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Reproductive traits of three species endemic to the Puglia region (south-eastern Italy)

M. Galié, R. Gasparri, E. Biondi, R.M. Perta, N. Biscotti, S. Pesaresi, S. Casavecchia
Department of Agricultural, Food and Environmental Sciences, Marche Polytechnic University, Via Brecce Bianche, I-60131 Ancona, Italy.

Abstract
The importance of the conservation of three species endemic to the Puglia region (*Centaurea subtilis*, *Centaurea diomedea*, *Campanula garganica* subsp. *garganica*) and of their habitats *sensu* Directive 92/43/EEC led us to carry out studies on seed viability assessments and germination of these species. Thus, the purpose of this study was to investigate the reproductive traits (seed germination behaviour and reproductive strategy in relation to temperature) of *Centaurea subtilis*, *C. diomedea* and *Campanula garganica* subsp. *garganica*. This preliminary study is fundamental to highlight the germinative potential of each of these species, on the basis of the different environmental conditions reproduced in the laboratory, and for the identification of any eco-physiological problems related to their germination, especially in terms of habitat conservation. Finally, these data will be very useful in the planning of seed multiplication strategies and reintroduction protocols.

Key words: *Centaurea subtilis*, *Centaurea diomedea*, *Campanula garganica* subsp. *garganica*, endemic species, habitat conservation, seed germination, seed viability.

Introduction

The importance of endemic species for defining conservation priorities is widely understood (Reid, 1998; Myers *et al*., 2000; Burlakova *et al*., 2011; Ungricht, 2004; Linder, 1995). Endemic species increase the amount of genetic diversity (Skarbek, 2008), capturing an unique evolutionary history (Cuttelod *et al*., 2008) and are related to the environmental and historical factors that influence their patterns of distribution (Casazza *et al*., 2008).

The present research focuses on seed viability assessments and germination studies of three Italian endemic species: *Campanula garganica* Ten. subsp. *garganica* and *Centaurea diomedea* Gasp., exclusively endemic to the Puglia region (south-eastern Italy) and *Centaurea subtilis* Bertol., endemic to the Puglia and Basilicata regions.

Centaurea subtilis, also known as cornflower of the Gargano (*Asteraceae*), is a suffruticous chamaephytes/scapose hemicryptophyte. Fruits (achenes) are 3 mm long and have a pappus that is 1/3-1/2 of achene in length (Pignatti, 2018).

It is reported in the IUCN category ‘Least Concern’ despite it is pressured by recreational/residential activities and natural system modifications (Orsenigo *et al*., 2018). It is a species of rocky garrigue and limestone cliffs with little slope, and it constitutes the association *Centaureetum subtilis*, which forms the junction between the rocky vegetation and that of the garrigue with thorny shrubs (Bianco *et al*., 1988). Moreover, *Centaurea subtilis* dominates some communities, such as *Matthiolo fruticulosae-Centaureetum subtilis* described by Di Pietro and Misano (2006) for the western ravines of the Arco Ionico, and *Centaureo-Thymetum capitati* described for the Murgia of Matera by Terzi and D’Amico (2006). It is also counted among the species of the habitat 8210 “Calcareous rocky slopes with chasmophytic vegetation” (Biondi *et al*., 2013). In the Gargano area, even if the species occurs in different localities, it forms isolated populations of small size and, therefore, the threat of genetic drift is high. Consequently, in the next future, it could be necessary to undertake restocking actions so that knowing the germination behavior of its seeds could be crucial to save the species from the extinction.

Centaurea diomedea, or cornflower of the Tremiti Islands (*Asteraceae*), is a suffruticous chamaephyte with summer flowering. Fruits (achenes) are 3.5 mm long with a pappus of 1.5 mm (Pignatti, 2018). It is exclusively endemic to the Tremiti archipelago, which originated through geographic isolation following the environmental changes that occurred in the Quaternary related to the formation of the islands. According to Orsenigo *et al*. (2018) *Centaurea diomedea* is an endangered species and its conservation is affected by multiple threats such as humane disturbance, residential/commercial development and biological resource use. It forms chamaephyte and sub-halophilous communities that relate to the association *Anthyllido-Centaureetum diomedeae*. These communities developed on the high limestone cliffs of the islands of San

Corresponding author: Simone Pesaresi. *Department of Agricultural, Food and Environmental Sciences, Marche Polytechnic University, Via Brecce Bianche, I-60131 Ancona, Italy; e-mail: s.pesaresi@staff.univpm.it*

Domino and San Nicola (Brullo & De Marco, 1989; Biondi *et al*., 2010, 2017), and they belong to the 5320 habitat "Low formations of *Euphorbia* close to cliffs" (Biondi *et al*., 2013). Once again, a deep knowledge on germination behavior can be very useful in case of active conservation actions.

Campanula garganica subsp. *garganica*, or Adriatic bellflower (*Campanulaceae*), is a scapose hemicriptophyte with late spring-summer flowering (May-July) that naturally occurs on rocks, but also colonises ancient walls. Seeds subglobose-ovoid, 0.45-0.5 mm, red-brown shiny (Bernini *et al*., 2002; Pignatti, 2018). It is reported in the IUCN Red list of the Italian endemic vascular flora (Orsenigo *et al*., 2018) as vulnerable species and mostly threatened by gathering. It is a characteristic species of the association *Aubrieto italicae-Campanuletum garganicae* described for Mount S. Angelo and the cliffs of the 'Vallone di Pulsano' up to Mattinatella (Gargano; Bianco *et al*., 1988). This is a relatively polymorphic vegetation in which *Campanula garganica* occupies the crannies and the recessed niches on compact limestone (Bianco *et al*., 1988; Biondi *et al*., 2017). These chasmophytic communities fall within habitat 8210 "Calcareous rocky slopes with chasmophytic vegetation" (Biondi *et al*., 2013; Wagensommer & Russo, 2013).

In order to effectively safeguard threatened habitats, it is important both the definition of the optimal germination protocols of their endemics (Estrelles *et al*., 2010) and the promotion of use of wild species germplasm for habitat restoration projects.

There are several studies on dispersion and germination (Viegi *et al*., 2003), molecular phylogeny (Hilpold *et al*., 2009) and taxonomy (Wagensommer *et al*., 2016) of some Italian *Centaurea* species but no previous study has investigated seed germination behaviour of *Centaurea diomedea* and *C. subtilis*. As regard as the *Campanulaceae* family, there are several seed morphology studies (Alçitepe, 2010; Mehrvarz & Kashi, 2015) and investigation on the effect of light on germination in 131 *taxa* (Koutsovoulou *et al*., 2014) but no seed germination studies of *Campanula garganica* subsp. *garganica* can be find in the literature.

Thus, this study was aimed at the investigation of these three species in terms of: (i) seed viability and seed macromorphological features; and (ii) seed germination behaviour and reproductive strategy in relation to temperature, useful for restoration purposes. The results of this study, although preliminary, are crucial for planning reintroduction strategies and subsequent analysis to identify the best seed multiplication protocols.

Materials and methods

The collection of the seeds (fruits for the two *Centaurea* species) necessary for this research was carried out on the Gargano Promontory and on the archipelago of the Tremiti Islands. The Gargano is a southern area on the Adriatic (eastern) side of the Italian peninsula. It has an area of around 2,000 km^2, and a geological matrix that is exclusively limestone. It is an important hotspot of biodiversity. Although it represents only 1% of the regional area, it contributes 31% of the Italian flora and 92.5% of Apulian flora (Biondi *et al*., 2017). The Tremiti Islands are the unique islands of the Italian Adriatic coast, along with Pianosa and Pelagosa Islands. From a geological point of view, the Tremiti Islands are the continuation of the calcareous dorsal of the Gargano promontory, and they are located at 12 nautical miles from the northern coast of the promontory, by Lesina and Varano Lagoons. The Gargano archipelago is composed of Tertiary calcareous rock, with three islands, San Domino (1.98 km^2), San Nicola (0.48 km^2) and Caprara (0.52 km^2), and an islet, Cretaccio (0.04 km^2), and a few rocks, and it has an overall surface area of 3 km^2. The biogeographic isolation of the two areas (i.e., Gargano promontory, Tremiti Islands) with their different environmental conditions has permitted the survival of plant and animal species that are rare in the rest of the regional territory (Pezzetta, 2011).

The mean annual temperature ranges from 16.3°C to 16.4°C for the Gargano collection sites and 16.9°C for the Tremiti Islands. The mean annual precipitation ranges from 638 mm and 665 mm for the Gargano collection sites and 371 mm for the Tremiti Islands. According to the bioclimatic classification of Rivas-Martínez *et al*. (2011), all collection sites belong to the Mediterranean macrobioclimate with lower mesomediterranean thermotype. The ombrotypes are upper dry for the Gargano sites collection and lower dry Tremiti Islands. Furthrmore the summer aridity is estimated by the summer bimonthly ombrothermic index (Ios_2; mm/°C). Ios_2 values lower than 2 testify a hydrical stress condition of at least two consecutive months during summer and are typical of Mediterranean environment Ios_2 ranges from 1.15 to 1.24 for the Gargano sites collection and 0.75 for the Tremiti Islands (Tab. 1). The climatic and bioclimatic data were derived from Pesaresi *et al*. (2014; 2017).

Seed collection

The seeds (achenes for the two *Centaurea* species) were collected according to the description of seed collection protocols (Bacchetta *et al*., 2006; International Seed Testing Association, 2006). A part of seed samples was properly preserved in the Amphiadriatic Species Seed Bank of the Botanical Garden "Selva di Gallignano" (Department of Agricultural, Food and Environmental Sciences, Marche Polytechnic University). Following their harvest, the seeds were extracted from the fruit or the inflorescence and processed using a blower to remove fruit and inflorescence residues and empty seeds (Agriculex CB1 Column Seed Cleaner; T.A. Baxall and Co., Ltd). Afterwards, the seeds were dried and stored in a dry room at 15 °C and 15% relative humidity, before being used for the germination studies and the morphological analysis.

Seed viability and quality analysis

The achenes of *Centaurea subtilis* and *C. diomedea* have a spatulate, erect embryo with two cotyledons that are generally well developed (Martin, 1946). The quality of the achene lots of *Centaurea subtilis* and *C. diomedea* was assessed via the cut test, the tetrazolium test and X-ray analysis. The cut test was carried out on a random sample of 70 seeds for each species, which were scored as healthy if the seed had a turgid, white and fully formed embryo (International Seed Testing Association [ISTA] 1999) and a well-developed endosperm. The seeds that were considered as healthy were then used for the tetrazolium test according to the protocol of the Association of Official Seed Analysts (2004) and specific for achenes of *Asteraceae*. Seeds were scored as viable when completely stained, while they were scored as not viable when the staining was incomplete or absent. A sample of 50 seeds was scanned in a digital X-ray cabinet (MX-20 Faxitron; Qados, Sandhurst, UK; settings: 22kV, 0.3 mA, 20 s) and counted as filled if the embryo and endosperm entirely filled the seed. After these analyses, the achene lots of *Centaurea subtilis* and *C. diomedea* underwent additional gravimetrical selection, with the lightest seeds eliminated, to reduce the proportion of empty and poorly developed seeds used in the macromorphological analysis and germination tests.

Seed macromorphological data and analysis

Ten samples of 50 air-dried seeds of each seed lot were weighed on a balance to four decimal places (BCA120; PBI International), thus with a precision of 0.1 mg. The lengths and widths of 20 randomly selected seeds were measured using a stereomicroscope (Nikon SMZ800). The seed volumes were calculated with the equation of Dias and Ganhão (2012). Means ±standard deviation was calculated for seed volumes and seed weights.

Germination assay and analysis

For the germination tests, the seeds were sown on 1% distilled water agar in 55-mm-diameter transparent polyethylene Petri dishes. The germination responses were evaluated using environmental chambers that were set at four constant temperatures, from 5 °C to 20 °C. The temperatures used in this study were selected based on the mean temperature of the seasons where there is enough moisture content in the soil to achieve germination (Tab. 1). Illumination was provided for 12 h each day using 30 W cool white fluorescent lights. Four replicates of 25 seeds were used in each germination test. The germinated seeds were removed when the radicle was at least 1 mm long (Bacchetta *et al*., 2006). These germination tests were considered completed when no additional seeds germinated over a period of at least 15 days. At the end of each germination test, the seeds that had not germinated were dissected (i.e., the cut-test) to determine whether they were viable (e.g., fresh), non-viable (e.g., mouldy) or empty. The tests at different temperatures allowed the determination of final germination percentage (FGP) and mean germination time (MGT) calculated according to equation reported in Ellis and Roberts (1980).

FGP and MGT response data constrained to temperature were analyzed by a second order orthogonal polynomial regression. The quadratic term explains the parabolic or unimodal pattern of the response variable, while the linear term allows to explain the global asymmetry of the curve. At first a full model including the linear and quadratic terms (orthogonal polynomials of degree of temperature) was fitted, then the full model was reduced by a stepwise selection. So

Tab. 1 - Seed collection sites of each population studied with GPS coordinates (WGS84); harvest period, P (mm): Mean annual precipitation; T (°C): Temperature; DJFT (°C): Mean seasonal temperature of winter where December, January and February (DJF) were taken as winter; MAM_T (°C): Mean seasonal temperature of spring where March, April and May (MAM) as spring; JJA_T (°C): Mean seasonal temperature of summer where June, July, and August (JJA) as summer; SON_T (°C): Mean seasonal temperature on autumn where September, October, and November (SON) as autumn.

Species	Collection site			Harvest period	P	T	DJF_T	MAM_T	JJA_T	SON_T	Ios_2
	Locality	X	Y								
Campanula garganica subsp. *garganica*	Gargano (Torre di Monte Pucci)	582085	4644190	Autumn 2010	665	16.3	9.4	14.2	23.9	17.7	1.24
Centaurea subtilis	Gargano (Calalunga)	587843	4643800	Autumn 2013	638	16.4	9.6	14.2	23.8	17.9	1.15
Centaurea diomedea	Tremiti Islands	542023	4663652	Autumn 2013	373	16.9	9.5	14.6	25	18.2	0.75

the final fitted response pattern can be quadratic, linear or the combination of the two. FGP and MGT were, respectively, analyzed by generalized linear model (GLM) (with a logit link function) and classical linear model (LM). The regression analysis was performed using software R (R Core Team, 2018).

Results

Seed viability and quality analysis

The cut test demonstrated that 56% of *Centaurea subtilis* seeds were empty or poorly developed. At the end of the tetrazolium test, 33% of the seeds were not stained (white: not viable), 13% were partially stained (pink: probably not viable), and 54% were completely stained (red: viable).

For *Centaurea diomedea*, 67% of the seeds were empty or poorly developed in the cut test, and the tetrazolium test showed that only 9% of the seeds were completely stained, and thus certainly viable. Most of the seeds used for this test did not show any chemical reaction with the tetrazolium, and remained white (78%), while 13% was only partially stained. On the basis of these data, for the seed lots used for these tests, there were only 16% and 3% 'certainly viable' seeds of *Centaurea subtilis* and *C. diomedea*, respectively (Fig. 1).

The X-ray analysis images of these seeds for *Centaurea subtilis* and *C. diomedea* (Fig. 2) showed that only 12 seeds out of 50 (24%) and 8 seeds out of 53 (15%), respectively, were considered as healthy.

For *Campanula garganica* subsp. *garganica* that reaches 99% of germination at the optimal temperature we considered not necessary to do the test because we assumed that all the seeds were vital.

Seed macromorphological analysis

Centaurea subtilis seeds are contained in an elliptic, flattish, yellowish, fine-haired achene, the tip of which has short brownish bristles. The mean achene weight is 2.12 ±0.179 mg, and the mean achene length (bristles excluded) and width are 2.97 ±0.41 mm and 1.37 ±0.14 mm, respectively. The mean seed volume is 2.20 ±0.77 mm^3. *Centaurea diomedea* seeds are included in an achene as well, which is very similar to that of C. subtilis, but it has whitish bristles. The mean achene weight is 1.74 ±0.21 mg, and the mean achene length (bristles excluded) and width are 3.35 ±0.41 mm and 1.40 ±0.14 mm, respectively. The mean seed volume is 2.70 ±0.87 mm^3. *Campanula garganica* seeds are very small, subspherical and brownish, with a smooth

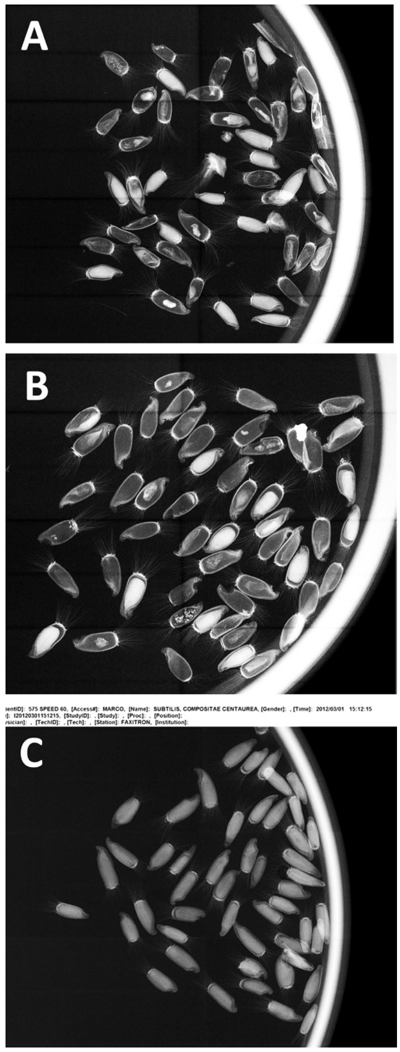

Fig. 2 - X-ray analyses of the seeds of *Centaurea subtilis* (A) and *C. diomedea* (B), and for the seeds of *Centaurea subtilis* after the additional gravimetric selection (C).

Fig. 1 - Tetrazolium tests on the seeds of *Centaurea subtilis* (left) and *C. diomedea* (right).

tegument. Their mean seed weight is 0.025 ±0.03 mg. The mean seed length and width are 0.241 ±0.001 mm and 0.194 ±0.005 mm, respectively. The mean seed volume is 0.003 ±0.0001 mm^3.

Germination tests

Final germination percentage (FGP) and mean germination time (MGT) of the three endemic species are reported in Table 2.

Centaurea subtilis shows a FGP range from 19% to 54% while MGT ranges from 5.8 days and 28.7 days (Tab. 2). The analysis of deviance for the binomial GLM fitted to *Centaurea subtilis* seed FGP shows the significant effects of the temperature with a total deviance explained of 68.5%. In particular FGP has a quadratic relationship with the temperature (Tab. 3; Fig. 3). FGP increases in the range of 5-10°C with an expected maximum percentage value of 58% at 12.5°C and it decreases in the range of 15-20°C while the expected germination is <5% with temperatures >23 °C and <2 °C.

The MGT has a significant relationship with temperature [$F(2,13)=163.7$, $P=6.056e-10$; $R^2=0.96$]. Both linear and quadratic terms are significant with respectively 77.2% and 18.9% of the total variance explained (Tab. 4). Then, the MGT response curve has a non-linear pattern with decreasing values between 5 and 15°C and constant up to 20°C (Fig. 3). The expected minimum MGT value is 5.09 days at 17°C.

Centaurea diomedea has a FGP ranging from 4% to 53% while MGT ranges from 7.3 days and 25.7 days (Tab. 2). The analysis of deviance for the binomial GLM fitted to *Centaurea diomedea* seed germination shows a significant effect of the temperature with a total deviance explained of 81%. Both linear and quadratic terms are significant as they explain respectively 13.3% and 67.8% of the total deviance (Tab. 3). Then, the FGP response curve is determined by a combination of linear and quadratic terms with a final unimodal and slightly asymmetrical pattern. The asymmetry is mainly determined by FGP values at 20°C significantly lower than those at 5°C. FGP curve has an expected maximum percentage value of 55% at 11.5°C while the expected germination is <5% with temperatures >23 °C and <2.5 °C (Fig. 4).

The MGT has a significant relationship with temperature [$F(2,12)=45.29$, $P=2.562e-06$; R2=0.883]. Both linear and quadratic terms are significant with respectively 13.2% and 75.0% of the total variance explained (Tab. 4). Then the FGP response curve is determined by a combination of linear and quadratic terms with a final unimodal and slightly asymmetrical pattern with an expected minimum MGT value of 6.37 days at 13°C (Fig. 4).

Campanula garganica subsp. *garganica* has a FGP ranging from 72% and 99% while MGT ranges from 27.9 days and 64.3 days (Tab. 2). The analysis of deviance for the binomial GLM fitted to *Campanula garganica* seed germination shows a significant effect of the temperature with a total deviance explained of 55.3% (Tab. 3). In particular, FGP has a quadratic relationship with the temperature (Tab. 3). FGP curve shows a unimodal pattern with an expected maximum percentage value of 97% at 12.5°C (Fig. 5).

Tab. 2 - Final germination percentage (FGP) and mean germination time (MGT) of *Centaurea subtilis*, *C. diomedea* and *Campanula garganica* subsp. *garganica* at the different temperature.

Species	Temperature	Germination characteristics	
	(°C)	FGP (%)	MGT (days)
Centaurea subtilis	5	30	28.7
	10	54	13.1
	15	52	5.8
	20	19	6.6
Centaurea diomedea	5	20	25.7
	10	47	9.2
	15	53	7.3
	20	4	19.3
Campanula garganica subsp. *garganica*	5	86	64.3
	10	94	34.1
	15	99	23.4
	20	72	27.9

Tab. 3 - Analysis of deviance table GLM (Signif. codes: 0 '***'; 0.001 '**'; 0.01 '*'; 0.05 '.'; 0.1 ' '; 1).

ANALYSIS DEVIANCE TABLE OF SEED GERMINATION								
SPECIES	SOURCE	Df	Deviance	Deviance (%)	Resid. Df	Resid. Dev.	Pr (>Chi)	pvalue
Centaurea subtilis	Deviance Tot.				15	28.462		
	T^2	1	19.514	68.5	14	89.492	3,90E-05	***
Centaurea diomedea	Deviance Tot.				15	60.154		
	T	1	8.004	67.8	14	52.150	0.001431	**
	T^2	1	40.807	13.3	13	11.343	6,08E-10	***
Campanula garganica subsp. *garganica*	Deviance Tot.				15	66.161		
	T^2	1	29.508	55.3	14	36.653	0.0001873	***

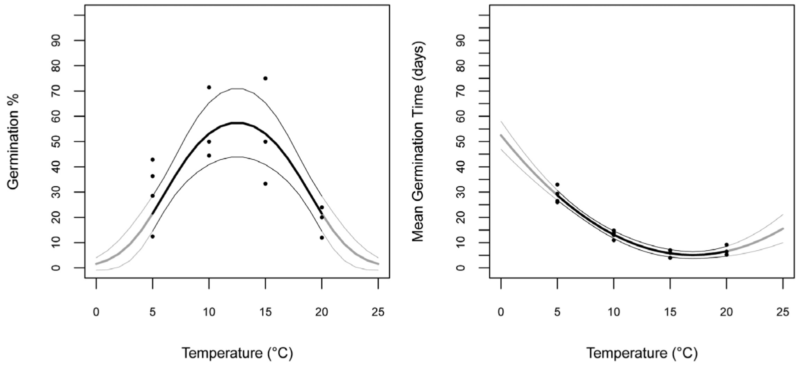

Fig. 3 - Germination behavior of *Centaurea subtilis* constrained to Temperature (°C). On the left the Final Germination Percentage response curve. On the right the Mean Germination Time (days) response curve. Fitted values (in bold) and 95% confidence bands (not in bold). In gray the values extrapolated for temperatures not analyzed here.

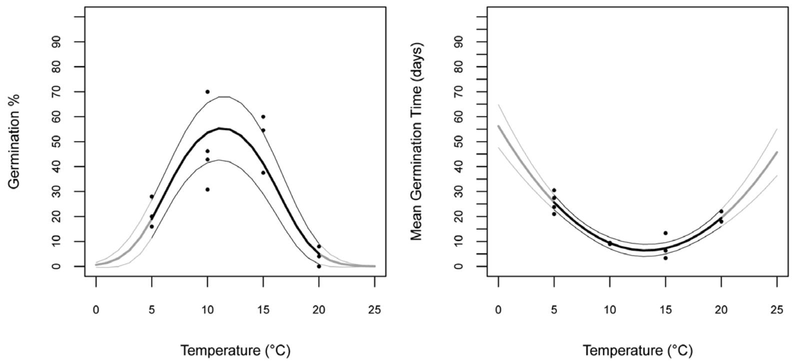

Fig. 4 - Germination behavior of *Centaurea diomedea* constrained to Temperature (°C). On the left the Final Germination Percentage response curve. On the right the Mean Germination Time (days) response curve. Fitted values (in bold) and 95% confidence bands (not in bold). In gray the values extrapolated for temperatures not analyzed here.

MGT shows a significant relation with the temperature [F(2,13)=194.3, P=2.073e-10; R2=0.9676]. Both linear and quadratic terms are significant with respectively 68.2% and 28.5% of the total variance explained (Tab. 4). Then the MGT response curve is determined by a combination of linear and quadratic terms with an asymmetrical pattern, with decreasing values between 5°C up to 16°C (minimum MGT value of 22.42 days at 16°C) and increasing values at higher temperatures (Fig. 5).

Discussion

The results of this study, although preliminary, have initiated a fundamental process of knowledge of the reproductive traits of three vulnerable/threatened Mediterranean species that are endemic to limited Italian territories and are fundamental for planning reintroduction strategies and subsequent analysis to identify the best seed multiplication protocols.

The germination behaviour of the three species is significantly affected by the temperature and follows the well-known pattern of Mediterranean species called "Mediterranean germination syndrome" (Mattana *et al*., 2016) with an optimal germination at the temperatures ranges of 5-15°C (Skordilis & Thanos, 1995) and a low germination rate (Thanos & Doussi, 2002). This germination response is a strategy to overcome the summer season where high temperature limits the moisture availability and then may limit seedling survival (Thompson, 1973; Berube & Myers, 1982; Estrelles *et al*., 2010).

Indeed, the three studied species showed a unimodal germination pattern with maximum germination between 10 and 15°C. It is interesting to note that *Centaurea diomedea* is the only species, among those studied, that showed a unimodal but asymmetrical germination pattern (Fig. 4). Indeed, such species "suffers" high temperatures (around 20°C) more than the low ones (around 5°C) and this phenomenon is more evident than in the other species. Such response is consistent with the climatic and bioclimatic features of the growth site. Indeed, Tremiti Islands have a mean summer temperature of 25°C and, above all, a very strong summer aridity, with an Ios_2 value remarkably lower than those of the growth sites of the other species.

In the case of *Centaurea subtilis* and *C. diomedea*, the germination tests produced very clear results testifying that germination is substantially stable between 10 °C and 15 °C and decreases significantly at 5 °C and more markedly at 20 °C. These data are in agree-

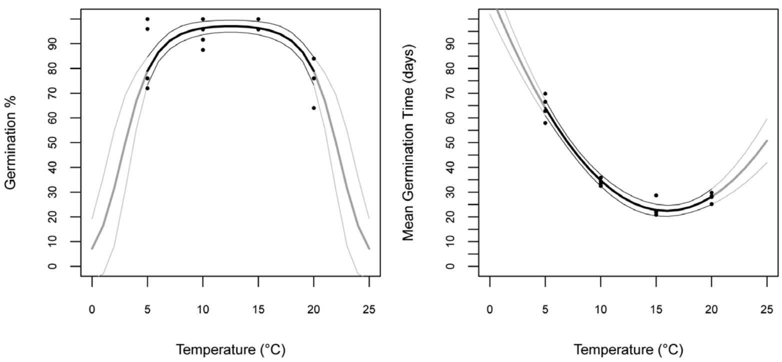

Fig. 5 - Germination behavior of *Campanula garganica* subsp. *garganica* constrained to Temperature (°C). On the left the Final Germination Percentage response curve. On the right the Mean Germination Time (days) response curve. Fitted values (in bold) and 95% confidence bands (not in bold). In gray the values extrapolated for temperatures not analyzed here.

Tab. 4 - Linear Regression ANOVA table (Signif. codes: 0 '***'; 0.001 '**'; 0.01 '*'; 0.05 '.'; 0.1 ' '; 1).

LINEAR REGRESSION ANOVA TABLE OF MGT							
SPECIES	**SOURCE**	**Df**	**Sum Sq**	**Mean Sq**	**F value**	**Pr (>F)**	**pvalue**
Centaurea subtilis	**T**	1	1090.6	1090.6	262.87	5.27e-10	***
	T^2	1	268.0	268.0	64.59	2.13e-06	***
	Residuals	13	53.9	4.1			
Centaurea diomedea	**T**	1	132.1	132.1	13.60	0.00311	***
	T^2	1	748.2	748.2	76.99	1.44e-06	***
	Residuals	12	116.6	9.7			
Campanula garganica subsp. *garganica*	**T**	1	2873.5	2873.5	273.8	4.09e-10	***
	T^2	1	1203.2	1203.2	114.7	8.09e-08	***
	Residuals	13	136.4	10.5			

ment with a similar study on *Centaurea melitensis*, which had the best germination at 15 °C (Bain, 2013), and on *Centaurea diffusa* and *C. maculosa*, where the final germination rates only reached 40% and 20%, respectively, and were enhanced by dry storage (Watson & Renney, 1974).

The seeds of *Campanula garganica* subsp. *garganica* showed high germination at all of the temperatures tested here, with the maximum values at 10 °C and 15 °C that appears to be the optimal temperature for *Campanula garganica* subsp. *garganica*, which showed the highest final germination rates and the lowest mean germination time at this temperature. This result is in agreement with the study of Koutsovoulou *et al*. (2014) on the requirements for germination of 131 *taxa* of *Campanulaceae*, among which several species of the genus *Campanula*, including *Campanula garganica* subsp. *cephallenica*, that had the highest germination rates at 15 °C.

The mean germination time (MGT) curves of the three species showed non linear patterns with optimal values around 15 °C. Besides, the three species were characterized by a slow germination rate that is especially evident for *Campanula garganica* subsp. *garganica* (Figs. 3, 4, 5). This slow germination rate could be an advantageous ecological adaptation (Doussi & Thanos, 2002) or could indicate the presence of a morpho-physiological dormancy (MPD) that can be broken with prechilling.

The reproductive biology of both *Centaurea subtilis* and *C. diomedea*, in particular the low production of viable seeds could be an important limit for the maintenance and conservation of these endemical species (LC/EN/VU). Seed viability is a parameter of paramount importance in quality assessment of any seed lot. A seed can be considered viable when it has the morphological, physiological and biochemical features that are essential for its germination (Bacchetta *et al*., 2006).

Our study confirmed a low production of fertile seeds (the estimated rate of viable seeds in these species was 16% and 3%, respectively) generally very common in numerous species of the *Asteraceae* family (ENSCONET, 2009); i.e., *Centaurea nivea* (Sözen & Özaydın, 2009), *Lamyropsis microcephala* (Mattana *et al*., 2012; Bacchetta *et al*., 2007), and *Centaurea cineraria* subsp. *circae* (Valletta *et al*., 2016). Moreover, the results of the X-ray analyses confirm the poor quality of these seed lots. Indeed, the images showed that only 24% of the seeds of *Centaurea subtilis* and 15% of those of *C. diomedea* were completely developed. X-rays are particularly useful to detect outwardly normal, but 'empty' seeds that regularly occur in collections of wild species, although this technique cannot give indications about the cell enzymatic activities. Thus, it can underestimate the proportions of non-viable seeds in seed lots. However, this analysis was very useful and convinces us of the need for better selection of seeds at the earlier stage with the blower, which could minimise the numbers of non-viable seeds used in the germination tests.

Achenes of *Centaurea subtilis* showed higher mean weight than those of *C. diomedea*, although *C. diomedea* had a higher mean seed volume. Although the mean seed width was substantially similar, the seeds of *Centaurea diomedea* were particularly longer (+12%). The seeds of *Campanula garganica* were instead notably smaller and lighter than those of the *Centaurea* species studied here. Thus, it is interesting to note that the seeds of *Campanula garganica* were those that showed the highest germination rate. Generally, seed weight is associated with fitness and population establishment, as seed traits are critical elements in the life history of plants. Indeed, seedlings that emerge from large seeds often survive longer than those from small seeds under adverse seedbed conditions, such as under low light (Simons & Johnston, 2000), low water (Hendrix & Trapp, 1992; Chacón & Bustamante, 2001), nutrient limitations (Vaughton & Ramsey, 1998), and deep buried depths (Yanful & Maun, 1996; Ruiz de Clavijo, 2002).

Conclusions

In conclusion, this study especially focused on seed viability and germination response to temperature of three species endemic to Southern Italy: *Centaurea diomedea*, *C. subtilis* and *Campanula garganica* subsp. *garganica*. The reproductive traits here identified are particularly important as they represent essential information in the design of *ex-situ* and *in-situ* conservation projects.

Indeed, the viability of seed accession is a measure of how many seeds are alive and can develop into plants that will then be able to reproduce themselves, given the appropriate conditions. Similarly, germination is a critical stage in the life cycle of weeds and crop plants, and often controls the population dynamics, with major practical implications (Radosevich *et al*., 1997).

Centaurea subtilis and *C. diomedea* showed a similar germinative behaviour, with their optimal temperatures at 15°C where the highest germination and the highest speed of germination were observed. However, *Centaurea diomedea*, showed a lower final germination percentage than *C. subtilis* at 20°C, probably due to the strong summer aridity of Tremiti Island. The models used in this study allow to extrapolate the values of germination percentage and mean germination time at the temperatures that were not tested here. Although such values must be interpreted with caution, they provide information that are useful for the restoration of these species and to implement future germination analyses. In particular, for the two *Centaurea* species, germination percentages lower than 5% (to be considered unsuccessful) were estimated at temperatures below 2.5°C and above 23°C.

It also emerged that these species adopted the same breeding strategies. This sees the production of a considerable proportion of non-viable seeds, although the plants probably have a genetic predisposition to the production of large quantities of seeds under more favorable environmental conditions. Such aspect must be taken into account in the definition of reintroduction protocols, indeed the seed-dose must be significantly increased to compensate for the high proportions of non-viable seeds.

Campanula garganica has germinative behaviour, with its optimal temperatures at 15°C, with the highest germination (about 90%) between 10 and 15°C and the lowest mean germination time between 15°C e 16°C (about 22 days). These seeds do not have germination difficulties although it could be promoted and accelerated with specific pre-treatments. Furthermore, this species showed quite high final germination (between 72% and 99%) at all the temperatures tested, thus the extrapolation of further data at the temperatures not tested in this study was considered unreliable.

Of course, further information is still needed on seed germination pattern of these species, their development in the growth site and the mechanisms that regulate the production of viable seeds in *Centaurea subtilis* and *C. diomedea*. Precisely, it could be useful to widen the range of temperature used for the tests to have more data and better represent germination pattern. In addition, collecting the seeds in different sites would enable to investigate the possible intra-specific variation in relation to local climatic conditions as observed for other species (Galiè *et al*., 2015; Gasparri *et al*., 2016).

Nevertheless, this study has been fundamental to highlight species germinative potential, on the basis of different environmental conditions reproduced in the laboratory, and for the identification of any eco-physiological problem related to germination, especially in terms of habitat conservation.

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