Habitat-correlated seed germination and morphology in populations of *Phillyrea angustifolia* L. (Oleaceae)

Sara Mira, Alberto Arnal and Félix Pérez-García*

Departamento de Biotecnología-Biología Vegetal, Escuela Técnica de Ingeniería Agronómica, Alimentaria y de Biosistemas, Universidad Politécnica de Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain

Abstract

The broad aim of this work was to study intraspecific variation of seed germination in *Phillyrea angustifolia* L. (Oleaceae), a species with a hard (water-permeable) endocarp. Germination of seeds from six different wild populations was correlated with traits related either to seed morphology or to environmental parameters. Germination of naked seeds (seeds without endocarp) at the optimum germination conditions was similar among populations and individuals, but great differences could be detected regarding the germination of seeds with endocarp both at inter- and intra-populational levels. Differences among populations could be related to climatic parameters and to morphometric variables of seeds with endocarp. A higher germination was associated with populations growing in habitats with more severe summer (higher temperature, lower precipitation and a longer drought period) and producing elongated seeds (lower Feret ratio and roundness). Moreover, seeds from eight different individuals within a population were tested independently, and great differences regarding the germination of seeds with endocarp could be detected among individuals. Our results suggest that the morphological variation found in *P. angustifolia* endocarp is both under strong maternal genetic control as well as influenced by environmental factors, as indicated by the high variability among individuals within one population and the significant correlation between climate variables and seed germination among populations. Finally, it is emphasized that standardization of plant propagation protocols should take into account the degree of intraspecific variation of Mediterranean species.

Keywords: climate change, hard endocarp, inter-population variability, intra-population variability, *Phillyrea angustifolia*, physiological dormancy, seed dormancy, seed germination

Introduction

Information on propagation strategies of Mediterranean plant species is most relevant for ecosystem conservation, especially in the present context of climate change (Manso *et al.*, 2014; Cochrane, 2016). Plants living in Mediterranean ecosystems must cope with extreme summer droughts and frequent fires that play an important role in such ecosystems (Chaves *et al.*, 2002). Climate change might alter the hydrological cycle in the Mediterranean region (Mariotti *et al.*, 2008), and models predict a reduction in total precipitation and drier summers (Christensen *et al.*, 2007), as well as an increase in fire hazard (Moriondo *et al.*, 2006; Pausas *et al.*, 2008; Moreno *et al.*, 2010). Seed germination is subject to strong selection pressure and, consequently, is likely to be highly sensitive to climatic changes (Walck *et al.*, 2011). In light of these forecast changes, a better understanding of inter- and intra-population variation in seed germination is of high importance since plasticity in its response among populations and individuals will provide a buffer against climate change (Lacerda *et al.*, 2004; Fernández-Pascual and Jiménez-Alfaro, 2014; Hudson *et al.*, 2015).

To produce seeds with different germination responses is the mechanism by which many plant species are able to adapt to changing environmental conditions, and it is a common strategy in Mediterranean wild species (Pérez-García, 1993; Kigel, 1995; Pérez-García, 2009; Martínez-Fernández *et al.*, 2014; Cochrane *et al.*, 2014). Phenotypic variation in a trait can be the result of genetic and environmental influences. Populations from similar habitats may have differences in seed morphology or germination, and these differences can arise from local climatic factors during seed maturation and from the effect of maternal genotype (Fenner, 1992; Wulff, 1995; Baskin and Baskin, 2014). Furthermore, intra-population variation can be attributed to genetic differences among individual parent plants, even within a small geographic area (Pérez-García, 1993; Bewley and Black, 1994; Qaderi and Cavers, 2002).

In this scenario, we examined the germination characteristics and seed morphology of wild populations and individuals of *Phillyrea angustifolia* L. (Oleaceae) – narrow leaf phillyrea, narrow-leaved mock privet, or evergreen privet – a small tree that grows in

*Correspondence
Email: felix.perez@upm.es

well-preserved Mediterranean habitats and plays an important role in the post-fire ecological dynamics (Herrera *et al.*, 1994; Vitale *et al.*, 2007). In recent years there has been a renewed interest in *P. angustifolia* plant production. It is increasingly used for landscaping purposes, for being thermophilic and low water demanding (De Marco *et al.*, 2005), but also in restoration programmes, since many of its natural populations are under decline. Germination in *P. angustifolia*, both in nature and in nurseries, is poor, erratic, and too gradual, resulting in a set of plants at different physiological phases (Catalán, 1991; Traveset *et al.*, 2007). These trees produce blue-black drupes, containing a single seed enclosed by a lignified endocarp. Previous investigations in the *Phillyrea* genus indicate that the endocarp may mechanically interfere with the emergence of the radicle but that it is at the same time water permeable (Mira *et al.*, 2015b, 2016). Therefore, and contrary to a previous suggestion (Takos and Efthimiou, 2003), seeds do not exhibit physical dormancy, defined as the result of a water-impermeable layer in the seed or fruit coat (Baskin and Baskin, 2004).

By collecting seeds from wild populations across a range of habitats and relating seed characteristics to climate parameters we intended to learn how germination strategies have maximized establishment probabilities for the species. The general aim of this work was to evaluate intraspecific variation of germination behaviour in *P. angustifolia* seeds. The specific aims were to investigate whether seed germination in *P. angustifolia*: (1) varied among populations, (2) varied among individuals of a population and (3) could be correlated with traits related either to seed morphology or to environmental parameters.

Materials and methods

Seed collection

The studied wild populations of *P. angustifolia* are all located in the Iberian Peninsula. Fruits from populations 1 and 5 (P1 and P5) were collected by the authors in September 2012, fruits from populations 2 and 3 (P2 and P3) were provided by Semillas Montaraz S.A., those of population 4 (P4) by Forestal Catalana S.A., and those of population 6 (P6) by Viveros Municipales Devesa-Albufera. Samples from populations 2, 3, 4 and 6 were also collected in September 2012. These six populations represent a broad range of the habitats of this species in the Iberian Peninsula (Table 1). Climatic data were obtained from the Spanish State Meteorological Agency (AEMET), from meteorological observatories close to the population sites. The climate data (Table 1) are mean values of 5 years (2008–2012). Fruit collection was performed

Table 1. Collection data and climatic parameters of six *Phillyrea angustifolia* populations

Population code	Collection site	UTM coordinates			Altitude (m)	Climatic parameters							
		Zone	X (m)	Y (m)		P annual (mm)	P summer (mm)	D (months)	T (°C)	T_w (°C)	T_c (°C)	Osc (°C)	F (months)
P1	Cáceres (Extremadura)	29	758389	4352716	570	607	40	3.9	16.2	34.8	3.3	18.6	0
P2	Ávila (Castilla-Leon)	30	329124	4457253	530	955	77	2.5	11.7	30.3	-0.7	18.0	2.2
P3	Cádiz (Andalucia)	30	250928	4070671	50	703	22	4.5	17.6	32.3	6.5	14.5	0
P4	Girona (Cataluña)	30	964204	4641031	490	738	143	1.2	14.9	28.9	2.6	15.7	0
P5	Madrid (Madrid)	30	437460	4477372	631	537	51	3.8	14.8	34.1	1.1	19.6	0.1
P6	Valencia (C. Valenciana)	30	732500	4355500	10	561	81	2.8	16.1	29.8	4.6	15.2	0

Abbreviations: total precipitation per year (P annual), precipitation June–August (P summer), number of drought months (D), annual mean temperature (T), mean temperature of the warmest month (T_w), mean temperature of the coolest month (T_c), annual mean of daily thermal oscillation (Osc), number of frost months (F), Universal Transverse Mercator (UTM). Climatic data from Spanish State Meteorological Agency (AEMET).

according to the following standards: sampling randomly and evenly from as many plants as possible, in order to capture the widest possible genetic diversity from the population, and never collecting more than 20% of the available fruits from each individual on the day of collection (ENSCONET, 2009).

In parallel, in the case of P1, fruit collection was carried out separately for eight individual trees, in order to study intra-population differences in germination. For each of the eight individuals in P1, at least 250 fruits were collected.

In all cases, the fleshy exocarp and mesocarp of the fruit were manually removed. To carry out the assays with naked seeds (seeds without endocarp), endocarp was completely removed using pliers and a scalpel. Seeds were stored for 1 month under laboratory conditions (at about 23°C, 35% relative humidity, in darkness) until the start of trials in October 2012.

Image analysis

Representative images of 25 seeds with endocarp from each population were obtained with a digital camera (Nikon Coolpix 4500) mounted on a copy stand table (Kaiser 5410 RS2) equipped with four lighting units. Light conditions were stable throughout the acquisition procedure to achieve reproducibility and comparability of all images. Measurements on the digital images were determined using software ImageJ version 1.43 (Rasband, 1997–2015) for the following parameters: area, perimeter, diameter maximum, diameter minimum, Feret ratio (diameter minimum/diameter maximum), shape factor (4π area/perimeter^2) and roundness factor (4 area/π diameter maximum^2). Feret ratio indicates an aspect ratio close to an equiaxed particle when values are close to 1; shape factor indicates maximum shape homogeneity when values are close to 1; and roundness indicates maximum circularity when values are close to 1.

Seed germination

For all germination trials, four replicates of 25 seeds were incubated in glass Petri dishes (9 cm diameter) on top of two sheets of filter paper previously moistened with 4 ml of distilled water. To avoid contamination, seeds were disinfected with 10% HCl for 5 min before starting the assay. Filter papers were re-wetted regularly with distilled water as required. Incubation conditions were 15°C with a 16-h photoperiod provided by cool white fluorescent tubes with an irradiance of 35 µmol m^2 s^{-1}. These incubation conditions were chosen as the most suitable for *P. angustifolia* seed germination in a previous study (Mira *et al*., 2015b). Samples were checked daily and germinated

seeds were counted and removed. The incubation period was 45 days for naked seeds and 65 days for seeds with endocarp. Emergence of the radicle was the criterion for germination. In all germination trials, non-germinated seeds were checked for viability by the Tetrazolium test. Seeds were cut in half and submerged in a 1% solution of tetrazolium chloride for 24 h in the dark at 25°C. The number of non-viable seeds (equal or less than 5% of the total seeds) and empty seeds (equal or less than 2%) were excluded from calculation of final germination percentage.

Seedling emergence

To determine the effects of different substrates on seed emergence, naked seeds from P1 were sown in three substrates: an equal volume mix of sand and vermiculite, soil from the original habitat, and sterilized soil from the original habitat. Soil was sterilized by autoclaving at 120°C and 1 atm for 1 h. For each treatment, 52 seeds were individually sowed in the cells of a seedbed. Cells were partially filled with gravel (previously disinfected with HCl at 10%) and then completed with the assayed substrate. Seedbeds were incubated in a greenhouse, with a minimum prefixed temperature of 10°C. Seedling emergence was measured (cotyledons visible at substrate surface), and seed vigour was evaluated measuring plant height and number of leaves after 4 and 6 months.

Data analysis

The statistical analyses of seed germination data were done using the approach proposed by Ritz *et al*. (2013) with the 'drc' package (Ritz and Streibig, 2005) for the software environment R (R Core Team, 2015). We considered a non-linear log-logistic model to relate the cumulative germination and to monitor time after initialization of the test:

$$F(t) = d/\left(1 + exp\left[b\left(\log(t) - \log(MGT)\right)\right]\right),$$

where d is the maximum germination percentage; MGT (mean germination time) is the time where 50% of the seeds that germinated during the experiment have germinated; and b is proportional to the slope of F at time t. The estimation of non-linear regression parameters was based on treating data as an event time; that is, considering the monitoring interval when seeds were germinated or the time interval of the entire experiment if they did not germinate. Thus we have a multinomial distribution across these intervals and this distribution was used to obtain the parameter estimates by maximum likelihood. The time-event model implemented in the 'drc' package allows parameter

comparison among germination curves for different treatment groups.

Also, one-way factorial ANOVA was used to test differences among populations in morphometric parameters of seeds and also differences on vigour parameters of seedlings in different substrates. Where ANOVA indicated a significant effect ($P < 0.01$), a multiple comparison test was carried out using the Tukey test.

Finally, data were subjected to principal component analysis (PCA) to assess associations between variables and to detect population separation trends based on the variables analysed. Twenty-one variables were included in the analysis of each population using a correlation matrix: climatic parameters of the population habitat, seed morphometric parameters, and germination and MGT of seeds with endocarp and naked seeds. Principal component analysis allowed us to condense as much of the original information contained in the 21 variables in orthogonal variables called principal components (PCs), which were weighed linear combinations of the original variables (Meglen, 1992; Wenning and Erickson, 1994). After data were normalized, the contribution of each variable to each PC was assessed. The adequacy of this analysis is verified by the amount of the total information of the original variables retained by the PCs. Therefore, the initial set of 21 variables become a reduced set of two new variables (PCs), which enabled the two-dimensional projection of the six populations on the PCs. Germination percentages were arcsine square-root transformed for PCA analysis. PCA analysis was performed using SPSS 20.0 (IBM Corporation, 2013).

Results

Inter-population variation

The morphometric analysis of seed samples with endocarp allowed us to obtain extremely precise data on endocarp size and shape (Table 2). Endocarp shape was subspherical in all populations, as determined by: Feret ratio (0.77–0.86), shape factor (0.81–0.87), and roundness (0.79–0.89). Shape factor values showed little differences among populations. Endocarp area, perimeter, diameter maximum and minimum were also similar in all populations except for P3, in which values were higher. However, Feret ratio and roundness values were significantly different among populations. Mass of seeds with endocarp in P5 was significantly lower than in the rest of the populations. Therefore, seeds with endocarp showed little variation in size or mass, but did show differences in circularity and aspect ratio: the seeds with endocarp of P2, P4 and P6 were more rounded, and those of P1, P3 and P5 were more elongated.

The germination of *P. angustifolia* seeds from six different populations was studied (Table 3, Fig. 1). The germination of seeds with endocarp was slow (34–54 days, MGT), and significant differences on final germination percentages were observed (Table 3, Fig. 1). P1 and P5 showed the highest germination (91–87%), and P4 the lowest (3%). Germination of naked seeds (without endocarp) was significantly faster in P1, P5 and P6 (15–16 days, MGT) than in P2, P3 and P4 (19–22 days), although there were no significant differences of germination percentages (78–88%) among populations (Table 3). To further study the germination behaviour of *P. angustifolia*, seedling emergence was tested. To do so, naked seeds from P1 were incubated in a greenhouse in three different substrates. No seedling emerged before 100 days (Fig. 2). After 200 days, seedling emergence was 50% in sand and vermiculite, 29% in soil, and 8% in the sterilized soil. Although the number of emerged seedlings varied among treatments, no significant differences ($P > 0.01$) were detected among the vigour parameters: average plant height was 4.0 ± 0.3 cm after 4 months, and 7.3 ± 0.4 cm after 6 months; and average number of leaves was 4.6 ± 0.4 after 4 months, and 12.2 ± 0.6 after 6 months.

PCA based on 21 climatic, morphometric and germination variables allowed us to separate the studied

Table 2. Morphometric parameters of seeds with endocarp from six *Phillyrea angustifolia* populations

	Seed morphometric parameters							
Population	Area (mm^2)	Perimeter (mm)	Diameter max (mm)	Diameter min (mm)	Feret ratio	Shape factor	Roundness	Mass (mg)
P1	5.4 ± 0.2a	8.8 ± 0.2a	3.1 ± 0.1b	2.4 ± 0.1a	0.77 ± 0.01a	0.86 ± 0.01b	0.79 ± 0.01a	101.6 ± 7.9a
P2	5.5 ± 0.1a	9.1 ± 0.1ab	3.0 ± 0.1b	2.5 ± 0.0ab	0.84 ± 0.01cd	0.84 ± 0.01b	0.86 ± 0.01bc	114.9 ± 2.6a
P3	6.6 ± 0.2b	9.7 ± 0.1b	3.4 ± 0.1c	2.7 ± 0.0b	0.80 ± 0.01abc	0.87 ± 0.01b	0.83 ± 0.01ab	118.8 ± 1.1a
P4	4.8 ± 0.3a	8.4 ± 0.3	2.7 ± 0.1a	2.4 ± 0.1a	0.86 ± 0.01d	0.85 ± 0.01b	0.89 ± 0.01c	114.3 ± 3.7a
P5	5.1 ± 0.1a	9.0 ± 0.1	3.1 ± 0.0b	2.4 ± 0.0a	0.78 ± 0.01ab	0.81 ± 0.01a	0.82 ± 0.01ab	073.2 ± 3.9b
P6	4.9 ± 0.2a	8.4 ± 0.2	2.8 ± 0.1ab	2.3 ± 0.0a	0.82 ± 0.01bcd	0.86 ± 0.00b	0.85 ± 0.00bc	113.5 ± 7.4a

Values are means ± standard error (SE). In each column, the mean values followed by the same letters are not significantly different ($P > 0.01$).
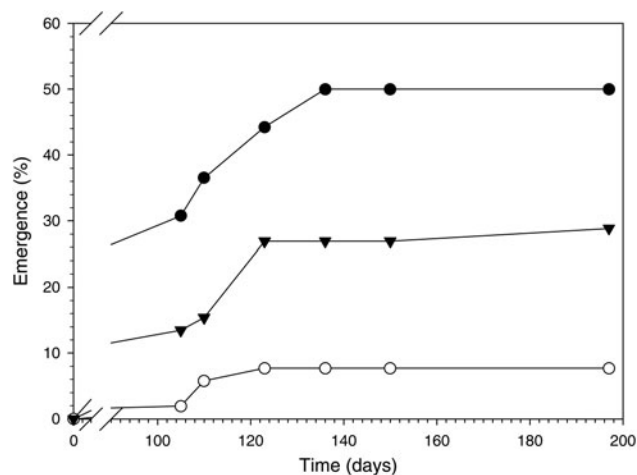
Table 3. Seed germination curve parameters of *Phillyrea angustifolia* seeds from six populations

Population	Germination (%)		MGT (days)	
	Seeds with endocarp	Naked seeds	Seeds with endocarp	Naked seeds
P1	87 ± 4a	88 ± 3a	34 ± 1a	15 ± 0a
P2	38 ± 10b	85 ± 4a	54 ± 7bc	22 ± 1b
P3	54 ± 7b	82 ± 4a	47 ± 2bc	21 ± 1b
P4	3 ± 2c	80 ± 4a	34 ± 4ab	19 ± 1c
P5	91 ± 6a	88 ± 5a	52 ± 2c	15 ± 1a
P6	50 ± 6b	78 ± 4a	51 ± 2c	16 ± 0a

Values are maximum germination percentage (mean ± standard error) and mean germination time (MGT) ± standard error. Parameters were estimated by maximum likelihood from a non-linear log-logistic model with a multinomial distribution. Within a column, values followed by the same letters are not significantly different ($P > 0.01$). For each treatment, four replicates of 25 seeds were tested.

Figure 2. Germination time courses for *Phillyrea angustifolia* naked seeds sown in three types of substrate: an equal volume mix of sand and vermiculite (●), soil from the original habitat (▼), and sterilized soil (○).

populations. The first three PCs explained 90% of the observed variation. Contribution of variables to PCs is presented in Table 4. The first principal component (PC1) accounted for 40% of the variation. In this component, the most relevant variables were germination of seeds with endocarp, seed morphometric parameters (such as diameter maximum, Feret ratio and roundness), and habitat characteristic (such as precipitation and temperature in summer) (Table 4). The second principal component (PC2) accounted for 29% of the observed variation. In this component, the most relevant variables were also seed morphometric parameters (such as area, diameter minimum and shape factor) and habitat characteristic (such as altitude, temperature in winter and daily thermal oscillation). The third principal component (PC3) explained 21% of the observed variation. The variables with most relevance in this third component were habitat characteristic (such as annual precipitation, temperature, and number of frost months). As PC3 is not clearly related to seed characteristics, like germination or morphometric parameters, discussion referred to this axis is not relevant for this study and was omitted.

Two-dimensional projections of populations as defined by PC1 and PC2 are shown in Fig. 3. PC1 separated P1, P3 and P5 from P2, P4 and P6. PC1 clustered populations P1, P3 and P5 towards the region of greater germination of seeds with endocarp, seeds with endocarp with higher values of diameter maximum, lower Feret and roundness ratio, and regarding the habitat: higher temperature of the warmest month, lower precipitation in summer (June to August) and
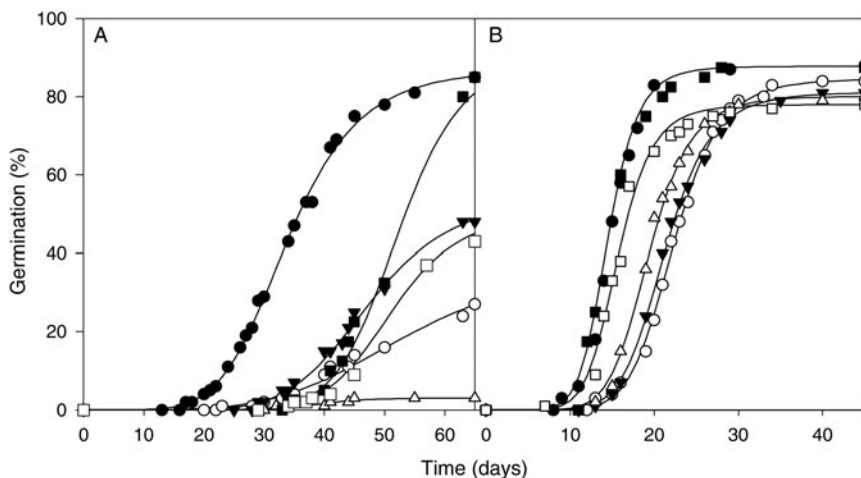

Figure 1. Germination time courses of *Phillyrea angustifolia* seeds with endocarp (A) and naked seeds (B) from six populations: P1 (●), P2 (○), P3 (▼), P4 (△), P5 (■) and P6 (□). Values are the averages of four replicates.

Table 4. Contribution of variables, variance and cumulative variance explained by the three principal components for climatic and seed morphometric parameters of six populations of *Phillyrea angustifolia*

	Component		
	1	2	3
Area	0.45	**0.77**	0.42
Perimeter	0.56	0.59	0.54
Diameter maximum	**0.77**	0.54	0.33
Diameter minimum	0.27	**0.78**	0.53
Feret ratio	**−0.98**	0.09	0.13
Shape factor	−0.27	**0.78**	−0.25
Roundness	**−0.94**	0.08	0.14
Mass	−0.59	**0.71**	0.06
Altitude	0.13	**−0.80**	0.36
Precipitation per year	−0.51	0.18	**0.80**
Precipitation summer	**−0.90**	−0.35	−0.17
Drought	**0.93**	0.35	−0.04
Temperature annual	0.41	0.54	**−0.71**
Temperature warmest month	**0.96**	−0.18	0.00
Temperature coolest month	0.20	**0.76**	−0.66
Daily thermal oscillation	0.47	**−0.81**	0.34
Frost days	−0.28	−0.13	**0.89**
Germination seeds with endocarp	**0.94**	−0.22	−0.02
Germination naked seeds	**0.72**	−0.50	0.39
MGT seeds with endocarp	0.11	0.05	0.47
MGT naked seeds	−0.45	0.55	0.68
Variance (%)	40.20	28.87	20.95
Cumulative variance (%)	40.20	69.07	90.02

Bold values are used to highlight those variables with a high contribution to each principal component.
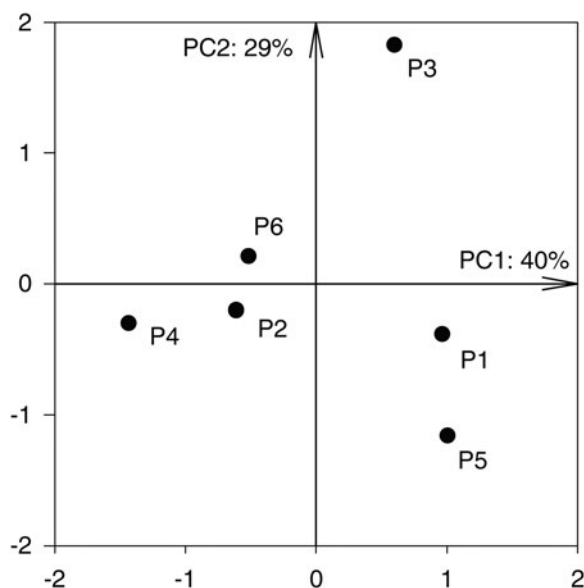
Figure 3. Projection of six populations of *Phillyrea angustifolia* on a bidimensional plane defined by principal components 1 (PC1) and 2 (PC2). Population numbers are indicated in Table 1.

longer drought. PC2 separated population P3 from the remaining populations, especially from P5. PC2 situates P3 towards the region of greater area and diameter minimum, and regarding the habitat: lower altitude, greater temperature of the coolest month and lower annual mean of daily thermal oscillation.

Correlations (coefficient of determination and *P*-value) among germination, morphometric variables, and habitat parameters of *P. angustifolia* seeds belonging to six populations were calculated (Table S1). Strong correlations between germination of seeds with endocarp and both Feret ratio and roundness (0.97 and 0.94, respectively, $P < 0.01$) and also with mean temperature of the warmest month (0.91, $P < 0.05$), were observed. Germination of naked seeds also correlated with mean temperature of the warmest month (0.84, $P < 0.05$), and with annual mean of daily thermal oscillation (0.90, $P < 0.05$).

Intra-population variation

The germination of *P. angustifolia* seeds from eight individuals of P1 was studied (Table 5, Fig. 4). Great differences were detected regarding germination of seeds with endocarp, with some individuals showing low germination (6–10%) and others showing high germination (81–88%) (Table 5). Differences were also detected among naked seeds, with most individuals showing high germination percentages (72–95%), but seeds from individual #3 achieved only 35% germination. Germination speed (MGT) was also significantly different among individuals, with values between 12 and 16 days for naked seed, and between 24 and 41 days for seed with endocarp.

Table 5. Germination parameters of *Phillyrea angustifolia* seeds from eight individuals of the same population (P1) after 45 days of incubation at 15°C

	Germination (%)		MGT (days)	
Individual	Seeds with endocarp	Naked seeds	Seeds with endocarp	Naked seeds
1	6 ± 2c	78 ± 5bc	36 ± 6b	12 ± 1a
2	61 ± 5b	72 ± 5c	24 ± 2abc	13 ± 1ab
3	10 ± 3c	35 ± 5d	29 ± 4a	16 ± 1de
4	81 ± 4a	89 ± 3ab	30 ± 1de	15 ± 0d
5	88 ± 3a	81 ± 4bc	32 ± 1c	16 ± 0e
6	65 ± 5b	95 ± 2a	41 ± 2ef	13 ± 0ab
7	67 ± 5b	87 ± 3ab	39 ± 1d	14 ± 0c
8	84 ± 4a	90 ± 4ab	35 ± 1e	15 ± 0de

Values are expressed by final germination percentages (mean ± standard error) and mean germination time (MGT ± standard error). In each column, the mean values followed by the same letters are not significantly different ($P > 0.01$).
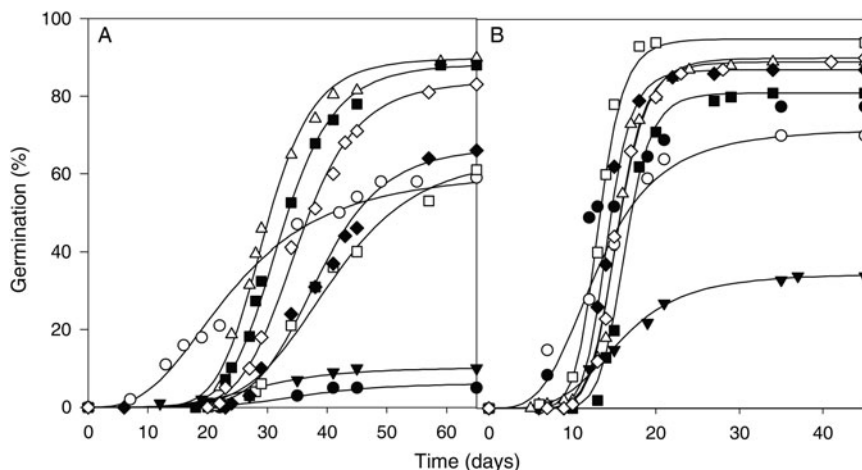
Figure 4. Germination time courses of *Phillyrea angustifolia* seeds with endocarp (A) and naked seeds (B) from eight individuals of the same population (P1): 1 (●), 2 (○), 3 (▼), 4 (△), 5 (■), 6 (□), 7 (◆) and 8 (◇). Values are the averages of four replicates.

Discussion

Phenotypic plasticity of seed traits will play a major role in plant responses to climate change (Nicotra *et al.*, 2010; Reed *et al.*, 2011). Our data provide useful information on inter- and intra-population variability of seed traits of a wild species. *Phillyrea angustifolia* is difficult to propagate in nurseries, with low, delayed and too gradual seed germination, due to the hardness of the lignified endocarp impeding radicle emergence. Previous work indicated that *Phillyrea* endocarp could be water permeable (Mira *et al.*, 2015b, 2016) and, therefore, seeds do not have physical dormancy *sensu stricto* (as defined by Baskin and Baskin, 2004). Nonetheless, the hardness of the endocarp mechanically interferes with the emergence of the radicle, being a major impediment for germination. When the endocarp is removed, we found that seed germination showed an average of 84% among populations. These germination results are in agreement with previous data on *Phyllirea* (García-Fayos *et al.*, 2001; Herranz *et al.*, 2006) and on seeds collected from P1 in 2011 previously studied by Mira *et al.* (2015b). Therefore, germination of naked seeds seems to be high, fast and homogeneous among populations and little influence of climate or year of collection could be inferred. However, great variability in germination percentage and speeds was found among populations when seed germination was assayed in the presence of the endocarp. Moreover, inter-population variability of morphometric parameters of seeds with endocarp was detected. Feret ratio and roundness, parameters that evaluate circularity, were significantly different among populations in seeds with endocarp. Also, while shape factor and seed mass were similar in most accessions, some populations were significantly different in these parameters. Ruprecht *et al.* (2015) have reported that more rounded seeds are more

tolerant to fire. Conversely, in our work, less rounded *P. angustifolia* seeds are related to climatic conditions (dry and hot summer) that favour fire occurrence in natural habitats.

Differences in seed germination among populations could be explained on the basis of inter-population variability of endocarp hardness, as previously suggested (Traveset *et al.*, 2007). In wild Mediterranean plants, inter-population differences in hardness and permeability of the dispersion unit, whether it be seed or fruit, have been previously detected, as in the seed coat of *Capsicum annuum* (Hernández-Verdugo *et al.*, 2001) and *Vicia villosa* (Renzi *et al.*, 2016) or in the pericarp of *Onopordum nervosum* (Pérez-García and Pita, 1989) and *Onopordum acanthium* (Pérez-García, 1993; Qaderi and Cavers, 2002). The inter-population variability of the endocarp could explain the difficulties faced by nurseries to propagate this plant (Catalán, 1991; Traveset *et al.*, 2007) and the contradictory results obtained with germination pre-treatments such as scarification with sulphuric acid (Bacchetta *et al.*, 2008; Mira *et al.*, 2015b, 2016). Likewise, field experiments showed that germination increased when fruits had been eaten by goats (Grande *et al.*, 2013) but decreased when eaten by birds (Traveset *et al.*, 2008).

Our results indicate that the origin of the plant material used in Mediterranean reforestation and forest restoration must be strongly considered. Standardization of plant propagation protocols should take into account the *P. angustifolia* intraspecific variation, and caution is advisable when considering the results of studies based on one single wild population. After germination, seedling emergence and plant development were slow with any of the substrates tested in this study. Substrate had no effect on plant growth but had a strong effect on seedling emergence. Sterilizing soil was detrimental for seedling emergence,

suggesting that the treatment might have killed mycorrhizal fungi in the soil. However, the artificial substrate of sand and vermiculite produced higher seedling emergence percentages (50%) than soil (29%) and would be, therefore, recommended for *P. angustifolia* plant propagation. The better performance of the artificial substrate is probably due to its higher water adsorption characteristics, since it is been stated by Lloret *et al.* (2004) that drier conditions can limit seedling emergence by decreasing germination in the related species *P. latifolia*.

Phenotypic variation of the hardness of the endocarp can be the result of maternal genetic and environmental influences (as reviewed by Hudson *et al.*, 2015) during embryo formation and fruit dispersal. Seed germination, the morphology of seed with endocarp and climatic variables allowed us to discriminate the six populations studied. We found that seeds with an endocarp that can be more easily broken by the radicle were more elongated in shape and collected originally from warmer and drier habitats: summer temperatures over 32.3°C, average summer rainfall under 51 mm, and over 3.8 drought months. This observation was supported by a significant correlation between germination of seeds with endocarp and Feret ratio, roundness, mean temperature of the warmest month and precipitation in summer. According to the correlation obtained among seed germination and climatic parameters, it could be hypothesized that less dormant seeds are able to germinate earlier in spring. Then, more developed seedlings are better able to withstand hot and dry summer conditions.

To elucidate the maternal genetic effect on seeds traits, intra-population variability of seed germination was studied. Seed germination of naked seeds differed only slightly among individuals. However, our data show greater differences in seed germination among individuals when the hard endocarp is not removed before planting. Great intra-population variability in germination has been detected in several wild Mediterranean species, such as *Erica australis* (Cruz *et al.*, 2003), *Ceratonia siliqua* (Pérez-García, 2009), *Astragalus gines-lopezii* (Martínez-Fernández *et al.*, 2014), *Senecio coiincyi* (Martínez-García *et al.*, 2012), *Gentiana lutea* (Pérez-García *et al.*, 2012), and several species of Cistaceae (Pérez-García, 1997; Tavşanoğlu and Çatav, 2012). In the Mediterranean climate, with variable and unpredictable environmental conditions, an important survival strategy of wild species would be variation in either germination requirements (Kigel, 1995; Cruz *et al.*, 2003; Pérez-García *et al.*, 2003; Pérez-García, 2005; Pérez-García *et al.*, 2006; Pérez-García and González-Benito, 2012; Pérez-García *et al.*, 2012; Baskin and Baskin, 2014; Copete *et al.*, 2014; Martínez-Fernández *et al.*, 2014), seed dormancy (Pérez-García *et al.*, 2012), seed longevity (Mira *et al.*, 2011a; Lazar *et al.*, 2014; Mira *et al.*, 2015a) or seed

characteristics (Pérez-García *et al.*, 2006; Mira *et al.*, 2011b; Pérez-García and González-Benito, 2012). In nature, *P. angustifolia* flowering takes place in March to June, fruits ripen in September to October and are dispersed from September to March. Seeds germinate with early spring temperature, from February to April (Herrera *et al.*, 1994; Andrés, 2011), after physiological dormancy of the embryo is broken and, therefore, the embryo becomes strong enough to break the endocarp. Our results suggest that endocarp phenotype has a high heritability, affecting intra-population variability in seed germination. The time when physiological dormancy of the embryo is broken and subsequent germination occurs will vary greatly among populations and also among individuals, seed germination being progressive along the season. Therefore, *P. angustifolia* plasticity would be an adaptative response to the enviromental variation of the Mediterranean climate.

Conclusions

Germination of *P. angustifolia* naked seeds was similar among populations and individuals, but great differences could be detected in the germination of seeds with an endocarp. Those diferences could be related to climatic and morphometric parameters of the population of origin, with higher germination associated with populations producing elongated seeds and habitats with a more severe summer. The phenotypic variation found in the endocarp is under both strong maternal genetic and environmental control, as indicated by the high variability among individuals within one population and the significant correlation between climate variables and seed germination. Selection pressures have shaped a high phenotypic plasticity in the species, which would allow it to adapt rapidly to the future scenario of climate change in which most Mediterranean areas are expected to become more stochastic (IPCC, 2014). Finally, the origin of the plant material used in Mediterranean reforestation must be strongly considered, while standardization of plant propagation protocols should take into account the intra-specific variation of Mediterranean species.

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Conflicts of interest

None.

References

Andrés, C. (2011) *Phillyrea* L. In Castroviejo, S., Aedo, C., Cirujano, S., Laínz, M., Montserrat, P., Morales, R., Muñoz, F., Navarro, C., Paiva, J. and Soriano, C. (eds), *Flora Iberica*, vol. 11, pp. 139–143. Madrid, Spain: Real Jardín Botánico de Madrid, CSIC.

Bacchetta, G., Bueno, A., Sánchez, G., Fenu, G., Jiménez-Alfaro, B., Mattana, E., Piotto, B. and Virevaire, M. (2008) *Conservación ex situ de plantas silvestres* [*Ex situ conservation of wild plants*]. Asturias, Spain: Anexo Digital I, Principado de Asturias/La Caixa.

Baskin, J.M. and Baskin, C.C. (2004) A classification system for seed dormancy. *Seed Science Research* 14, 1–16.

Baskin, C.C. and Baskin, J.M. (2014) *Seeds: Ecology, Biogeography, and Evolution of Dormancy and Germination* (2nd edition). New York, USA: Academic Press.

Bewley, J.D. and Black, M. (1994) *Seeds: Physiology of Development and Germination*. New York, USA: Plenum Press.

Catalán, G. (1991) *Phillyrea*. In Catalán, G. (ed), *Semillas de árboles y arbustos forestales* [*Forest tree and shrub seeds*], pp. 265–266. Madrid, Spain: Instituto Nacional para la Conservación de la Naturaleza.

Chaves, M.M., Pereira, J.S., Maroco, J., Rodrigues, M.L., Ricardo, C.P.P., Osorio, M.L., Carvalho, I., Faria, T. and Pinheiro, C. (2002) How plants cope with water stress in the field. Photosynthesis and growth. *Annals of Botany* 89, 907–916.

Christensen, J.H., Hewitson, B., Busuioc, A., Chen, A., Gao, X., Held, I., Jones, R., Kolli, R.K., Kwon, W.T., Laprise, R., Magaña Rueda, V., Mearns, L., Menéndez, C.G., Räisänen, J., Rinke, A., Sarr, A. and Whetton, P. (2007) Regional climate projections. In Solomon, S. *et al.* (eds), *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change.* Cambridge, UK: Cambridge University Press.

Cochrane, A. (2016) Can sensitivity to temperature during germination help predict global warming vulnerability? *Seed Science Research* 26, 14–29.

Cochrane, A., Hoyle, G L., Yates, C.J., Wood, J. and Nicotra, A.B. (2014) Predicting the impact of increasing temperatures on seed germination among populations of Western Australian *Banksia* (Proteaceae). *Seed Science Research* 24, 195–205.

Copete, E., Herranz, J.M., Copete, M.A. and Ferrandis, P. (2014) Interpopulation variability on embryo growth, seed dormancy break, and germination in the endangered Iberian daffodil *Narcissus eugeniae* (Amaryllidaceae). *Plant Species Biology* 29, 72–84.

Cruz, A., Pérez, B., Velasco, A. and Moreno, J. (2003) Variability in seed germination at the interpopulation, intrapopulation and intraindividual levels of the shrub *Erica australis* in response to fire-related cues. *Plant Ecology* 169, 93–103.

De Marco, A., Gentile, A.E., Arena, C. and De Santo, A.V. (2005) Organic matter, nutrient content and biological activity in burned and unburned soils of a Mediterranean maquis area of southern Italy. *International Journal of Wildland Fire* 14, 365–377.

European Native Seed Conservation Network (ENSCONET) (2009) *Seed Collecting Manual for Wild Species*. Kew, UK: Royal Botanic Gardens; Madrid, Spain: Universidad Politécnica de Madrid.

García-Fayos, P., Gulias, J., Martínez, J., Marzo, A., Melero, J.P. and Traveset, A. (2001) *Bases ecológicas para la recolección, almacenamiento y germinación de semillas de especies de uso forestal de la Comunidad Valenciana* [*Ecological basis for forest tree seed collection, storage and germination of the Valencian Community*]. Comunidad Valenciana, Spain: IMEDEA, Banc de Llavors Forestals.

Grande, D., Mancilla-Leyton, J.M., Delgado-Pertinez, M. and Martin-Vicente, A. (2013) Endozoochorus seed dispersal by goats: recovery, germinability and emergence of five Mediterranean shrub species. *Spanish Journal of Agricultural Research* 11, 347–355.

Hernández-Verdugo, S., Oyama, K. and Vázquez-Yanes, C. (2001) Differentiation in seed germination among populations of *Capsicum annuum* along a latitudinal gradient in Mexico. *Plant Ecology* 155, 245–257.

Herranz, J.M., Ferrandis, P., Copete, M.A., Duro, E.H. and Zalacain, A. (2006) Effect of allelopathic compounds produced by *Cistus ladanifer* on germination of 20 Mediterranean taxa. *Plant Ecology* 184, 259–272.

Herrera, C.M., Jordano, P., Lopezsoria, L. and Amat, J.A. (1994) Recruitment of a mast-fruiting, bird-dispersed tree-bridging frugivore activity and seedling establishment. *Ecological Monographs* 64, 315–344.

Hudson, A.R., Ayre, D.J. and Ooi, M.K. (2015) Physical dormancy in a changing climate. *Seed Science Research* 25, 1–16.

IBM Corporation (2013) *IBM SPSS Statistics for Windows*, version 22.0. Armonk, New York, USA: IBM Corporation.

Intergovernmental Panel on Climate Change (IPCC) (2014) Summary for policymakers. In Field, C.B., Barros, V.R., Dokken, D.J., Mach, K.J., Mastrandrea, M.D., Bilir, T.E., Chatterjee, M., Ebi, K.L., Estrada, Y.O., Genova, R.C., Girma, B., Kissel, E.S., Levy, A.N., MacCracken, S., Mastrandrea, P.R. and White, L.L. (eds), *Climate Change 2014: Impacts, Adaptation and Vulnerability. Part A: Global and sectoral aspects. Contribution of working group II to the 5th assessment report of the Intergovernmental Panel on Climate change*. Cambridge, UK: Cambridge University Press.

Fenner, M. (1992) *Seeds: The Ecology of Regeneration in Plant Communities*. Wallingford, UK: CABI Publishing.

Fernández-Pascual, E. and Jiménez-Alfaro, B. (2014) Phenotypic plasticity in seed germination relates differentially to overwintering and flowering temperatures. *Seed Science Research* 24, 273–280.

Kigel, J. (1995) *Seed germination in arid and semiarid regions*. In Kigel, J. and Galili, G. (eds), *Seed Development and Germination*, pp. 645–699. New York, USA: Marcel Dekker.

Lacerda, D.R., Filho, J.P.L., Goulart, M.F., Ribeiro, R.A. and Lovato, M.B. (2004) Seed-dormancy variation in natural populations of two tropical leguminous tree species: *Senna multijuga* (Caesalpinoideae) and *Plathymenia reticulata* (Mimosoideae). *Seed Science Research* **14**, 127–135.

Lazar, S.L., Mira, S., Pamfil, D. and Martínez-Laborde, J.B. (2014) Germination and electrical conductivity tests on artificially aged seed lots of 2 wall-rocket species. *Turkish Journal of Agriculture and Forestry* **38**, 857–864.

Lloret, F., Penuelas, J. and Ogaya, R. (2004) Establishment of co-existing Mediterranean tree species under a varying soil moisture regime. *Journal of Vegetation Science* **15**, 237–244.

Manso, R., Pukkala, T., Pardos, M., Miina, J. and Calama, R. (2014) Modelling *Pinus pinea* forest management to attain natural regeneration under present and future climatic scenarios. *Canadian Journal of Forest Research* **44**, 250–262.

Mariotti, A., Zeng, N., Yoon, J.-H., Artale, V., Navarra, A., Alpert, P. and Li, L.Z.X. (2008) Mediterranean water cycle changes: transition to drier 21st century conditions in observations and CMIP3 simulations. *Environmental Research Letters* **3**, 4.

Martínez-Fernández, V., Martínez-García, F. and Pérez-García, F. (2014) Census, reproductive biology, and germination of *Astragalus gines-lopezii* (Fabaceae), a narrow and endangered endemic species of SW Spain. *Turkish Journal of Botany* **38**, 686–695.

Martínez-García, F., Guerrero-García, S. and Pérez-García, F. (2012) Evaluation of reproductive success and conservation strategies for *Senecio coincyi* (Asteraceae), a narrow and threatened species. *Australian Journal of Botany* **60**, 517–525.

Meglen, R.R. (1992) Examining large databases: a chemometric approach using principal component analysis. *Marine Chemistry* **39**, 217–237.

Mira, S., Arnal, A. and Pérez-García, F. (2016) Seed germination of *Phillyrea angustifolia* L., a species with difficult propagation. *Forest Systems* (in press).

Mira, S., Estrelles, E. and González-Benito, M.E. (2015a) Effect of water content and temperature on seed longevity of seven Brassicaceae species after 5 years storage. *Plant Biology* **17**, 153–162.

Mira, S., Estrelles, E., González-Benito, M.E. and Corbineau, F. (2011a) Biochemical changes induced in seeds of Brassicaceae wild species during ageing. *Acta Physiologiae Plantarum* **33**, 1803–1809.

Mira, S., González-Benito, M.E., Ibars, A.M. and Estrelles, E. (2011b) Dormancy release and seed ageing in the endangered species *Silene diclinis*. *Biodiversity and Conservation* **20**, 345–358.

Mira, S., Veiga-Barbosa, L. and Pérez-García, F. (2015b) Seed germination characteristics of *Phillyrea angustifolia* L. and *P. latifolia* L. (Oleaceae), two Mediterranean shrub species having lignified endocarp. *Annals of Forest Research* **58**, 27–37.

Moreno, J.M., Zavala, G., Martín, M. and Millán, A. (2010) Forest fire risk in Spain under future climate change. In Settele, J., Georgiev, T., Grabaum, R., Grobelnik, V., Hammen, V., Klotz, S., Kotarac, M. and Kuehn, I. (eds), *Atlas of Biodiversity Risk*. Sofia, Bulgaria: Pensoft Publishers.

Moriondo, M., Good, P., Durao, R., Bindi, M., Giannakopoulos, C. and Corte-Real, J. (2006) Potential impact of climate change on fire risk in the Mediterranean area. *Climate Research* **31**, 85–95.

Nicotra, A.B., Atkin, O.K., Bonser, S.P., Davidson, A.M., Finnegan, E.J., Mathesius, U., Poot, P., Purugganan, M.D., Richards, C.L.,Valladares, F. and van Kleunen, M. (2010) Plant phenotypic plasticity in a changing climate. *Trends in Plant Science* **15**, 684–692.

Pausas, J., Llovet, J., Rodrigo, A. and Vallejo, R. (2008) Are wildfires a disaster in the Mediterranean basin? – a review. *International Journal of Wildland Fire* **17**, 713–723.

Pérez-García, F. (1993) Effect of the origin of the cypsela on germination of *Onopordum acanthium* L. (Asteraceae). *Seed Science and Technology* **21**, 187–195.

Pérez-García, F. (1997) Germination of *Cistus ladanifer* seeds in relation to parent material. *Plant Ecology* **133**, 57–62.

Pérez-García, F. (2005) Seed germination of different populations of wild (n=9) *Brassica montana* and *B. oleracea*. *Spanish Journal of Agricultural Research* **3**, 331–334.

Pérez-García, F. (2009) Germination characteristics and intrapopulation variation in carob (*Ceratonia siliqua* L.) seeds. *Spanish Journal of Agricultural Research* **7**, 398–406.

Pérez-García, F. and González-Benito, M.E. (2012) Intrapopulation variation in seed germination of six rock-rose (*Cistus* L.) species. *Acta Horticulturae* **937**, 379–384.

Pérez-García, F., Hornero, J. and González-Benito, M.E. (2003) Interpopulation variation in seed germination of five Mediterranean Labiatae shrubby species. *Israel Journal of Plant Science* **51**, 117–124.

Pérez-García, F., Huertas, M., Mora, E., Peña, B., Varela, F. and González-Benito, M.E. (2006) *Hypericum perforatum* L. seed germination: intrapopulation variation and effect of light, temperature, presowing treatments and seed desiccation. *Genetic Resources and Crop Evolution* **53**, 1187–1198.

Pérez-García, F. and Pita, J.M. (1989) Mechanical resistance of the seed coat during germination of *Onopordum nervosum* Boiss. *Seed Science and Technology* **17**, 277–282.

Pérez-García, F., Varela, F. and González-Benito, M.E. (2012) Morphological and germination response variability in seeds of wild Bellow gentian (*Gentiana lutea* L.) accessions from northwest Spain. *Botany* **90**, 731–742.

Qaderi, M.M. and Cavers, P.B. (2002) Effects of dry heat on the germinability and viability of Scotch thistle (*Onopordum acanthium*) cypselas: interpopulation and interposition variation. *Canadian Journal of Botany* **81**, 684–697.

Rasband, W.S. (1997–2015) ImageJ, US National Institutes of Health, Bethesda, Maryland, USA. Available at: http://imagej.nih.gov/ij/

Reed, T.E., Schindler, D.E. and Waples, R.S. (2011) Interacting effects of phenotypic plasticity and evolution on population persistence in a changing climate. *Conservation Biology* **25**, 56–63.

R Core Team (2015). R: A language and environment for statistical computing. Vienna, Austria: Foundation for Statistical Computing. Available at: http://www.R-project.org/

Renzi, J.P., Chantre, G.R. and Cantamutto, M.A. (2016) Effect of water availability and seed source on physical dormancy break of *Vicia villosa* ssp. *villosa*. *Seed Science Research* (online). doi: http://dx.doi.org/10.1017/S096025851600012X

Ritz, C., Pipper, C.B. and Streibig, J.C. (2013) Analysis of germination data from agricultural experiments. *European Journal of Agronomy* **45**, 1–6.

Ritz, C. and Streibig, J.C. (2005) Bioassay analysis using R. *Journal of Statistics Software* **12**, 1–22.

Ruprecht, E., Fenesi, A., Fodor, E., Kuhn, T. and Tökölyi, J. (2015) Shape determines fire tolerance of seeds in temperate grasslands that are not prone to fire. *Perspectives in Plant Ecology, Evolution and Systematics* **17**, 397–404.

Tavşanoğlu, C. and Çatav, S. (2012) Seed size explains within-population variability in post-fire germination of *Cistus salviifolius*. *Annales Botanici Fennici* **49**, 331–340.

Takos, I.A. and Efthimiou, G.S.P. (2003) Germination results on dormant seeds of fifteen tree species autumn sown in a northern greek nursery. *Silvae Genetica* **52**, 67–71.

Traveset, A., Robertson, Q.W. and Rodríguez-Pérez, J. (2007) A review on the role of endozoochory in seed germination. In Dennis, A.J., Schupp, E.W., Green, R.A. and Westcott, D.A. (eds), *Seed Dispersal, Theory and its Application in a Changing World*, pp. 78–103. London, UK: CABI Publishing.

Traveset, A., Rodríguez-Pérez, J. and Pias, B. (2008) Seed trait changes in dispersers' guts and consequences for germination and seedling growth. *Ecology* **89**, 95–106.

Vitale, M., Capogna, F. and Manes, F. (2007) Resilience assessment on *Phillyrea angustifolia* L. maquis undergone to experimental fire through a big-leaf modelling approach. *Ecological Modelling* **203**, 387–394.

Walck, J., Hidayati, S.N., Dixon, K.W., Thompson, K. and Poschlod, P. (2011) Climate change and plant regeneration from seed. *Global Change Biology* **17**, 2145–2161.

Wenning, R.J. and Erickson, G.A. (1994) Interpretation and analysis of complex environmental data using chemometric methods. *Trends in Analytical Chemistry* **13**, 446–457.

Wulff, R.D. (1995) Environmental maternal effects on seed quality and germination. In Kigel, J. and Galili, G. (eds), *Seed Development and Germination*, pp. 491–506. New York, USA: Marcel Dekker.