourable for seedling establishment.

H. amplexicaulis seeds germinated with NaCl in the substrate at all tested concentrations (up to 600 mM NaCl), although in salt substrate lower germination percentages than under control

conditions were observed. Many studies reported that germination decreased with increased salinity stress and highest germination occurs in absence of NaCl in the substrate (Khan & Ungar, 1984; El-Keblawy *et al*., 2010; Vallejo *et al*., 2010). NaCl did not affect seed viability and all ungerminated seeds showed high recovery responses, independently to salinity concentrations. This pattern highlights the capability of this species to tolerate high salinities in the soil and germinate when salt level is lowered by the autumn rainfalls. During the study, a seedling emergence in the field was observed in late November (Figure 11) as already reported by Silletti (2012) for an Apulian population (South Italy).

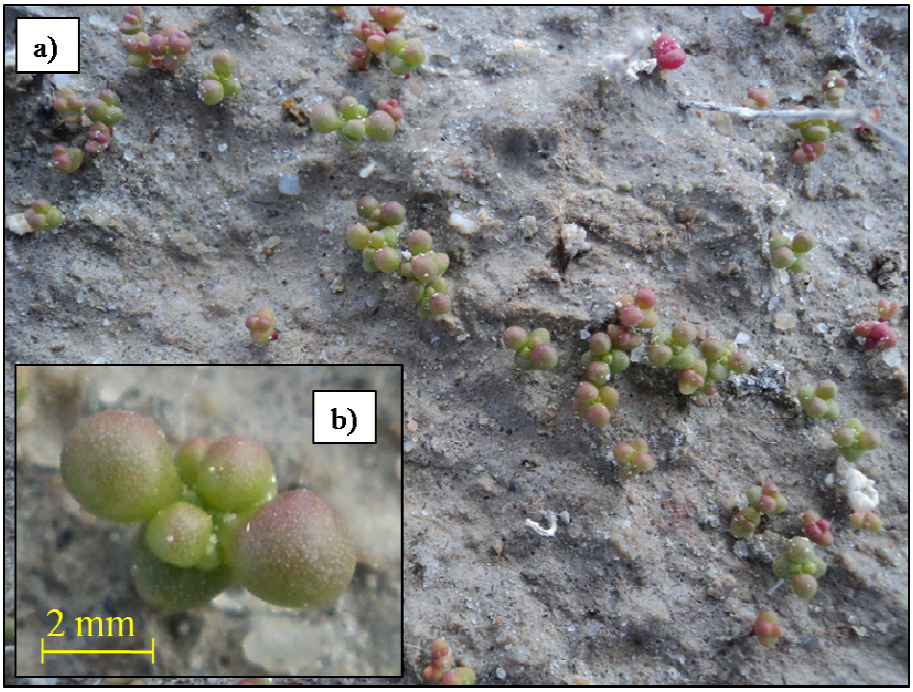

Figure 11 - *H. amplexicaulis* **seedlings in field (a) and particular of a seedling (b).**

All these data confirmed the halophytic behavior of *H. amplexicaulis*, as previously highlighted by several authors (Tremblin, 1982; Tremblin & Binet, 1984; Tremblin & Ferard, 1994). The capability of a species to tolerate high salinities is reflected both on the maximum salt

concentration at which seeds may germinate, both to tolerate high salinity concentrations and then to have recovery after this NaCl exposure (Ungar, 1995), as here reported for *H. amplexicaulis*.

In coastal communities, the distribution of species can sometimes be tied to their tolerance of salt spray (Sykes & Wilson, 1988; Wilson & Sykes, 1999). Salt spray deposition levels can vary greatly depending not only on the proximity to the shore, but also on wind intensity and direction, topography, and the timing of rainfall episodes (Boyce, 1954; Barbour, 1978; Cheplick & Demetri, 1999). Salt spray experiments highlighted a salt aerosol tolerance for *H. amplexicaulis* seedlings with almost all the seedling surviving at the lowest nebulization frequencies, and more than 30% of seedlings surviving in the three/days per week treatment. In solar salterns, salinity levels can reach high levels and salt crusts are often present. In this habitat *H. amplexicaulis* is highly subjected to high soil salinity values, although the salt spray impact is quantitatively reduced respect to that in coastal environments as cliffs or sandy dunes near the sea. Therefore, according to our results of soil analysis and field observations during all the length of the present study, *H. amplexicaulis* appears to be adapted to high soil salinity levels and moderate salt spray impact, with its germination starting from autumn, when under a Mediterranean pluviseasonal climate, rainfalls leach salts on soil surface and allow seed germination and consequent seedling establishment.

The distribution of *H. amplexicaulis* Sardinian population in its peculiar habitat, sheltered by the high influence of marine salt spray, confirm the tolerance for this species to low quantities of salt spray, and may reflect an environmental adaptation to its habitat and abiotic factors which influence it.

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Conclusions

In this study, seed germination requirements and salt stress responses of seven species were investigated. Light did not affect germination percentages in any of the studied species enabling seed germination also under soil surface and highlighting that seeds were not photo-inhibited for germination, contrary to other Mediterranean coastal species, as reported by Thanos *et al.* (1989, 1991, 1995) and Doussi and Thanos (2002). This germination pattern was confirmed for all the species by the seed mass as seeds < 0.1 mg in weight are largely light-requiring for germination and the incidence of light-dependence declines with increasing seed size (Grime *et al*., 1981; Pearson *et al*., 2002).

Seed germination of *Phleum sardoum* and *Rouya polygama* (see Chapters 1 and 2, respectively), as well as that of *Lavatera agrigentina*, *L. triloba* ssp. *pallescens* and *L. triloba* ssp. *triloba* (Chapter 4), reflected the optimal range of temperatures of "typical" Mediterranean species (Thanos *et al*., 1989, 1995; Doussi & Thanos, 2002; Kadis & Georghiou, 2010), suggesting germination in autumn-winter, when water availability, soil moisture and rainfalls are high, and temperatures are not excessively prohibitive for germination and consequent seedlings establishment (Thanos *et al*., 1995; Maun, 2009; Kadis & Georghiou, 2010). *Brassica insularis* differed from other "typical" Mediterranean plants, for which germination at low temperatures is a widely extended trait (Thanos *et al*., 1995), demonstrating that germination of this species may occur in a wide time window during the year (see Chapter 3), as highlighted by Thanos *et al.* (1991) for another species of rocky coastal habitats (*Crithmum maritimum*). *H. amplexicaulis* seed germination, was highly influenced by the daily fluctuation of temperatures, while germination at constant temperatures was sensibly lower, as previously reported by Albert *et al.* (2002) and Royal Botanic Gardens Kew (2008) (see Chapter 5).

Salinity tests conducted at different concentrations (0-600 mM NaCl) showed that higher germination percentages were detected for all the species in the non-saline control. Many studies reported that final germination decreases with increasing salinity stress and the highest germination occurs with the absence of NaCl in the substrate (Khan & Ungar, 1984, Pujol *et al*., 2000; Vallejo *et al*., 2010). Several studies highlighted that the limits of tolerance to salt vary among different species (Ungar, 1982, 1995). In this study, *P. sardoum* was the most NaCl sensitive species and its highest seed germination in NaCl occurred at 100 mM and only at the temperature of 10°C (see Chapter 1). The NaCl tolerance was slightly higher for *R. polygama*, *B. insularis* and *L. triloba* ssp. *triloba* seeds (Table 1), for which the tolerated limit was 200 mM (although for all the three species inter-population variability was detected and at least one population for each species did not tolerate NaCl concentrations higher than 100 mM; see Chapter 2, 3 and 4, for each species, respectively). *L. triloba* ssp. *pallescens* and *H. amplexicaulis* germinated at all tested concentrations, up to 600 mM NaCl (see Table 1), showing an high salt tolerance in seed germination (see Chapter 4 and 5). For all species seed mortality increased proportionally with NaCl concentrations and temperatures. *P. sardoum* and *H. amplexicaulis* were exceptions to this pattern, in fact for these two species, seed mortality was independent from NaCl concentration to which seeds were exposed and did not increase with salinity. Ungar (1995) argued that salinity-temperature interactions may have significant eco-physiological implications in terms of time of germination under field conditions. Several studies reported that salt stress negatively affected seed germination, with consequent seed mortality, either osmotically (through reduced water absorption) or ionically (through the accumulation of Na^+ and Cl^-, causing an imbalance in nutrient uptake and toxicity effect; Baskin & Baskin, 1998; Li, 2008; Shokohifard *et al.*, 1989).

Intra-specific variability in germination patterns has been reported for several species and investigated in various studies (Bischoff *et al*., 2006; Kremer *et al*., 2009; Bischoff & Müller-Schärer, 2010). Differences in salt stress response were showed among populations of several species (Hester *et al*., 1996; El-Keblawy *et al*., 2010). According Gutterman (1994) and Kigel (1995) the variability of germination characteristics could be interpreted as one of the most important survival strategies for species growing under unpredictable environmental conditions. The results of low salt tolerance in seed germination obtained in this study for the two psammophytes (see Table 1) were coherent with a delayed field germination in early winter for *P. sardoum*, whereas highlighted the capability of *R. polygama* seeds to germinate from autumn to spring, when temperatures and salinity concentrations in the sandy soil are low. Therefore, these species typical of sandy dunes avoid seedlings establishment during the dry summer period, and their germination pattern represents an advantageous ecological adaptation towards the unpredictable Mediterranean rainfall pattern (Doussi & Thanos, 2002). *B. insularis* and *L. triloba* ssp. *pallescens*, showed different salt tolerance in seed germination (200 mM and 600 mM, respectively; see Table 1). Both species, grow in a habitat where spindrifts by marine waves and salt spray tend to accumulate, into rocks fissures, small quantities of marine water and consequent NaCl. While *L. pallescens* appeared to be a species highly able to germinate even to salt concentrations equal to seawater, *B. insularis* demonstrated limited capability for seed germination and consequent recovery at high salt concentrations. *L. triloba* with a higher distance from seashore appeared adapted to its habitat, with germination at low salt concentrations and high capability to recover from saline conditions, when salt concentration level is reduced by rainfalls. In its habitat *H. amplexicaulis* is highly subjected to high soil salinity values, and often salt crusts are present on soil surface, so this species appeared to be adapted to these high salinity levels (see Table 1).

Table 1 - NaCl tolerance limit in seed germination for the species investigated in this study. * indicate inter-population variability in salt stress response. Colors indicate: red (species of coastal sandy dunes), green (rupestrian species) and blue (species with high salt concentration in their habitat).

NaCl concentration (mM)		
≤ 100	**≤ 200**	**600**
Phleum sardoum	*Rouya polygama* * *Brassica insularis** *Lavatera triloba ssp. triloba**	*Lavatera triloba ssp. pallescens* *Halopeplis amplexicaulis*

The species for which salt spray experiments were conducted showed different response on seedling growth to salt aerosol tolerance. Probably, this different tolerance was strongly connected to the habitat of each species and to its distance from the sea. The ability of plants to tolerate and possibly adapt to airborne saltwater sprays may be critical to the maintenance of coastal plant populations (Maun, 1994; Greipsson & Davy, 1996). In coastal communities, the distribution of species can sometimes be tied to their tolerance of salt spray (Sykes & Wilson, 1988; Wilson & Sykes, 1999). *B. insularis* and *L. triloba* ssp. *pallescens* showed the highest salt spray tolerance and lowest seedling mortality (see Chapter 3 and 4, for each species, respectively), probably due to their habitat, coastal cliffs highly influenced by wind and salt spray. Seedling survival of *L. triloba* ssp. *triloba* and *H. amplexicaulis* (see Chapter 4 and 5, respectively) was inversely proportional to the nebulization frequency increase. The distribution of the only *H. amplexicaulis* Sardinian population in its peculiar habitat, sheltered by the high influence of marine salt spray, confirm the tolerance for this species to low quantities of salt spray, and may reflect an environmental adaptation to its habitat and abiotic factors which influence it. *L. triloba* ssp. *triloba* resulted the most sensitive species to the salt spray factor, demonstrating as distance and/or interposed vegetation may determine a lower impact of marine aerosol for this species.

The results of this study lead to a better knowledge on the autoecology of the investigated species and to their limits of tolerance to abiotic factors such as temperature, soil salinity and salt spray.

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The following annexes were elaborated in 2012 during the project “Schede per una lista rossa della flora vascolare e crittogamica italiana”, upon request of “Ministero dell’Ambiente e della Tutela del Territorio e del Mare” (MATTM), in order to evaluate and update the conservation *status* of certain selected species of Italian flora. IUCN red lists criteria were followed for the attribution of the species to the categories. These annexes were submitted and accepted for publication to “Informatore Botanico Italiano”.

Annex 1

Rouya polygama (Desf.) Coincy

A. SANTO, G. FENU, G. BACCHETTA

Nomenclatura:
Specie: *Rouya polygama* (Desf.) Coincy
Basionimo: *Thapsia polygama* Desf.
Famiglia: Apiaceae
Nome comune: Firrastrina bianca

Descrizione. Pianta erbacea perenne, alta 15-30(50) cm, con fusti generalmente ascendenti e flessuosi. Foglie inferiori bipennatosette, con segmenti di secondo ordine generalmente trifidi o pennatopartiti, lunghi 5-10 mm, acuti, glabri di sopra. Ombrelle a 10-20 raggi; brattee numerose, spesso trifide e ripiegate verso il basso; petali bianchi. Il frutto è un achenio di 8-9-mm, con ali lunghe 2 mm, ondulate (PIGNATTI, 1982; BACCHETTA, 2001; GAMISANS, JEANMONOD, 2007).

Biologia. Emicriptofita scaposa con fioritura da giugno a luglio e fruttificazione da settembre a ottobre (PIGNATTI, 1982; TUTIN *et al.*, 1993; BACCHETTA, 2001). L'unità di dispersione è costituita da un achenio alato, adattato alla dispersione anemocora. La biologia riproduttiva non è ancora stata indagata e non si hanno ad oggi informazioni circa la vitalità e l'effettiva capacità germinativa delle diaspore.
Il numero cromosomico è 2n=20, calcolato su materiale proveniente dalla Corsica (CONSTANCE *et al.*, 1976).

Ecologia. Specie psammofila ed eliofila, caratteristica degli ambienti dunali costieri, prevalentemente nelle depressioni retrodunali su sabbie consolidate. Raramente, lungo le coste centro-orientali della Sardegna, si rinviene su dune semistabili, dune d'arresto e pendii pietrosi fronte mare (BACCHETTA, 2001).
Dal punto di vista bioclimatico si ritrova in ambito Mediterraneo Pluvistagionale Oceanico, con termotipo termomediterraneo superiore e ombrotipi variabili dal secco superiore al subumido inferiore (BACCHETTA, 2001).
Le cenosi cui partecipa non sono ancora state indagate dal punto di vista fitosociologico, ma in maniera preliminare possono essere riferite all'alleanza *Crucianellion maritimae* Riv.-God. *et* Riv.-Mart. 1963. Tali formazioni rientrano nell'habitat di interesse comunitario "Dune fisse del litorale del *Crucianellion maritimae*" (2210) e, secondariamente, nell'habitat "Dune costiere con *Juniperus* ssp. (2250)".

Distribuzione in Italia.
Regione biogeografica. Sulla base della classificazione ecoregionale proposta da BLASI, FRONDONI (2011), le popolazioni sarde di *R. polygama* ricadono nella Provincia del Blocco Sardo-Corso ed in particolare nelle sezioni delle Montagne del Gennargentu e delle Montagne dell'Iglesiente. Sulla base della classificazione biogeografica di RIVAS-MARTÍNEZ (2004, 2007), le stazioni ricadono nella Regione biogeografica Mediterranea, Subregione Mediterraneo Occidentale, Provincia Italo-Tirrenica, Subprovincia Sarda; BACCHETTA *et al.* (2009) hanno modificato tale inquadramento, individuando una Superprovincia Italo-Tirrenica, una Provincia Sardo-Corsa e una Subprovincia Sarda.
Regione amministrativa. in Italia la specie è presente esclusivamente in Sardegna.
Numero di stazioni. il *taxon* risulta presente in cinque stazioni nella parte sud-occidentale dell'isola: a Portoscuso (CI), Is Solinas-Masainas (CI), Porto Pino (Sant'Anna Arresi, CI), oltre che sulle isole di

Sant'Antioco e di San Pietro (DE MARCO, MOSSA, 1973; MILIA, MOSSA, 1977; ATZEI, 1981). Altre quattro stazioni si rinvengono nella parte centro-orientale e in particolare in Ogliastra: Dune di Girasole, Lido di Orrì, Il Golfetto e Arbatax (BACCHETTA, 2001). Recentemente, FILIGHEDDU *et al.* (2011) ne hanno segnalato la presenza sull'Isola di Tavolara (Olbia, OT).

Tipo corologico e areale globale. *R. polygama* è un *taxon* a distribuzione SW-Mediterranea (PIGNATTI, 1982; BACCHETTA, 2001), con distribuzione limitata a Sardegna, Corsica (PARADIS, GÉHU, 1992; POZZO DI BORGO, PARADIS, 2000), Algeria (QUEZEL, SANTA, 1963) e Tunisia (POZZO DI BORGO, PARADIS, 2000).

Minacce. Per l'identificazione delle categorie di minaccia è stata utilizzata la versione 3.1 delle Major Threats IUCN (www.iucn.org).
Minaccia 1: *Residential and commercial development*, in particolare Minaccia 1.1: *Housing and Urban Areas* e Minaccia 1.3: *Tourism and Recreation Areas*. La progressiva perdita di habitat, dovuta allo sviluppo urbano, rappresenta una delle principali minacce per la specie, come osservato nell'area di Portoscuso e nelle aree costiere di Arbatax e Porto Pino.
Minaccia 4: *Trasportation and Service Corridors* ed in particolare Minaccia 4.1: *Roads and railroads*. La realizzazione di infrastrutture per trasporti e servizi in prossimità di spiagge e litorali, ha portato alla riduzione della superficie occupata dal *taxon*, determinando inoltre una frammentazione delle popolazioni.
Minaccia 6: *Human intrusions and disturbance*, ed in particolare Minaccia 6.1: *Recreational activities*. La notevole pressione turistica durante i mesi estivi, che insiste in molte delle stazioni (Is Solinas-Masainas, Porto Pino, Lido di Orrì, Il Golfetto), determina un'importante minaccia per le popolazioni.
Minaccia 9: *Pollution*, ed in particolare Minaccia 9.4: *Garbage and solid waste*. Nell'area di Portoscuso, gran parte degli individui sono localizzati ai margini di una strada, nei pressi del porto industriale, in un'area ampiamente degradata per la presenza di rifiuti e inerti.

Criteri IUCN applicati.
L'assegnazione di *R. polygama* ad una categoria di rischio è stata effettuata sulla base del criterio B, relativo all'ampiezza dell'areale geografico.

Criterio B

Sottocriteri

B1-Areale regionale (EOO): 7230 km²
B2-Superficie occupata (AOO): 36 km²
Superficie occupata effettiva: 0,89 km²

Opzioni
a) Popolazione frammentata o Numero di location: In base alle minacce osservate (inquinamento, sviluppo residenziale e commerciale, realizzazione di infrastrutture per trasporti e servizi, disturbo antropico legato alla fruizione turistica dei siti) è possibile identificare quattro distinte *locations*. La specie presenta inoltre una distribuzione estremamente frammentata.
b) (ii) Declino della superficie occupata: a causa delle minacce osservate è possibile ipotizzare una diminuzione della superficie occupata dalla specie.
b) (iii) Declino della qualità dell'habitat: Le modificazioni dell'habitat stanno determinando un costante declino della qualità degli ecosistemi dunali costieri.

Categoria di rischio.
In base al criterio B, il *taxon* può essere considerato come minacciato. Categoria di rischio: *Endangered*, EN B2ab(ii,iii).

Interazioni con la popolazione globale.
Non si dispone di informazioni relative a possibili interazioni con le popolazioni della Corsica e del Nord-Africa.

***Status* alla scala "regionale/globale":** EN B2ab(ii,iii);
- *status* alla scala globale: *Not evaluated* (NE)
- precedente attribuzione a livello nazionale: VU (CONTI *et al.*, 1992, 1997; SCOPPOLA, SPAMPINATO, 2005), EN (BACCHETTA, 2001).

Strategie/Azioni di conservazione e normativa.
R. polygama è una specie di grande interesse sistematico, fitogeografico ed ecologico, inserita nella Convenzione di Washington (CITES), nell'Allegato I della Convenzione di Berna e nell'Allegato II della Direttiva "Habitat" 92/43/CEE.
Alcune stazioni di *R. polygama* ricadono all'interno di Siti di Importanza Comunitaria (SIC), quali "Stagno di Porto Botte" (ITB042226), "Promontorio, Dune e Zone Umide di Porto Pino" (ITB040025); "Lido di

Orrì" (ITB022214), "Isole Tavolara, Molara e Molarotto" (ITB010010).
Parte delle stazioni ricadono all'interno dei seguenti siti d'importanza internazionale per le piante (IPAs), individuati per la Sardegna (BLASI *et al.,* 2010): "Stagno Santa Caterina, Porto Pino, Capo Teulada e M. Lapanu" (SAR4), "Isole Tavolara, Molara e Molarotto" (SAR16) e "Lido di Orrì" (SAR32).
Già a partire dal 2005 è stata avviata, presso la Banca del Germoplasma della Sardegna (BG-SAR), la conservazione *ex situ* a lungo periodo del germoplasma, mediante la conservazione di undici lotti di semi, relativi a quattro popolazioni sarde e due della Corsica. Inoltre sono stati inviati *duplicata* presso la Millenium Seed Bank (Royal Botanic Gardens of Kew).
Presso BG-SAR sono attualmente in corso studi sull'ecofisiologia della germinazione, volti a identificare i requisiti ottimali, in termini di fotoperiodo, temperatura e salinità.

Note.
R. polygama è considerata un paleoendemismo (VERLAQUE *et al*., 1993) che dal Nord Africa (Algeria e Tunisia) si sarebbe irradiato in Sardegna e Corsica (CONTANDRIOPOULOUS, 1962; PARADIS, GÉHU, 1992).

Ringraziamenti – Si ringrazia la Provincia di Cagliari, Assessorato Tutela Ambiente, per il sostegno fornito alle attività scientifiche del CCB ed in particolare alle attività di conservazione *ex situ* attuate da BG-SAR.

LETTERATURA CITATA

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Annex 2

Brassica insularis Moris

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Nomenclatura:

Specie: *Brassica insularis* Moris
Sinonimi: *Brassica oleracea* subsp. *insularis* (Moris) Rouy *et* Foucad; *Brassica oleracea* var. *insularis* (Moris) Cosson; *Brassica cretica* subsp. *atlantica* (Cosson) Onno; *Brassica oleracea* var. *insularis* (Moris) Cosson subvar. *atlantica* Cosson.
Famiglia: Brassicaceae
Nome comune: Cavolo di Sardegna, Colza di Sardegna.

Descrizione. Pianta perenne, alta 40-100(180) cm. Fusti eretto-ascendenti, ramificati nel terzo distale, a volte contorti e non completamente lignificati. Foglie 10-20(35) × 5-12(15) cm, glabre, verdi-glauche, alterne, le basali in rosetta, quelle caulinari generalmente pennatifide, con robusto picciolo e lamina carnosa, espansa, increspata ed irregolare al margine, da orbicolare-obovata a ovato-lanceolata e nervature molto pronunciate sulla pagina inferiore. Infiorescenze terminali in racemo, con 50-100 fiori fortemente profumati, provvisti di peduncoli lunghi (8)12-24(30) mm all'antesi, da eretto-patenti a patenti; calice a sepali verdi, eretti o parzialmente divergenti, lunghi 9-13 mm, caduchi; petali in numero di quattro, all'antesi solitamente patenti, più raramente deflessi, bianchi, ovato-spatolati, lunghi 10-16 mm; stami 6, con filamenti bianchi lunghi 1-1,2 mm ed antere gialle lunghe (3)3,5-4,5 mm. Frutti in forma di silique lomentacee, (3)4-6 × 30-70(90) mm, a sezione circolare, con pedicelli fruttiferi di (12)20-30(40) mm e becco di (3)5-20(23) mm. I frutti si aprono per due valve che lasciano scoperto il setto centrale (*replum*) a cui sono adesi i semi in numero di 15-35 per loculo, sferici, di diametro 1-2 mm, generalmente bruno-scuri (PIGNATTI, 1982; TUTIN *et al.*, 1993; BACCHETTA, 2001).

Biologia. Camefita suffruticosa o, più raramente, fanerofita cespitosa semicaducifoglia. La fioritura si verifica da febbraio a metà maggio e la fruttificazione da fine maggio agli inizi di agosto (BACCHETTA, 2001). La dispersione dei semi è barocora e, secondariamente, anemocora. L'ovulo è campilotropo e la nucella crassinucellata; i granuli pollinici sono trinucleati (CORSI, 1963).
Il numero cromosomico è 2n=18, calcolato su materiale proveniente da Pantelleria (LENTINI *et al.*, 1988) e su piante coltivate in vaso nell'Orto Botanico dell'Università di Pisa, prodotte a partire da germoplasma raccolto sull'Isola Rossa, presso Teulada, nella Sardegna sud-occidentale (CORSI, 1963).

Ecologia. Specie rupicola, eliofila, xerofila e indifferente edafica, che si rinviene in aree costiere e, meno frequentemente, in quelle interne, su pendii, falesie e pareti verticali, a quote comprese tra il livello del mare e 1200 m (BACCHETTA, 2001). In Sardegna e Corsica si rinviene con maggiore frequenza su substrati di natura carbonatica, a Pantelleria è presente su vulcaniti, mentre in Tunisia ed Algeria si rinviene su substrati di diversa natura.
Dal punto di vista bioclimatico, in Sardegna, si ritrova in ambito Mediterraneo pluvistagionale oceanico, con termotipi variabili dal termomediterraneo inferiore al mesomediterraneo superiore e ombrotipi compresi tra il secco inferiore e il subumido superiore (BACCHETTA, 2001).
Per Pantelleria il piano bioclimatico è quello inframediterraneo semiarido (GIANGUZZI, 1999).
Dal punto di vista sintassonomico la specie è caratteristica dell'alleanza *Brassicion insularis* Gamisans 1991 (BACCHETTA, 2001). In Sardegna, sulle falesie costiere di Capo Caccia (Alghero, SS), partecipa anche a cenosi riferibili alla classe *Crithmo-Limonietea*, (CHIAPPINI, DIANA, 1978). Sull'Isola dei Cavoli (Villasimìus, CA) forma popolamenti quasi monospecifici, e caratterizza la subassociazione *brassicetosum* Mossa *et* Tamponi 1978, dell'associazione *Oleo-Lentiscetum* Br.-Bl. *et* Maire *in* Maire 1924 (BACCHETTA, 2001).

B. insularis si rinviene all'interno di vari habitat di interesse comunitario, tra i quali: "Scogliere con vegetazione delle coste mediterranee con *Limonium* spp. endemici" (1240), "Pareti rocciose calcaree con vegetazione casmofitica" (8210), e "Pareti rocciose silicee con vegetazione casmofitica" (8220).

Distribuzione in Italia.
Regione biogeografica. Le popolazioni sarde, dal punto di vista ecoregionale, ricadono nella Provincia Sardo-Corsa, mentre quella di Pantelleria ricade nella Provincia del blocco Pelagico, sezione delle isole di Pantelleria e Linosa (BLASI, FRONDONI, 2011).
Sulla base della classificazione biogeografica di RIVAS-MARTÍNEZ (2004, 2007), le stazioni sarde ricadono nella Regione biogeografica Mediterranea, Subregione Mediterraneo Occidentale, Provincia Italo-Tirrenica, Subprovincia Sarda; tale inquadramento, modificato da BACCHETTA *et al.* (2009), individua una Superprovincia Italo-Tirrenica, una Provincia Sardo-Corsa e la Subprovincia Sarda. La popolazione di Pantelleria ricade invece nella Regione biogeografica Mediterranea, Subregione Mediterraneo Occidentale, Provincia Italo-Tirrenica, Subprovincia Siciliana (RIVAS-MARTÍNEZ, 2004, 2007).
Regione amministrativa. In Italia la specie è presente in Sardegna e Sicilia.
Numero di stazioni. In Sardegna la specie risulta presente in 36 stazioni. Lungo la costa è presente in vari siti [Capo Caccia (Alghero, SS), Capo Figari (Olbia, OT), Capo Teulada (Teulada, CA), Planu Sartu (Buggerru, CI), Porto Flavia (Iglesias, CI), San Nicolò (Buggerru, CI)] oltre che in molti sistemi insulari circumsardi [Asinara (Porto Torres, SS) Figarolo (Golfo Aranci, OT), Foradada (Alghero, SS), Isola dei Cavoli (Villasimìus, CA), Isola Rossa (Teulada, CA), Isola San Macario (Pula, CA), Pan di Zucchero (Iglesias, CI), Sa Tuarredda (Teulada, CA), Isola Tavolara (Olbia, OT)]. Nelle aree interne si rinviene a Domus sa Medusa (Samugheo, OR), Gutturu Cardaxius (Iglesias, CI), Gutturu Pala (Fluminimaggiore, CI), La Cartiera (Cuglieri, OR) Marganai (Iglesias, CI), Monte Arcuentu (Arbus, VS), Monte Padenteddu (Pula, CA), Monte San Giovanni (Gonnesa, CI) Monte Tiscali (Dorgali, NU), S'atta e Bidda (Oliena, NU) (BACCHETTA, 2001). Lungo i versanti SE dell'isola di Pantelleria (TP), dove per la prima volta venne segnalata da CATANZARO (1968), è presente un'unica popolazione con cinque stazioni: Cala Tramontana, Contrada Dietro Isola, Contrada Kania, Punta del Cultignolo e Punta del Formaggio (GIARDINA *et al.*, 2007).

Tipo corologico e areale globale. *B. insularis* può essere considerato un endemismo SW Mediterraneo (SNOGERUP *et al.*, 1990) e più precisamente tirrenico-nordafricano (BACCHETTA, PONTECORVO, 2005). Oltre che in Italia, è presente in Corsica, Tunisia ed Algeria (SNOGERUP *et al.*, 1990; GLEMIN *et al.*, 2006). In Corsica si rinviene sui Monti Rossi, a Teghime (Brando), Caporalino e Francardo (Omessa), Penta Frascaja (Piano), sull'Alpa Mariuccia (Bocognano), sulle Gole dell'Inzecca (Ghisonaccia), sulle pareti del Rio Stretto (Ghisoni) e in due stazioni meridionali a Punta d'Aquella (Lecci) e Punta di Calcina (Conca) (CORSI, 1963). In Tunisia la specie è segnalata lungo la costa settentrionale per le isole de La Galite, Zembra e Zembretta e per il Monte Ressas (POTTIER-ALAPETITE, 1979) mentre in Algeria si ritrova in diverse aree costiere ed interne della Cabilia (MAIRE, 1965).

Minacce. Per l'identificazione delle categorie di minaccia è stata utilizzata la versione 3.1 delle Major Threats IUCN (www.iucn.org).
Minaccia 2: *Agriculture* & *Aquaculture* ed in particolare Minaccia 2.3: *Livestock farming* & *ranching* e Minaccia 2.3.1: *Nomadic grazing*. Il pascolo, nelle stazioni non rupicole (come ad esempio l'Isola dei Cavoli), soprattutto in tempi passati, ha costituito una minaccia per questa specie.
Minaccia 6: *Human intrusions and disturbance,* e in particolare Minaccia 6.1: *Recreational activities*. In Sardegna l'unico fattore di minaccia è legato all'arrampicata sportiva, principalmente nelle aree di Gutturu Cardaxius (Iglesias, CI) e Gutturu Pala (Fluminimaggiore, CI), dove negli ultimi anni si è osservato un declino delle popolazioni a causa dell'impatto determinato da questa attività. L'isola di Pantelleria è meta di turismo nel periodo estivo e data la accessibilità di alcuni dei siti in cui si rinviene la specie, il pericolo è rappresentato dalla modificazione dell'habitat dovuta al calpestio o all'apertura di nuovi sentieri.
Minaccia 7: *Natural system modifications* ed in particolar modo Minaccia 7.1: *Fires* & *Fire Suppression* e Minaccia 7.1.1: *Increase in fire frequency/intensity*. Sull'isola di Pantelleria gli incendi estivi possono ridurre drasticamente il numero di individui della popolazione.

Criteri IUCN applicati.
Per l'assegnazione di *B. insularis* ad una categoria di rischio, considerando i dati a disposizione, è stato valutato il criterio B, relativo all'ampiezza dell'areale geografico.

Criterio B
B1-Areale (EOO): 22.874 km²
B2-Superficie occupata (AOO): 140 km² (griglia di 2 x 2 km).

Opzioni
I valori relativi all'ampiezza dell'areale geografico rientrano nel range individuato per la categoria VU, tuttavia non essendo stato osservato un declino, non è possibile attribuibile tale categoria alla specie.

Categoria di rischio.
L'assenza di minacce serie per la conservazione della specie e la mancanza di declino, indicano che *B. insularis* deve essere considerata come non minacciata a livello nazionale.
Categoria di rischio: *Near Threatened* (NT).

Interazioni con la popolazione globale.
A causa dell'isolamento geografico delle popolazioni sardo-corse, siciliane e nord-africane, non si ritengono possibili fenomeni di scambio genico.

***Status* alla scala "regionale/globale.**
-*status* alla scala globale: *Near Threatened* (NT) (BILZ *et al.*, 2011).
-precedente attribuzione a livello nazionale: A livello regionale la specie è stata considerata *Endangered* (EN) per la Sicilia (CONTI *et al.*, 1997, RAIMONDO *et al.*, 2011).

Strategie/Azioni di conservazione e normativa.
B. insularis, specie di interesse fitogeografico, sistematico e conservazionistico, è inserita nell'Allegato I della Convenzione di Berna e nell'Allegato II della Direttiva "Habitat" 92/43/CEE.
Alcune delle stazioni sarde di *B. insularis* ricadono all'interno di Siti di Importanza Comunitaria (SIC), ed in particolare nel SIC "Isole Tavolara, Molara e Molarotto" (ITB010010), "Capo Figari e Isola Figarolo (ITB010009), "[Capo Caccia (con le isole Foradada e Piana) e Punta del Giglio (ITB010042)], "Isola dell'Asinara" (ITB010082), "Isola dei Cavoli, Serpentara e Punta Molentis (ITB040020), "Isola Rossa e Capo Teulada" (ITB040024), "Costa di Nebida" (ITB040029) e "Monte Arcuentu e Rio Piscinas" (ITB040031). Sull'Isola di Pantelleria (TP), l'unica popolazione ricade all'interno del SIC "Isola di Pantelleria, area costiera, falesie e bagno dell'acqua" (ITA010020).
Inoltre, la popolazione dell'Isola dell'Asinara ricade all'interno dell'omonimo Parco Nazionale, istituito in seguito al D.P.R 3 ottobre 2002, mentre quella di Capo Caccia si trova all'interno del "Parco Naturale Regionale di Porto Conte", istituito con la L.R. n°4 del 26 febbraio 1999.
Popolazioni sarde di *B. insularis* ricadono anche all'interno di Aree Marine Protette (AMP), quali AMP "Isola dell'Asinara", "Tavolara-Punta Coda Cavallo", "Capo Caccia-Isola Piana" e "Capo Carbonara".
La popolazione di Pantelleria ricade all'interno dell'istituenda AMP "Isola di Pantelleria".
Alcune delle stazioni sarde di *B. insularis* sono anche incluse all'interno delle *Important Plant Areas* (IPAs) individuate per la Sardegna (BLASI *et al.,* 2010) e in particolare nelle seguenti aree: "Isola Asinara e Punta Rumasinu" (SAR14), "Isole Tavolara, Molara e Molarotto" (SAR16), "Capo Figari e Isola Figarolo" (SAR22), "Punta Maxia e Monte Arcosu" (SAR5), "Isola dei Cavoli, Serpentara, Campu Longu e Monte Macioni" (SAR6), "Monte Linas, Costa di Nebida e Capo Pecora" (SAR7), "Capo Caccia, Monte Rodedo e Punta Argentiera (SAR13).
Già a partire dal 2005 è stata avviata presso la Banca del Germoplasma della Sardegna (BG-SAR) la conservazione *ex situ* a lungo periodo del germoplasma (BACCHETTA *et al.*, 2007) e attualmente sono conservati in banca ventidue lotti di semi relativi a otto popolazioni sarde. Sono stati inoltre inviati *duplicata* alla Millenium Seed Bank (Royal Botanic Gardens of Kew). Presso BG-SAR sono attualmente in corso studi sull'ecofisiologia della germinazione, volti a identificare i requisiti ottimali in termini di fotoperiodo, temperatura e salinità.

Note.
B. insularis Moris appartiene al gruppo di *B. oleracea* L., insieme a *B. balearica* Pers., *B. rupestris* Raf., *B. villosa* Biv. e *B. tyrrhena* Giotta, Piccito *et* Arrigoni. Quest'ultima in passato veniva inclusa all'interno di *B. insularis,* dalla quale differisce sia per numero cromosomico ($2n=20$) che per la morfologia, in quanto possiede silique a sezione quadrangolare, petali gialli, sepali bianco-giallastri e dimensioni più modeste (ARRIGONI, 2006).

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