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in three Tyrrhenian coastal species of the *Silene mollissima*
aggregate (Caryophyllaceae)**

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Complete List of Authors:	<p>Murru, Valentina; Centro Conservazione Biodiversità (CCB), Università degli Studi di Cagliari, , Dipartimento di Scienze della Vita e dell'Ambiente (DISVA)</p> <p>Santo, Andrea; Centro Conservazione Biodiversità (CCB), Università degli Studi di Cagliari, Dipartimento di Scienze della Vita e dell'Ambiente (DISVA)</p> <p>Piazza, Carol; Conservatoire Botanique National de Corse, Office de l'Environnement de la Corse</p> <p>Hugot, Laetitia; Conservatoire Botanique National de Corse, Office de l'Environnement de la Corse</p> <p>Bacchetta, Gianluigi; Centro Conservazione Biodiversità (CCB), Università degli Studi di Cagliari, Dipartimento di Scienze della Vita e dell'Ambiente (DISVA)</p>
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Seed germination, salt stress tolerance and effect of nitrate in three Tyrrhenian coastal species of the *Silene mollissima* aggregate (Caryophyllaceae)

Murru¹ Valentina, Santo^{1*} Andrea, Piazza² Carol, Hugot² Laetitia, Bacchetta¹ Gianluigi

¹ Centro Conservazione Biodiversità (CCB), Dipartimento di Scienze della Vita e dell'Ambiente (DISVA), Università degli Studi di Cagliari, V.le S. Ignazio da Laconi 11-13, 09123, Cagliari, Italy.

² Conservatoire Botanique National de Corse, Office de l'Environnement de la Corse, Avenue Jean Nicoli 20, 250, Corte, France.

Murru Valentina: va.murru1@studenti.unica.it;

Santo Andrea: andreasanto85@gmail.com;

Piazza Carole: Carole.piazza@oec.fr;

Hugot Laetitia: laetitia.hugot@oec.fr;

Bacchetta Gianluigi: bacchet@unica.it

*Corresponding author: Andrea Santo; andreasanto85@gmail.com; Centro Conservazione Biodiversità (CCB), Dipartimento di Scienze della Vita e dell'Ambiente (DISVA), Università degli Studi di Cagliari, V.le S. Ignazio da Laconi 11-13, 09123, Cagliari, Italy; Phone: +39 070 6753681; Fax: +39 070 6753509.

Abstract

Silene mollissima aggregate is part of the section *Siphonomorpha* and currently comprises 11 narrow endemic species of the Western Mediterranean Basin. Three of these taxa (*S. velutina*, *S. ichnusae* and *S. badaroi*) have a distribution range centred in the Northern Tyrrhenian area and occurring in coastal habitats. Inter- and intra-specific variability in the responses to light, constant (5-25°C) and alternating temperatures (25/10°C), NaCl (0–600 mM), KNO₃ (20 mM) under salinity stress and recovery of seed germination were evaluated for these species to more effectively support their *in situ* and *ex situ* conservation. Our results highlighted that the seeds of these three taxa were non-dormant, and light significantly improved their germination, which showed high percentages (> 80%) at low temperatures (5–15°C) and under the alternating temperature regime (25/10°C), decreasing significantly at the highest temperature (25°C). *Silene velutina* and *S. ichnusae* seeds germinated in up to 300 mM NaCl, and *S. badaroi* germinated until 100 mM. For all species except *S. badaroi*, salt did not affect seed viability and recovery did not decrease with increasing salinity and temperature, except for *S. badaroi*. Inter-population variability both in salt tolerance and recovery was detected for *S. velutina*. The addition of KNO₃ did not affect germination or recovery under salt conditions. The lack of effect of KNO₃ suggests that nutrient availability is not a requirement for seed germination in these species. Our results show that all species experience optimum period of germination during autumn-winter, when water availability is highest and soil salinity levels are minimal due to the Mediterranean rainfalls, but *S. velutina* and *S. ichnusae* are also able to germinate until spring.

Keywords: endemism, germination ecology, Mediterranean, NaCl, potassium nitrate

Introduction

The Mediterranean vascular flora is characterised by the presence of high species richness and high rates of endemism and shows elevated frequencies of disjoined distributions of related species (Thompson 2005).

These distributions frequently consist of narrow endemics (often located on islands) or a widespread species with some restricted endemics outside of its distribution area (Küpfer 1974; Verlaque et al. 1991; Cañadas et al. 2014). Many such taxa are identified as “schizo-endemics” with distribution patterns mainly ascribed to allopatric speciation (Gielly et al. 2001). In the Western Mediterranean, the *Silene mollissima* (L.) Pers. aggregate may be considered an effective example of this phenomenon (Boquet et al. 1978; Jeanmonod 1984).

This aggregate is included in the section *Siphonomorpha* Otth. and currently comprises 11 narrow endemic species of the Western Mediterranean Basin. Five of these species (*S. badaroi* Bestr., *S. ichnusae* Brullo, De Marco & De Marco f., *S. hicesiae* Brullo & Signorello, *S. oenotriae* Brullo and *S. velutina* Pourr. ex Loisel.) have a distribution centred in the Tyrrhenian area. For the purposes of this study, only the species with North Tyrrhenian distribution (*S. velutina*, *S. ichnusae*, *S. badaroi*) were considered. Among these species, *S. velutina* is the only one under protection, although it is present in a greater number of stations with respect to the other species.

Seed germination is one of the most important and complex stages of a plant's life cycle, and a detailed understanding of how environmental factors influence this process is important to ensure the conservation of rare and/or endangered species (Baskin and Baskin 1998).

Several studies highlight the presence of inter- and intra-specific variations in seed germination and dormancy (e.g. Andersson and Milberg 1998; Keller and Kollman 1999; Santo et al. 2015a, 2015b), attributing this phenomenon to environmental differences, genetic variations or both (Degreef et al. 2002; Cruz et al. 2003). In phylogenetically related species, such as congeneric species, it is well known that several environmental factors (e.g. light, temperature, moisture, and soil composition) have a direct impact on germination behaviour (Ellison 2001), which reflects their ecological adaptations and therefore may explain their distribution and rarity (Ramírez-Padilla and Valverde 2005). This aspect is even more significant for plants growing in coastal habitats, where harsh environmental conditions occur (e.g. high insolation, temperature and soil moisture fluctuations, strong winds, high salt concentrations, low nutrient

availability) (Maun 2009; Thanos et al. 1991). In these complex ecosystems several species adopt the strategy of seed dormancy, waiting for favourable conditions to germinate (Baskin and Baskin 1998). Although light and temperature are the main factors that influence seed germination, salinity is also considered as one of the selective forces (Baskin and Baskin 1998). Salt stress can cause changes in the mechanisms producing the balance of germination regulators, thereby inducing a physiological secondary dormancy (Ungar 1978; 1984). In particular, salt may inhibit seed germination either by creating a low osmotic potential, which prevents water uptake, or through the toxic effects of Na^+ and Cl^- ions on the metabolic processes (Khajeh-Hosseini et al. 2003; Kaya et al. 2006). In the literature, nitrogenous compounds, and more specifically potassium nitrate (KNO_3), are reported to alleviate salt stress and promote germination under saline conditions (Khan 2003; Zehra et al. 2013). Seeds that are unable to germinate at high salinity levels might survive during salt exposure and maintain the ability to germinate later (recovery), when salinity decreases due to various environmental events, such as autumnal rainfall (Baskin and Baskin 1998). Seeds of several species treated with high salt concentrations recovered their germination following transfer to distilled water. The ability of seeds to recover is species-specific (Song et al. 2005), and variations in seed recovery percentages were often attributed to differences in the temperature regimes to which they were exposed (Pujol et al. 2000).

Several studies on the seed germination ecology of *Silene* L. species were conducted with the aim to investigate their responses to light, temperature, and the effect of seed scarification (Thompson 1970, 1975; Flocca et al. 2004; Menges 2005; Camelia 2011). However, there are few studies on the effect of NaCl and nitrates (Woodell 1985). Despite this, these compounds could play an important role on the germination of three species highlighted in the present study, which are exposed to a salt gradient in their habitats, due to their proximity to the sea and to nitrates in the soil, originating both from the decomposition of *Posidonia oceanica* (L.) Delile leaves and the guano produced by seabirds (seagulls, shearwaters, etc.). In particular, the presence of seabird colonies and their excrement was considered as a threat for *S. velutina* (Paradis et al. 2001), the only species, among the three considered in this work, that has already been the object of other studies (Bacchetta 2001; Paradis 2006). Nevertheless, in the literature, the relationship between nitrates and salinity is poorly understood and varies among species. Moreover, no published works exist about the ecology and biology of the studied species, or the other species of the aggregate.

Therefore, the present study was conducted with the aim to investigate and compare: 1) seed germination requirements in terms of light and temperature, 2) the effect of NaCl and KNO₃ on seed germination and its recovery at different temperatures and 3) intra- and inter-specific variability in seed germination under the different treatments, for the three different species (*S. velutina*, *S. ichnusae* and *S. badaroi*).

The responses to these factors could be useful to better understand the distribution and rarity of these schizo-endemic species and to plan appropriate *in situ* and *ex situ* conservation actions.

Materials and Methods

Study species

The three investigated species belonging to the *S. mollissima* aggregate are perennial chamaephytes characterised by dense rosettes, flowering from April to June and fruiting from May to July. Moreover, *Silene* species produce orthodox seeds with a peripheral embryo (Martin 1946; Royal Botanic Gardens Kew 2015). All three species occur in coastal habitats of the Northern Tyrrhenian area. In particular, *S. badaroi* has a narrow and scattered range. It is endemic to sandy and rocky areas of the Provence region (France), Liguria and the Tuscan Archipelago (Italy), therefore having a disjunct distribution. *S. ichnusae* is a narrow endemic occurring only on rocky and glareicolous habitats in North Western Sardinia (Italy). *S. velutina* is a Sardinian-Corse endemic species of rocky and sandy habitats in the Northern Sardinia (Italy), Central-Southern Corse (France) and some surrounding islets. *Silene velutina* is listed in the Habitats Directive 92/43/EEC as a priority species, in the Bern Convention and it is included both in the IUCN International Red Lists as near threatened (NE) (IUCN 2013) and in the French and Italian Red Lists, respectively as vulnerable (VU) and endangered (EN) (Olivier et al. 1995, Conti et al. 1997, Rossi et al. 2015).

Seed lot details

Capsules of each species were collected from 30 individuals randomly selected in their natural populations at the time of natural dispersal (Table 1). Seed collections of *S. velutina* in Sardinia were carried out after obtaining permits from the “Ministero dell’Ambiente e della Tutela del Territorio e del Mare (MATTM)”, as required by the European and Italian laws for the species listed in the appendices of Habitat Directive 92/43/EEC, while seeds from Corsica were collected and provided after obtaining permits from the

“Direction Régionale de l'Environnement, de l'Aménagement et du Logement (DREAL). Seeds were separated from the fruits, selected by hand and stored under controlled conditions (20°C and 50% of relative humidity) for two weeks before starting germination tests. The mean seed mass (± 1 standard deviation, hereafter SD) was calculated by weighing 10 replicates of 20 seeds each (Table 1).

Germination tests

Germination tests were performed under laboratory conditions at the Sardinian Germplasm Bank (BG-SAR). A preliminary test was carried out for each seed lot in order to evaluate the effect of light on seed germination. Seeds were sown on 1% water agar substrate in plastic Petri dishes of 60 mm diameter and then incubated in growth chambers (SANYO MLR-351, Japan) at 15°C, both in the light (12 h of irradiance per day) and in total darkness. Light in each growth chamber was provided by nine fluorescent lamps with white light (Mitsubishi OSRAM 40; 53 Watt for each), and darkness was achieved by wrapping the dishes in two layers of aluminium foil, immediately after the seeds were sown and before water imbibition. For each condition, four replicates of 25 seeds each were used. The criterion for germination was visible protrusion of the radicle (≥ 2 mm). Seeds incubated in the light were scored daily, and germinated seeds discarded. 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, tests were stopped, and the viability of each remaining seed was checked by a cut test using a scalpel and subsequent observation of the endosperm under a binocular microscope. In order to evaluate the effect of temperature and inter- and intra-population variability, germination tests were conducted for each seed lot only in the light. Four replicates of 25 seeds per each treatment were incubated in growth chambers in a range of constant (5, 10, 15, 20, 25°C) and alternating (25/10°C) temperatures. In particular, in this latter temperature regime, the higher temperature coincided with the light period.

To evaluate the effect of salt stress on seed germination, seeds from two *S. velutina* seed lots and from the unique seed lots of both *S. ichnusae* and *S. badaroi* (Table 1) were sown and treated with different NaCl concentrations (0, 100, 200, 300, 400, 500, 600 mM) and incubated in a range of constant temperatures (5, 10, 15 and 20°C) in the light. In order to evaluate the effect of potassium nitrate (KNO₃) on the seed germination of each species, seeds from selected seed lots (Table 1) were sown in different NaCl

concentrations (0, 100, 200, 300 mM) with the addition of 20 mM of KNO₃ and incubated at the set of constant temperatures of 10, 15 and 20°C.

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 an additional 30 days period (recovery phase) at the same incubation temperatures. Seeds treated with KNO₃ were compared using the correspondent germination in the same NaCl concentration at the same incubation temperature as the control.

Data analysis

Final germination percentages were calculated as the mean of four replicates ($\pm$ 1SD), and recovery percentages (hereafter RP) were calculated according to the following equation (1) (Pujol et al. 2000):

$$(1) \text{ RP} = \{[(a-b)/(c-b)] \times 100\},$$

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

Germination percentages and RP were analysed using the non-parametric Kruskal–Wallis test, followed by a Mann–Whitney *U* test. Graphs were realised using Sigmaplot 11.0 software (Systat Software Inc., London, UK), and all statistical analyses were carried out using Statistica 7.0 for Windows (Software Statsoft Release 7).

Results

Effect of light on seed germination

The preliminary light experiment was performed for all the seed lots at a temperature of 15°C. *Silene velutina* seed germination in the light ranged from $98.0 \pm 2.3\%$ (Svel3) to $99.0 \pm 5.2\%$ (Svel2), while in the dark from 0% (Svel2) to $30.0 \pm 11.5\%$ (Svel3). *Silene ichnusa* germinated with $99.0 \pm 2.3\%$ and $26.0 \pm 6.9\%$, in the light and in the dark, respectively. *Silene badaroi* seeds germinated with percentages of $99.0 \pm 9.8\%$ in the light and $40.0 \pm 10.8\%$ in the dark. The Kruskal–Wallis test for all the seed lots showed that final germination percentages were significantly higher ($p < 0.05$) in the light (ca. 100%) than in the dark (up to ca. 40%). Therefore, all subsequent germination tests were conducted in the light.

Effect of temperature and inter-population variability in seed germination

At 5°C final germination differed among the seed lots and the highest germination percentages (up to ca. 99%) were detected for the three *S. velutina* populations. The lowest germination was detected for Sbad1 and it was significantly different ($p < 0.05$) from all others. At 10 and 15°C, germination was higher than 85% for all the seed lots (Figure 1). At 20°C the highest germination was detected for Svel2, Svel3 and Sich1 (with percentages of ca. 90%), which was significantly different ($p < 0.05$) from the lowest germination observed for Svel1 and Sbad1 (ca. 40%; Figure 1). At 25°C the highest germination occurred for Svel3 and Sich1 (ca. 20%) while the lowest was detected for Sbad1 (ca. 5%). No germination at this temperature was observed for seed lots Svel1 and Svel2. At the alternating temperature regime of 25/10°C, the highest germination occurred for Svel3 (ca. 95%), and this value was not significantly different ($p > 0.05$) only from Sich1. Seeds from Svel1, Svel2 and Sbad1 germinated with percentages of ca. 80%.

For all the seed lots, final germination at 25°C was significantly lower than at all other temperatures, or in some cases (Svel1 and Svel2) no germination occurred. Significantly lower germination percentages were also detected at 20°C for Svel1 and Sbad1 (ca. 50% and 35%, respectively). High germination ($> 80\%$) occurred at 5, 10, 15 and 25/10°C for all populations.

NaCl and recovery on seed germination

Svel2

The highest germination percentages were observed under control conditions (0 mM NaCl), and significant differences ($p < 0.05$) were detected among the tested temperatures. Seed germination decreased with increasing salinity at all temperatures; however, germination at 100 mM NaCl at 10 and 15°C did not show significant differences ($p > 0.05$) with that detected at 0 mM (ca. 90%). At 100 mM, the lowest germination occurred at 5 and 20°C (ca. 70% for both). At concentrations above 200 mM, germination was completely inhibited at all temperatures. Significant differences ($p < 0.01$) among germination percentages under the different NaCl concentrations were detected at each tested temperature (Table 2A).

Independent of the tested temperature, RP did not show significant differences ($p > 0.05$) at 100, 200 and 300 mM NaCl. At 400 mM, RP were higher than 80% at all temperatures and showed the highest values at 10

and 15°C (ca. 93%). At 500 mM, the highest recovery occurred at 15°C (ca. 90%) while the lowest RP at 5 and 20°C (ca. 45% and 70%, respectively) were observed. At 600 mM RP did not differ among the tested temperatures and showed values above 90%. Significant differences ($p < 0.05$) were detected among RP at 5 and 20°C, and RP did not differ at 10 and 15°C ($p > 0.05$).

Svel3

The highest germination percentages ($> 70\%$) were detected at 0 and 100 mM NaCl (Table 2B). Significant differences ($p < 0.01$) were detected under the control among the four tested temperatures. This seed lot germinated at a concentration of 300 mM at 15°C (ca. 6%), and germination at all other temperatures was inhibited at concentrations above 100 mM. No significant differences ($p > 0.05$) were detected at 100, 200 and 300 mM for germination at different temperatures.

RP did not show significant differences ($p > 0.05$) among the tested temperatures, with the exceptions of 200 and 400 mM, at which RP ranged from ca. 90% to ca. 100% ($p < 0.05$). At all temperatures, RP did not show significant differences at different NaCl concentrations.

Sich1

Under control conditions (0 mM NaCl), no significant differences ($p > 0.05$) were observed among the tested temperatures (Table 3A). Significant differences ($p < 0.05$) were detected at 100 mM and germination ranged from ca. 80% to ca. 95%. The highest germination occurred at 0 mM and 100 mM but, at 5°C, germination at 100 mM was significantly ($p < 0.05$) lower than at 0 mM. At 15°C, seeds showed their ability to germinate up to 300 mM, while germination at all other temperatures was inhibited at concentrations above 100 mM. RP at all NaCl concentrations showed no significant differences ($p > 0.05$) among the tested temperatures.

Sbad1

Under control conditions (0 mM NaCl) significant differences ($p < 0.01$) were detected among the tested temperatures, and germination ranged from ca. 50% to ca. 95%. At 100 mM, the lowest germination occurred at 5°C (ca. 20%) while the highest occurred at 10 and 15°C (ca. 85%). Germination was inhibited at concentrations above 100 mM at all tested temperatures (Table 3B). Significant differences were detected

among RP at all NaCl concentrations, except at 200 mM. At each of the tested temperatures, RP showed significant differences ($p < 0.05$) among the NaCl concentrations.

Effect of KNO_3 on seed germination under salinity

For each seed lot, germination both under control conditions (only NaCl) and with the addition of potassium nitrate (NaCl + KNO_3) was inhibited at all concentrations above 100 mM NaCl, except for Svel3 and Sich1 under control conditions at 15°C. For all seed lots, no significant differences in germination percentages ($p > 0.05$) were detected between the control conditions and nitrate treatment (Figure 2). The only exceptions were for Svel3 (0 mM NaCl at 15°C), where KNO_3 improved seed germination, and at 100 mM NaCl (Svel2 at 20°C, Svel3 at 15°C, Sich1 and Sbad1 at 10°C), where the addition of nitrate negatively affected germination.

Effect of KNO_3 under salinity on recovery

For the four tested seed lots and at all temperatures, germination recovery under control conditions (NaCl) increased significantly ($p > 0.05$) following exposure to 100 mM to 200 and 300 mM NaCl, except for Sich1 at 20°C, which did not show significant differences ($p > 0.05$) at different salt concentrations (Figure 3).

Recovery at 15°C for all seed lots (except Sbad1) was not performed because all seeds germinated or died under the previous phase of treatment.

Germination recovery at 200 and 300 mM was also significantly higher ($p < 0.05$) than at 100 mM NaCl, with the exception of Sich1 at 10°C (for which there were no differences), and 20°C (for which RP at both 100 and 300 mM NaCl were significantly lower than at 200 mM NaCl).

In most cases, no significant differences ($p > 0.05$) were detected in the RP between control conditions and nitrate treatment. The only differences were observed for Svel3 at 200 mM NaCl at 15°C and Sich1 and Sbad1 at 100 mM at 10° and 20°C, respectively.

Discussion

Thanos et al. (1989; 1995) found that the germination of several Mediterranean coastal species is photo-inhibited, highlighting a surface avoidance mechanism that enables seeds to avoid germinating under the

harsh conditions of the soil surface. However, seeds of the three *Silene* species investigated in the present study, which achieved high germination percentages in the light, did not display this kind of response, therefore they were not photo-inhibited for germination. Moreover, for all species, the higher germination percentages detected in the range of 5-15°C and the significant decrease in germination at the highest temperature (25°C) are in accordance with Thanos et al. (1989; 1995), who observed that germination at low temperatures is a widely extended trait in many Mediterranean coastal species. However, for *S. badaroi*, the germination response at 20°C was significantly lower than that detected at the colder temperatures. *S. velutina* and *S. ichnusa* seeds also showed their capability to germinate at high percentages at 20°C. Therefore these results may correspond to field germination in the period from autumn until late spring for *S. velutina* and *S. ichnusa*, and mainly during winter months for *S. badaroi*. Germination during the period from autumn to spring (when water availability, soil moisture and rainfall are high, and temperatures are not prohibitive for germination and the establishment of consequent seedlings) may ensure ecological success in an unpredictable climate such as that found in the Mediterranean Basin (Thanos et al. 1995; Kadis and Georgioui 2010, Santo et al. 2014b).

Probert (1992) suggested that responding to alternating temperatures represents an adaptation of small-seeded species, which ensures that germination occurs only close to the soil surface because light is only able to penetrate 4-5 mm into the soil in physiologically significant quantities (Tester and Morris 1987). Seeds of all three *Silene* species responded positively to the tested alternating temperature regime, showing that field germination occurs preferably in the soil layers near to the surface, where the effect and influence of the alternate temperatures are greater.

The tested *Silene* species showed different levels of tolerance to salinity, which were 300 mM NaCl for *S. velutina* and *S. ichnusa* and 100 mM for *S. badaroi*. For all the species, lower germination percentages were observed in the salt substrate, compared to the higher germination percentages observed under control conditions (0 mM NaCl). Temperature did not influence germination under salt stress in any of the species. Many studies report that percentages of germination decrease with increasing salinity stress, and the highest germination occurs in the absence of NaCl in the substrate (Khan and Ungar 1984; Baskin and Baskin, 1998; Santo et al. 2014b). The limit of tolerance to salt varies among different species (Ungar 1995), for example, 200 mM NaCl in *Halopyrum mucronatum* (L.) Stapf and *Sporolobus arabicus* Boiss. (Gulzar et al. 2001),

300 mM in *Silene maritima* With. (Woodell 1985), 310 mM in *Brixia maxima* L. (Lombardi et al. 1998), 344 mM in *Puccinellia nuttalliana* (Schult.) Hitchc. (Macke and Ungar 1971) and *Hordeum vulgare* L. (Badger and Ungar 1989), 400 mM in *Diplachne fusca* (L.) Beauv. (Morgan and Myers 1989), 500 mM in *Urochondra setulosa* (Trin.) C.E. Hubb. (Gulzar et al. 2001) and up to 1712 mM NaCl in *Neokochia americana* (S.Wats.) G.L. Chu & S.C. Sand. (Clarke and West 1969). Differences in tolerance to salinity were detected in the present study between the two tested populations of *S. velutina* (100 mM for Svel1 and 300 mM for Svel2). However 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 and Müller-Schärer 2010). Moreover, differences in salt stress response were also observed among populations of *Panicum turgidum* Forssk. (El-Keblawy et al. 2010), *Spartina patens* (Aiton) Muhl (Hester et al. 1996) and *Rouya polygama* (Desf.) Coincy (Santo et al. 2014a), but not in *Crucianella maritima* L. (Del Vecchio et al. 2012). According to Gutterman (1994) and Kigel (1995), the variability of germination requirements could be interpreted as one of the most important survival strategies for species growing under unpredictable environmental conditions.

Similarly to *Silene maritima* (Woodell 1985), *S. velutina* and *S. ichnusa* showed a high capability to recover their germination after exposure to salt, independent of the temperatures and NaCl concentrations to which the seeds were exposed in the previous salt phase. Conversely, *S. badaroi* seed recovery after salt conditions decreased with increasing salinity at the extreme temperatures of 5°C and 20°C. The different NaCl tolerance and recovery response behaviour among the three *Silene* species confirm the assertion of Khan and Ungar (1984) that tolerance and recovery from salinity and temperature stress are species-specific. Seeds of some species do not recover or show little recovery response when subjected to high salinity and temperature stress (Khan and Gul 2006). However, for all three species the effects of NaCl did not influence seed mortality, inasmuch as seeds of each species also survived high salt concentrations, demonstrating their ability to wait for favourable germination conditions, after high salt exposure.

Many studies have demonstrated that nitrates are capable of alleviating salinity stress in several species. For example, Gull and Weber (1998) found that the addition of nitrate (20 mM) alleviated the inhibitory effect of salt on germination in *Allenrolfea occidentalis* (S. Watson) Kuntze at different salt concentrations (200, 400, 600 and 800 mM NaCl); similarly, an equal concentration of nitrate promoted seed germination in *Atriplex*

prostrata Boucher ex DC. at 100, 200, 300 mM NaCl (Khan et al. 2003), and in *Crithmum maritimum* L., 10 mM KNO₃ enhanced germination at 100 and 200 mM NaCl (Atia et al. 2009). On the contrary, nitrate did not alleviate seed dormancy and germination in *Suaeda salsa* (L.) Pallas, *Descurainia sophia* (L.) Webb ex Prantl (Li et al. 2005), *Halogeton glomeratus* (M.Bieb.) C.A.Mey., *Lepidium latifolium* L. and *Peganum harmala* L. (Ahmed et al., 2013) under various salinity treatments (from 0 until 400 mM NaCl). We observed a similar response in the three *Silene* species investigated in this study, for which, at any tested NaCl concentration, germination was not significantly promoted or affected by nitrate.

Our results highlight that the investigated species experience optimum germination during autumn-winter, when, water availability is highest and soil salinity levels are minimal under the Mediterranean climate. *S. ichnusa* and *S. velutina* are also able to germinate in spring, while *S. badaroi*, responding less to the constant temperature of 20°C, showed a narrower germination window, demonstrating the ability to germinate mainly during winter months. All three species demonstrated that their germination in the field may occur when, due to rainfall, salt concentration decreases in the soil. However, their seeds may tolerate relatively high salinity (600 mM) values in the substrate, recovering their germination after the salt exposure. While they cannot be considered halophilous species, this property may be considered a way to avoid ecological competition with other coastal plant species. The addition of nitrate in the substrate did not affect seed germination of the three species, suggesting both that the nutrient availability is not a requirement for seed germination and that the presence of KNO₃ in the habitat due to seabirds does not constitute necessarily a threat for them. Further studies are needed, possibly extended to the whole *S. mollissima* aggregate, to better understand the ecology of all species and explain their distribution and rarity.

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Table 1 - Population data and seed lot details. In the last column the different experiments carried out for each seed lot are reported (L = Light; T = Temperature; NaCl = Salinity; KNO₃ = Nitrate under salinity).

Species	Population code	Coordinates (UTM, WGS84)	Substrate	Distance from sea (m)	Date of collecting	Mean seed mass (mg $\pm$ SD)	Experimental trials
<i>S. velutina</i>	Svel1	41°14' N 09°24' E	Aeolian sands	10	09/08/2013	0.98 $\pm$ 0.06	L; T
<i>S. velutina</i>	Svel2	41°35' N 09°18' E	Conglomerates	2	28/07/2013	1.09 $\pm$ 0.09	L; T ; NaCl ; KNO ₃
<i>S. velutina</i>	Svel3	41°11' N 09°26' E	Granites	2	11/08/2013	1.26 $\pm$ 0.09	L; T ; NaCl; KNO ₃
<i>S. ichnusae</i>	Sich1	40°57' N 08°11' E	Metamorphytes	10	29/07/2013	0.98 $\pm$ 0.08	L; T; NaCl; KNO ₃
<i>S. badaroi</i>	Sbad1	44°11' N 08°25' E	Limestones	25	01/07/2013	0.74 $\pm$ 0.06	L; T ; NaCl; KNO ₃

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Table 2 - Germination (G) and recovery percentages (RP) at each temperature regime (5-20°C) and under different saline conditions (0-600 mM NaCl) for *Silene velutina* (A: Svel2 and B: Svel3).

Kruskal-Wallis tests were conducted to detect the effect of the same temperature and salinity concentration 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 mean of four replicates ($\pm$ 1SD). Capital letters in columns are related to the same NaCl concentration, 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). See Table 1 for the explanation of the population code.

(A)

Temperature (°C)	Percentage (%)	NaCl concentration (mM)							
		0	100	200	300	400	500	600	
5	G	96.0 $\pm$ 3.3 Aa	72.0 $\pm$ 7.3 Ab	0 Ac	0 Ac	0 Ac	0 Ac	0 Ac	**
	RP	-	25.1 $\pm$ 23.0 a	82.0 $\pm$ 10.1 bc	90.0 $\pm$ 2.3 bd	81.0 $\pm$ 3.8 Acd	44.0 $\pm$ 17.0 Aa	91.0 $\pm$ 7.6 bcd	*
10	G	91.0 $\pm$ 6.0 ABa	91.0 $\pm$ 3.8 Ba	0 Ab	0 Ab	0 Ab	0 Ab	0 Ab	**
	RP	-	0	82.0 $\pm$ 12.0	89.0 $\pm$ 5.0 b	92.0 $\pm$ 5.7 BC	86.0 $\pm$ 2.3 B	95.0 $\pm$ 2.0	ns
15	G	83.0 $\pm$ 3.8 Ba	89.0 $\pm$ 3.8 Ba	0 Ab	0 Ab	0 Ab	0 Ab	0 b	**
	RP	-	0	94.0 $\pm$ 5.2	91.0 $\pm$ 3.8	94.0 $\pm$ 4.0 B	91.0 $\pm$ 2.0 C	91.0 $\pm$ 3.8	ns
20	G	96.0 $\pm$ 3.3 Aa	72.0 $\pm$ 7.3 Ab	0 Ac	0 Ac	0 Ac	0 Ac	0 Ac	**
	RP	-	25.1 $\pm$ 23.0 a	97.0 $\pm$ 3.8 b	92.0 $\pm$ 6.5 bc	83.0 $\pm$ 3.8 ACc	67.0 $\pm$ 10.5 Ad	91.0 $\pm$ 8.2 bc	*
	G	*	**	sn	sn	sn	sn	sn	
	RP	-	sn	sn	sn	*	**	sn	

(B)

Temperature (°C)	Percentage (%)	NaCl concentration (mM)							
		0	100	200	300	400	500	600	
5	G	90.0 $\pm$ 2.3 Aa	92.0 $\pm$ 10.8 Aa	0 Ab	0 Ab	0 Ab	0 Ab	0 Ab	**
	RP	-	88.9 $\pm$ 19.2	90.0 $\pm$ 5.2	82.0 $\pm$ 9.52	88.0 $\pm$ 3.3 A	96.0 $\pm$ 4.6	88.0 $\pm$ 3.3	ns
10	G	92.0 $\pm$ 5.7 Aa	98.0 $\pm$ 2.3 Aa	0 Ab	0 Ab	0 Ab	0 Ab	0 b	**
	RP	-	-	100.0 $\pm$ 0.0	93.3 $\pm$ 7.9	91.0 $\pm$ 3.8 A	93.0 $\pm$ 2.0	90.0 $\pm$ 7.7	ns
15	G	70.5 $\pm$ 13.8 Ba	96.0 $\pm$ 0 Ab	1.0 $\pm$ 2.0 Ac	6.0 $\pm$ 9.52 Ac	0 Ac	0 Ac	0 Ac	**
	RP	-	-	99.0 $\pm$ 2.1	77.7 $\pm$ 16.5 A	100.0 $\pm$ 0 B	89.0 $\pm$ 8.2	69.0 $\pm$ 21.3	ns
20	G	99.0 $\pm$ 2.0 Ca	92.0 $\pm$ 5.7 Ab	0 Ac	0 Ac	0 Ac	0 Ac	0 Ac	**
	RP	-	87.5 $\pm$ 25.5	96.0 $\pm$ 5.7	86.0 $\pm$ 9.5	87.0 $\pm$ 7.6 A	92.0 $\pm$ 6.5	69.0 $\pm$ 21.3	ns
	G	**	sn	sn	sn	sn	sn	sn	
	RP	-	sn	*	sn	*	sn	sn	

Table 3 - Germination (G) and recovery percentages (RP) at each temperature regime (5-20°C) and under different saline conditions (0-600 mM NaCl) for *Silene ichnusa* (A: Sich1) and *S. badaroi* (B: Sbad1). The statistical tests were the same used to analyze the data of Table 2. Data are the mean of four replicates (± 1SD). Capital letters in columns are related to the same NaCl concentration, 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). See Table 1 for the explanation of the population code.

(A)

Temperature (°C)	Percentage (%)	NaCl concentration (mM)							
		0	100	200	300	400	500	600	
5	G	90.0 ± 4.0 Aa	80.0 ± 3.3 Ab	0 Ac	0 Ac	0 Ac	0 Ac	0 Ac	**
	RP	-	75.8 ± 19.5	89.0 ± 3.8	81.0 ± 6.0	88.0 ± 8.6	70.0 ± 9.5	89.0 ± 14.4	ns
10	G	90.0 ± 5.16 Aa	91.0 ± 3.8 Ba	0 Ab	0 Ab	0 Ab	0 Ab	0 Ab	**
	RP	-	33.0 ± 0	82.0 ± 12.0	89.0 ± 5.0	92.0 ± 5.7	86.0 ± 2.3	95.0 ± 2.0	ns
15	G	97.0 ± 3.8 Aa	95.0 ± 3.8 Ba	1.0 ± 2.0 Ab	2.0 ± 2.3 Ab	0 Ab	0 Ab	0 Ab	**
	RP	-	-	93.9 ± 4.1	85.7 ± 9.8	94.0 ± 2.3	88.0 ± 9.8	89.0 ± 6.0	ns
20	G	93.0 ± 6.8 Aa	94.0 ± 5.2 Ba	0 Ab	0 Ab	0 Ab	0 Ab	0 Ab	**
	RP	-	83.3 ± 28.9	98.0 ± 2.3	87.0 ± 6.0	92.0 ± 7.3	88.0 ± 9.8	89.0 ± 6.0	ns
	G	sn	*	sn	sn	sn	sn	sn	
	RP	-	sn	sn	sn	sn	sn	sn	

(B)

Temperature (°C)	Percentage (%)	NaCl concentration (mM)							
		0	100	200	300	400	500	600	
5	G	48.0 ± 14.2 Aa	18.0 ± 5.2 Ab	0 Ac	0 Ac	0 Ac	0 Ac	0 Ac	**
	RP	-	85.4 ± 3.5 a	78.0 ± 6.9 b	74.0 ± 6.9 Ab	25.0 ± 13.2 Ac	25.0 ± 15.8 Ac	30.0 ± 18.0 Ac	*
10	G	95.0 ± 5.0 Ba	84.0 ± 6.5 Bb	0 Ac	0 Ac	0 Ac	0 Ac	0 Ac	**
	RP	-	25.0 ± 28.9 a	88.0 ± 3.3 b	83.0 ± 7.6 Abc	71.0 ± 6.8 Bc	77.0 ± 3.8 Bc	75.0 ± 10.5 Bc	*
15	G	70.0 ± 9.52 ACa	84.0 ± 6.5 Ba	0 Ab	0 Ab	0 Ab	0 Ab	0 Ab	**
	RP	-	25.0 ± 28.9 a	79.0 ± 11.2 b	83.0 ± 7.6 Abc	70.0 ± 15.0 Bb	77.0 ± 3.8 Bb	78.0 ± 14.8 Bb	*
20	G	78.0 ± 7.7 Ca	44.0 ± 11.8 Cb	0 Ac	0 Ac	0 Ac	0 Ac	0 Ac	**
	RP	-	75.5 ± 17.4 a	75.0 ± 10.5 a	42.0 ± 14.0 Bb	52.0 ± 5.7 Bb	39.0 ± 13.2 Ab	43.0 ± 16.1 Ab	*
	G	**	**	sn	sn	sn	sn	sn	
	RP	-	*	sn	*	**	**	**	

Figure 1 - Germination in the light (12/12 h) at constant (5-25°C) and alternating (25/10°C) temperatures for all seed lots of the species investigated in this study. Kruskal-Wallis tests were conducted to detect differences among populations at the same temperature (capital letters, by Mann Whitney *U*-test) and the effect of different temperatures for each 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 mean of four replicates ($\pm 1SD$). See table 1 for the explanation of the population codes.

Figure 2 - Effect of KNO₃ (20 mM) on seed germination under NaCl (100-300 mM) at constant temperatures (10-20°C) for *S. velutina* (Svel2, Svel3), *S. ichnusae* (Sich1) and *S. badaroi* (Sbad1). Kruskal-Wallis tests were conducted to detect differences between the nitrate treatment and the control (capital letters, by Mann Whitney *U*-test) and the effect of different temperatures for each 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 mean of four replicates ($\pm 1SD$). See Table 1 for the explanation of the population codes.

Figure 3 - Recovery percentages (RP) of seeds exposed to KNO₃ (20 mM) under different salinities (100-300 mM) at constant temperatures (10-20°C) for *S. velutina* (Svel2, Svel3), *S. ichnusae* (Sich1) and *S. badaroi* (Sbad1). Kruskal-Wallis tests were conducted to detect differences between treatment and control (capital letters, by Mann Whitney *U*-test) and the effect of different NaCl concentrations for each 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 of four replicates ($\pm 1SD$). See table 1 for the explanation of the population codes.

Figure 1 - Germination in the light (12/12 h) at constant (5-25°C) and alternating (25/10°C) temperatures for all seed lots of the species investigated in this study. Kruskal-Wallis tests were conducted to detect differences among populations at the same temperature (capital letters, by Mann Whitney U-test) and the effect of different temperatures for each 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 mean of four replicates ($\pm 1SD$). See table 1 for the explanation of the population codes.

208x237mm (300 x 300 DPI)

Figure 2 - Effect of KNO₃ (20 mM) on seed germination under NaCl (100-300 mM) at constant temperatures (10-20°C) for *S. velutina* (Svel2, Svel3), *S. ichnusae* (Sich1) and *S. badaroi* (Sbad1). Kruskal-Wallis tests were conducted to detect differences between the nitrate treatment and the control (capital letters, by Mann-Whitney U-test) and the effect of different temperatures for each 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 mean of four replicates ($\pm$ 1SD). See Table 1 for the explanation of the population codes.

196x219mm (300 x 300 DPI)

Figure 3 - Recovery percentages (RP) of seeds exposed to KNO₃ (20 mM) under different salinities (100-300 mM) at constant temperatures (10-20°C) for *S. velutina* (Svel2, Svel3), *S. ichnusae* (Sich1) and *S. badaroi* (Sbad1). Kruskal-Wallis tests were conducted to detect differences between treatment and control (capital letters, by Mann Whitney U-test) and the effect of different NaCl concentrations for each 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 of four replicates ($\pm 1SD$). See table 1 for the explanation of the population codes.
170x162mm (300 x 300 DPI)