

## RESEARCH PAPER

# Germination sensitivity to water stress in four shrubby species across the Mediterranean Basin

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## Keywords

Cistaceae; *Erica arborea*; Fabaceae; fire cues; heat shock; hydrotime analysis; PEG; smoke.

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## ABSTRACT

Mediterranean shrublands are generally water-limited and fire-driven ecosystems. Seed-based post-fire regeneration may be affected by varying rainfall patterns, depending on species sensitivity to germinate under water stress. In our study, we considered the germination response to water stress in four species from several sites across the Mediterranean Basin. Seeds of species with a hard coat (*Cistus monspeliensis*, *C. salviifolius*, Cistaceae, *Calicotome villosa*, Fabaceae) or soft coat (*Erica arborea*, Ericaceae), which were exposed or not to a heat shock and smoke (fire cues), were made to germinate under water stress. Final germination percentage, germination speed and viability of seeds were recorded. Germination was modelled using hydrotime analysis and correlated to the water balance characteristics of seed provenance. Water stress was found to decrease final germination in the three hard-seeded species, as well as reduce germination speed. Moreover, an interaction between fire cues and water stress was found, whereby fire cues increased sensitivity to water stress. Seed viability after germination under water stress also declined in two hard-seeded species. Conversely, *E. arborea* showed little sensitivity to water stress, independent of fire cues. Germination responses varied among populations of all species, and hydrotime parameters were not correlated to site water balance, except in *E. arborea* when not exposed to fire cues. In conclusion, the species studied differed in germination sensitivity to water stress; furthermore, fire cues increased this sensitivity in the three hard-seeded species, but not in *E. arborea*. Moreover, populations within species consistently differed among themselves, but these differences could only be related to the provenance locality in *E. arborea* in seeds not exposed to fire cues.

## INTRODUCTION

The germination process is the beginning of the autonomous life of a plant, and is controlled by water availability provided that suitable temperatures are present. Germination starts with the imbibition of the seed, which prompts the initiation of metabolic processes that will result in radicle elongation. The uptake of water is triphasic (Finch-Savage & Leubner-Metzger 2006), while the length of each phase depends on species and environmental conditions, although seed germination occurs only when seeds hold 30–35% water (Roberts & Ellis 1989). If water in the seed environment is limiting, water uptake may start, but the germination process cannot be concluded if all three phases of imbibition are not completed. Water availability is, therefore, an important limiting factor for germination, affecting the final germination percentage as well as the rate and uniformity of emergence (Bewley & Black 1994).

Seeds in the field will be exposed to various water potentials depending on their position in the soil profile, soil characteristics and weather conditions following a rainfall event. Seeds in the soil may hydrate once a rain event occurs, after which seeds start to dehydrate as the soil dries out until a new rain event eventually allows re-imbibition of the seeds (Batlla & Benech-Arnold 2006). As the soil dries out, its water potential declines, thereby potentially limiting germination (Koller & Hadas 1982; Downs & Cavers 2000). Seeds at the soil surface or in the very upper soil layers are subjected to significant fluctuations in water content, with more rapid wetting after the rain, but also faster drying. In contrast, seeds buried deeper in the soil will not be moistened until larger amounts of rain have fallen. Once moistened, they will remain wet for a longer time due to the exponential decay of the evaporation rate in relation to soil depth (Allen *et al.* 1998; Xiao *et al.* 2011).

In Mediterranean areas, germination occurs after autumn rains once soils are wetted (Espigares & Peco 1993; Céspedes *et al.* 2012). Duration and timing of the wet season have important consequences for seed germination and recruitment, with delayed and shorter wet seasons leading to lower final germination and lower richness and diversity of species (Miranda *et al.* 2009; Céspedes *et al.* 2012; Joët *et al.* 2013). In these areas, fire occurs mainly during the summer dry season (Urbietta *et al.* 2015). In post-fire environments, temporal germination patterns show large variability among species, and are closely related to variations in rainfall (Quintana *et al.* 2004; Moreno *et al.* 2011). Germination is highest during wet years, only occurring during the first post-fire year; during dry years, however, germination is lower and extends over several years (Moreno *et al.* 2011). Seeds that germinate in the second or later years post-fire have little chance of establishing (Quintana *et al.* 2004; Moreno *et al.* 2011).

Germination response to gradients of water stress has been studied under laboratory conditions by exposing seeds to polyethylene glycol (PEG), an inert, water-binding polymer with a non-ionic impermeable long chain that correctly simulates drought stress under dry soil conditions. Many of these studies typically address the response of a single species, with greater focus on species of agricultural interest, such as crop species or weeds (Almansouri *et al.* 2001; Zhang *et al.* 2010). Few studies have focused on a larger number of species and, when they do, significant interspecific variation is often reported. Interspecific variation to water stress has been related to habitat characteristics (Evans & Etherington 1990; Sy *et al.* 2001; Schütz *et al.* 2002), climate characteristics (Köchy & Tielbörger 2007) and life-history traits (Kos & Poschlod 2008), although generalisations are largely inconclusive. Similarly, research relating to the intraspecific population variation of germination patterns to habitat characteristics has not produced consistent results (Boydak *et al.* 2003; Raccaia *et al.* 2004; Tilki & Dirik 2007; Petrů & Tielbörger 2008; Atia *et al.* 2011; Cochrane *et al.* 2015a).

Fire plays a major role in Mediterranean ecosystems. After a fire event, many species regenerate solely from seeds, which are most often stored in the soil seed bank and resistant to high temperatures (Luna *et al.* 2007). Species with hard-coated seeds (*i.e.* with physical dormancy) are common, with dormancy-breaking being cued to fire (heat with or without smoke; Ne'eman *et al.* 2012). Many of these species dominate the various types of shrubland in the Mediterranean region; these include species of the Cistaceae and woody shrubs of the Fabaceae. Other dominant species in shrublands on more mesic and acidic substrates include Ericaceae. Seeds in this last family are soft-coated and can have physiological dormancy, while the role of fire in promoting germination is less clear (Mésleard & Lepart 1991; Crosti *et al.* 2006; Moreira *et al.* 2010). While Cistaceae shrubs are generally obligate seeders, shrubs in the Fabaceae and Ericaceae often also resprout after fire.

Knowledge on seed germination sensitivity to water stress is of the utmost importance in dry areas such as the Mediterranean region and other areas with a similar climate in the world having similarly alternating periods of dry and wet soils. In these environments, droughts are common and rainfall is highly variable from year to year; with more droughts the lower the rainfall (Lionello *et al.* 2006). This implies that after a fire

event, when recruitment of obligate seeders is most vulnerable, germination might proceed under reduced rainfall. Additionally, in the context of changing climate, this situation can be even more critical. In the Mediterranean region, global warming is projected to increase mean surface temperatures more than the mean global average, and modify the precipitation regime, with a lengthened and more intense drought period during the year (Ruffault *et al.* 2014). Rainfall is projected to be concentrated in the autumn and winter months, with fewer, but more intense precipitation events (Giorgi & Lionello 2008), which is consistent with recent observations (Bindoff *et al.* 2013). Plant establishment will likely be affected by limited water availability in addition to high temperatures. Notwithstanding, a small number of studies have anticipated the likely impact of changes in climate, rainfall and drought, in particular, upon germination (see Walck *et al.* 2011).

In this study, we analysed the effects of water stress and fire-related cues (*i.e.* exposure of seeds to heat shock and smoke) on seed germination and viability of four woody species that are widespread across the Mediterranean Basin. In so doing, we asked the following questions: (i) do species differ in their germination sensitivity to water stress; (ii) is the response affected by exposing the seeds to fire cues; (iii) do water stress response patterns vary across the Mediterranean Basin; and (iv) are germination response patterns related to the climate conditions of the locality of the seed provenance?

## MATERIAL AND METHODS

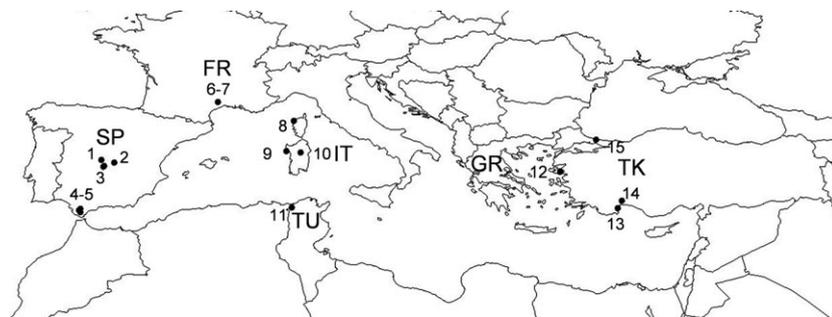
### Study species and seed collection

Seeds of four typical Mediterranean shrubland woody species were collected from across the Mediterranean Basin. These were: *Cistus monspeliensis* L., *C. salviifolius* L. (Cistaceae), *Calicotome villosa* (Poir.) Link. (Fabaceae) and *Erica arborea* L. (Ericaceae). The first three species have hard-coated seeds (*i.e.* the seeds have physical dormancy), while *E. arborea* has soft seeds (*i.e.* the seeds do not have physical dormancy; Baskin & Baskin 2014). Seeds were collected on ripening in summer 2010 (from July to August) from at least 20 plants per site, in order to make a single species–site sample, at sites spanning 3237 km, in Spain, France, Tunisia, Italy, Greece and Turkey (Fig. 1, Table 1). To avoid spurious effects due to selecting sites close together that might have high intraspecific variability (Moreira *et al.* 2012), the minimum distance between sites for a given species was 387 km, such that climate- and other fire-related pressures would be unique to each site. Seeds were stored in paper bags at room temperature until the germination experiments began in January of the following year.

### Germination experiments

In fire-prone environments, germination is triggered by both heat and smoke (Keeley & Fotheringham 2000). Smoke cannot stimulate the germination of seeds with an impermeable coat until this coat has been broken with fire or other scarifying agents (Moreira *et al.* 2010). Prior to incubation, half of the seeds were heated at 100 °C for 10 min, which is a common temperature and timeframe in shrubland fires (Moreno *et al.* 2011; Céspedes *et al.* 2012), and then exposed to smoke for 20 min, in order to simulate the effects of fire. Seeds were

**Fig. 1.** Geographic locations from which seeds were collected. Seeds were harvested from six countries (Spain, France, Italy, Tunisia, Greece and Turkey) and 15 populations across the Mediterranean Basin (see Table 1 for further details on specific locations sampled).



**Table 1.** Characteristics of locations where seeds were harvested. Population number (Pop) refers to Fig. 1. Code refers to the first initials of each country and, in the case of Spain, we used additional coding related to the geographic position (C: centre refers to populations 1–3; S: south refers to populations 4–5) since several populations were studied. Climate data was obtained for the climate reference period 1961–1990 from the WATCH climate dataset (<http://www.eu-watch.org>). Duration (days) and intensity (mm) of the period of vulnerability (*i.e.* the period between maximum drought until continuous positive water balance) for germination are indicated for each population. Additionally, the Standardised Precipitation–Evapotranspiration Index (SPEI 10) (<http://sac.c-sic.es/spei/index.html>) is provided for 2010, when seeds were harvested. This is a drought index based on the climatic water balance ( $P - PET$ ), with positive values indicating drought being less intense than the historical trend and negative values being the opposite (Beguería *et al.* 2014).

Country	Code	Pop	Lat (N)	Long (E)	Alt (m)	$T$ (°C)	P (mm)	SPEI 10	Period of vulnerability	
									Duration (days)	Intensity (mm)
<i>C. monspeliensis</i>										
Spain	SP (C)	1	39.64°	−3.39°	820	14.5	422.2	0.63	146	406.5
	SP (S)	4	36.30°	−5.68°	145	17.6	754.5	1.77	119	287.9
France	FR	6	43.74°	3.59°	270	13.2	1303.9	−0.37	84	85.0
Italy	IT	9	40.61°	8.15°	43	14.9	921.7	0.93	105	181.6
Tunisia	TU	11	36.61°	8.56°	520	17.2	991.4	0.76	116	330.9
<i>C. salviifolius</i>										
Spain	SP (C)	2	39.82°	−4.24°	533	14.7	480.6	1.53	141	384.3
	SP (S)	5	36.52°	−5.66°	399	17.6	652.5	1.79	129	364.2
France	FR	7	43.61°	3.40°	174	11.9	1298.2	−0.41	76	60.6
Italy	IT	10	40.33°	9.12°	347	13.2	966.5	0.65	99	154.9
Greece	GR	12	39.02°	26.61°	97	16.2	716.3	2.11	91	95.1
Turkey	TK	13	36.60°	30.48°	70	12.3	696.4	1.24	95	358.9
<i>C. villosa</i>										
Spain	SP (S)	4	36.30°	−5.68°	170	17.6	754.5	1.77	119	287.6
France	FR	8	42.47°	8.69°	43	12.7	1079.0	0.55	97	131.5
Tunisia	TU	12	36.61°	8.56°	520	17.2	991.4	0.76	116	330.9
Turkey	TK	14	37.01°	30.76°	125	15.0	748.9	1.25	122	377.0
<i>E. arborea</i>										
Spain	SP (C)	3	39.42°	−4.07°	917	14.0	605.3	1.29	132	357.4
	SP (S)	5	36.52°	−5.66°	399	17.6	652.5	1.79	129	364.2
France	FR	7	43.61°	3.40°	174	11.9	1298.2	−0.41	76	60.6
Turkey	TK	15	41.17°	29.01°	50	14.1	763.5	1.93	110	184.3

heated in an electric oven. Smoke was produced by burning a mixture of fine fuel from several species, including *Cistus* spp. and *E. arborea*. Smoke was continuously funnelled for 20 min through a box containing the seeds laid out in trays. Seeds were then incubated at 20 °C with a photoperiod of 12 h/12 h for 60 days in plastic Petri dishes (5.5-cm diameter) over two filter papers (Whatman no. 1). Seeds were germinated under different levels of water stress by moistening the Petri dishes with either 1.2 ml deionised water or the appropriate polyethylene glycol (PEG) solution in order to produce four levels of water potential: 0, −0.15, −0.30 and −0.45 MPa. Filter papers were replaced weekly and the corresponding PEG solution added to avoid changes in the germinating solution. PEG is routinely

used as a water stressor agent (Baskin & Baskin 2014). The required water potential was produced with PEG 6000 and deionised water according to the formula  $\Psi = 0.130[\text{PEG}]^2 T - 13.7[\text{PEG}]^2$ , in line with Michel & Kaufmann (1973) and additional adjustments of Hardegree & Emmerich (1990). Six replicates of 25 seeds per species and site were used in each of the treatments. All Petri dishes were sealed with Parafilm in order to prevent desiccation. Petri dishes were placed at random on the plate of a temperature- and humidity-controlled chamber (Model G-21, Ibercex). Germination was recorded every day for the first 30 days and every 3 days until the end of the experiment, with radicle emergence used as the criterion for scoring a seed as germinated. When the experiment ended,

the viability of each non-germinated seed was checked using the tetrazolium test for Cistaceae and Fabaceae. The tetrazolium test was undertaken after the seeds were cut into half and incubated in 1% 2,3,5-triphenyl tetrazolium chloride for 48 h in the dark (Moore 1985). Given their small size, the tetrazolium test could not be conducted with *E. arborea* seeds. Instead, in the case of *E. arborea*, a 1% solution of gibberellic acid ( $GA_3$ ) was added to non-germinated seeds until germination was completed. Seeds that were infected by fungi were considered non-viable.

Four variables were obtained: final germination percentage at the end of the experiment corrected for viability (FG; *i.e.* germination percentages were estimated in relation to viable seeds and not in relation to the total number of seeds), germination speed characterised by the time to initiate germination ( $T_0$ ; *i.e.* the time until the first seed germinated) and the time to produce 50% of the total germination obtained ( $T_{50}$ ), and, finally, seed viability ( $V$ ; viability percentages were assessed by considering germinated seeds plus non-germinated, but tetrazolium-coloured, seeds).

### Data analyses

Final germination percentage,  $T_0$ ,  $T_{50}$  and seed viability data were analysed with generalised linear models (GLMs). Based on error structure, we used a binomial error distribution and logit link function for final germination and seed viability. In the case of  $T_0$  and  $T_{50}$ , a Poisson error distribution with identity link function was considered most appropriate in relation to the data. Each species was tested for the effects of population provenance (*i.e.* site of collection) and germination treatments were nested within populations. In cases where no differences among populations emerged, a non-nested model with three factors was fitted. Germination treatments were fire cues (two levels, with and without heat + smoke) and water stress (four levels, 0 to  $-0.45$  MPa), which were considered as fixed factors. The population of the provenance was also considered as a fixed factor on the assumption that a population would reflect the long-term effects of a local climate (Bolker *et al.* 2008). Where water stress treatment effects were significant, pair-wise comparisons among treatments were performed using the Bonferroni correction. All statistical analyses were performed using the SPSS Statistics version 19.0 (SPSS, Chicago, IL, USA).

Hydrottime analyses were carried out on the basis that they allow for a unifying model that is useful for describing the patterns of germination occurring in response to water potential (Bradford 1990). Hydrottime analysis quantifies the speed of germination ( $\theta_H$ ), the stress tolerance of germination ( $\Psi_b$ ) and the uniformity of germination ( $\sigma_{\Psi_b}$ ) (Bradford & Still 2004).  $\theta_H$  is the hydrottime constant (MPa h) for the population, defined as  $\theta_H = (\Psi - \Psi_b(g))t_g$  where  $\Psi$  is the seed water potential (MPa),  $\Psi_b(g)$  is the base or threshold water potential (MPa) defined for a specific germination fraction  $g$ , and  $t_g$  is the time required for germination of percentage  $g$ . Base water potential,  $\Psi_b$ , is the minimum water potential permitting germination and the  $\Psi_b$  of individual seeds varies as a normal distribution in a population of seeds (Gummerson 1986; Bradford 1990) with a median  $\Psi_b$  (50) and the corresponding SD  $\sigma_{\Psi_b}$  (50).

Values of  $\theta_H$ ,  $\Psi_b$  (50) and  $\sigma_{\Psi_b}$  (50) were determined using repeated probit regression analysis in order to align the time

courses to the hydrottime model, as described previously by Bradford (1990). Higher  $\theta_H$  indicates a longer time needed for germination (MPa h; *i.e.* slower germination). Lower (*i.e.* more negative) values of  $\Psi_b$  mean that seeds will germinate across a wider range of water potentials. Finally, higher values of  $\sigma_{\Psi_b}$  (50) indicate higher germination variability within the population.

Hydrottime analysis requires appropriate germination percentages over a range of  $\Psi$ s, with high germination percentages at 0 MPa and in at least one other level of  $\Psi$ . In the case of species with hard-coated seeds, germination percentages without fire cues were very low, which precluded hydrottime modelling. Differences in hydrottime parameters between species were analysed with univariate GLMs and, in the case of *E. arborea*, differences between seeds exposed or otherwise to fire cues were analysed with repeated measures GLMs.

We were interested in determining the relationship between germination sensitivity to water stress and the local water balance characteristics at the sites where seeds were harvested. The germination sensitivity to water stress was described as  $\theta_H$  and  $\Psi_b(50)$ . The water balance characteristics at each site were characterised by what we called the 'period of vulnerability' for germination. This period of vulnerability was determined from a daily water balance, which was calculated as the difference between precipitation and potential evapotranspiration ( $P - PET$ ). The daily potential evapotranspiration was calculated in line with the FAO-56 Hargreaves equation (Allen *et al.* 1998), and the daily temperature and precipitation data were obtained for the climate reference period 1961–1990 from the WATCH climate dataset at  $0.5^\circ$  latitude/longitude resolution (<http://www.eu-watch.org/>). Mean water balance was calculated for each day and then applied to a quadratic model. The period of vulnerability was defined as the period between the time when the water balance was at its minimum (*i.e.* maximum drought) and when it became continuously positive, and described with the following two variables: duration (number of days between the height of drought and continuous wetness; *i.e.* positive water balance) and intensity (cumulative water balance throughout the days of the period of vulnerability). Overall, the period of vulnerability is expected to occur following seed dispersal during the dry season (*i.e.* from mid-summer) when water stress is maximum, until early autumn, depending on patterns of seasonal rain. The relationship between germination sensitivity to water stress and both variables of the period of vulnerability was determined by least squares regression. The dependent variables were  $\theta_H$  and  $\Psi_b$ , while the duration and intensity of the period of vulnerability were the independent variables.

### RESULTS

The four species differed in their germination responses to the treatments. *Erica arborea* was the species with the highest final germination, in seeds both exposed and not exposed to fire cues (Table S1). Germination of the other species was increased considerably by fire cues, with *C. monspeliensis* being the species with the lowest final germination values. Final germination was significantly different among populations in all species (Table 2). Species with hard-coated seeds were significantly affected by both fire cues and water stress (Table 2). Final germination increased with fire cues and decreased with water

stress. Moreover, a significant interaction between these two factors emerged (Table 2; Fig. 2), whereby the negative effect of water stress was largest in seeds exposed to fire cues. Lastly, final germination in *E. arborea* was significantly affected by fire

**Table 2.** *P*-values from the GLM for effects of population (P), fire cues (Fc) and water stress (Ws) nested within population, on final germination (FG);  $T_0$  (time to initiate germination),  $T_{50}$  (time to reach 50% of total germination) and seed viability (V). More information on GLM results can be found in Table S5.

	FG	$T_0$	$T_{50}$	V
<i>C. monspeliensis</i>				
P	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>0.005</b>	<b>&lt;0.001</b>
Fc [P]	<b>&lt;0.001</b>	0.203	<b>0.004</b>	0.069
Ws [P]	<b>&lt;0.001</b>	<b>0.002</b>	<b>&lt;0.001</b>	0.164
Fc × Ws	<b>0.040</b>	0.217	<b>0.004</b>	0.461
<i>C. salviifolius</i>				
P	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>
Fc [P]	<b>&lt;0.001</b>	<b>0.002</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>
Ws [P]	<b>&lt;0.001</b>	<b>0.036</b>	0.192	<b>0.019</b>
Fc × Ws	<b>0.009</b>	0.800	<b>&lt;0.001</b>	<b>&lt;0.001</b>
<i>C. villosa</i>				
P	<b>&lt;0.001</b>	<b>0.002</b>	0.579	<b>&lt;0.001</b>
Fc [P]	<b>0.001</b>	<b>0.048</b>	<b>&lt;0.001</b> <sup>a</sup>	<b>&lt;0.001</b>
Ws [P]	<b>0.023</b>	0.841	<b>&lt;0.001</b> <sup>a</sup>	<b>0.011</b>
Fc × Ws	<b>&lt;0.001</b>	0.112	0.479	0.544
P × Fc			0.799	
P × Ws			0.969	
P × Fc × Ws			0.958	
<i>E. arborea</i>				
P	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>
Fc [P]	<b>0.045</b>	0.289	0.694	<b>0.003</b>
Ws [P]	0.422	<b>&lt;0.001</b>	<b>&lt;0.001</b>	0.201
Fc × Ws	0.717	0.354	0.310	<b>0.016</b>

Significant *P*-values are shown in bold ( $P < 0.05$ ).

<sup>a</sup>In the case of *C. villosa*  $T_{50}$ , populations were not significantly different and, consequently, a non-nested model with three factors was fitted (see Table S5).

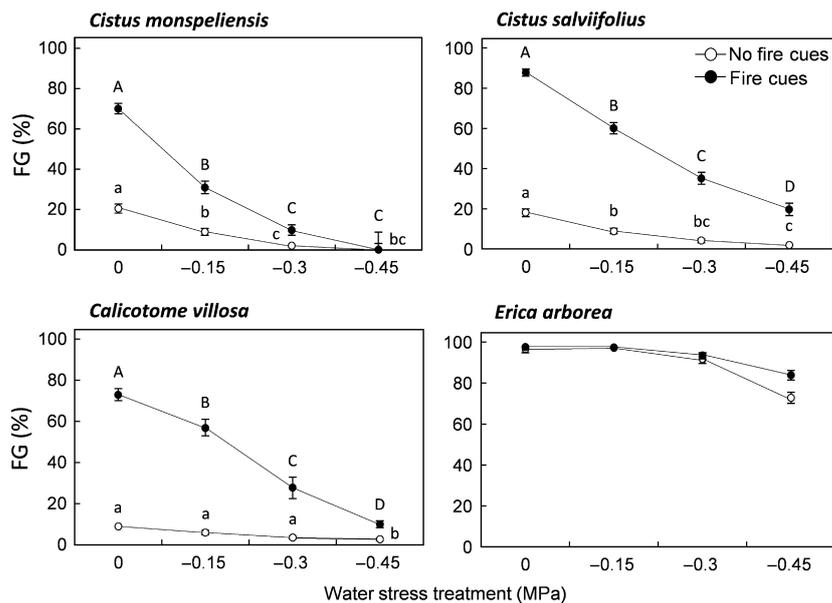
cues, albeit with a minor positive effect, and not significantly affected by water stress. No interaction between the two treatments was ascertained (Table 2).

Time to initiate germination ( $T_0$ ) in seeds not treated with fire cues was lowest and significantly homogeneous among populations in *E. arborea* (8–9 days for non-water-stressed seeds), and higher and more variable among populations in the other three species, with *C. villosa* being the most extreme (from 5 to 25 days in the non-water-stressed seeds; Table S2). Population was a significant factor in all four species.  $T_0$  generally decreased in seeds exposed to fire cues, with significant effects observed in *C. salviifolius* and *C. villosa*. Water stress significantly increased  $T_0$  in all species except *C. villosa*.

The time to reach 50% of final germination ( $T_{50}$ ) was significantly different among populations in all species except *C. villosa*. Exposing seeds to fire cues significantly increased  $T_{50}$  in hard-coated species, but was unchanged in *E. arborea*. Water stress increased  $T_{50}$  in all species except *C. salviifolius*, where such an increase was only observed in seeds that had previously been exposed to fire cues (Table 2, Fig. S1). Fire cues and water stress interaction was also significant in *C. monspeliensis* (Table 2).

Significant differences in seed viability (V) among populations were also found for all species (Tables 2, S4). In the case of *C. monspeliensis*, seed viability was not affected by any of the treatments, unlike *C. villosa* and *C. salviifolius*, which were affected by both (Table 2). Viability of *E. arborea* seeds was only affected by fire cues treatment (Table 2). Additionally, an interaction between fire cues and water stress treatments emerged for *C. salviifolius* and *E. arborea*. Contrary to *E. arborea*, seed viability of *C. salviifolius* was not affected by water stress in the absence of fire cues, although seed viability decreased with increased water stress after exposure to fire cues. *Erica arborea* displayed an opposite pattern, showing a decrease in viability with water stress only in seeds not exposed to fire cues (Fig. S1).

The hydrotime model was generally compatible with the timeline of germination (with  $r^2$ -values ranging from 0.69 to 0.95; Table 3). Overall, hydrotime parameters showed great



**Fig. 2.** Final germination percentage (FG) for each species and water stress treatment. Means  $\pm$  SE are presented for seeds not exposed to fire cues (heat shock + smoke) or exposed to them. When the interaction between water stress treatment and fire cues was statistically significant, differences among treatments were assessed. Therefore, different letters (lowercase for seeds not exposed to fire cues, and uppercase for seeds exposed to them) show significant differences among water stress treatments from pair-wise comparisons with the Bonferroni correction ( $P < 0.05$ ) after GLM analysis (see Table 2).

**Table 3.** Hydrotime model parameters for each species and population studied. In the case of species with hard-coated seeds, hydrotime analyses were only possible for seeds exposed to fire cues (heat shock + smoke). In the case of *E. arborea*, hydrotime parameters are shown for seeds both not exposed and exposed to fire cues.

	$\theta_H$	$\Psi_b(50)$	$\sigma_{\Psi_b(50)}$	$r^2$
<i>C. monspeliensis</i>				
SP (C)	63	-0.01	0.16	0.95
SP (S)	162	-0.11	0.16	0.78
FR	107	-0.11	0.21	0.69
IT	81	-0.25	0.21	0.75
TU	88	-0.16	0.11	0.91
<i>C. salviifolius</i>				
SP (C)	69	-0.10	0.22	0.89
SP (S)	199	-0.40	0.26	0.91
FR	137	-0.42	0.21	0.86
IT	123	-0.37	0.25	0.86
GR	75	-0.30	0.19	0.90
TK	94	-0.13	0.16	0.77
<i>C. villosa</i>				
SP (S)	231	-0.32	0.34	0.75
FR	47	-0.03	0.24	0.73
TU	76	-0.06	0.26	0.76
TK	159	-0.08	0.24	0.80
<i>E. arborea</i>				
Fire cues				
SP (C)	219	-1.06	0.28	0.89
SP (S)	162	-0.67	0.24	0.87
FR	286	-1.09	0.49	0.90
TK	216	-0.84	0.27	0.91
No fire cues				
SP (C)	221	-0.94	0.28	0.88
SP (S)	243	-0.78	0.26	0.91
FR	111	-0.49	0.17	0.91
TK	212	-0.79	0.29	0.81

variability among populations within a species in all four species (Table 3). Species differed in  $\theta_H$  and  $\Psi_b(50)$  among them ( $F_{3,14} = 3.997$ ,  $P = 0.030$ ;  $F_{3,14} = 26.553$ ,  $P < 0.001$ , respectively). *Erica arborea* had the highest values of  $\theta_H$  and the lowest (i.e. more negative)  $\Psi_b(50)$ . *Post-hoc* analysis showed that  $\theta_H$  in *E. arborea* was significantly different from *C. monspeliensis*, with the other two neither differing from these two species nor among themselves. On the other hand, *post-hoc* analysis for  $\Psi_b(50)$  indicated that *E. arborea* was significantly different from the other three species. Furthermore,  $\theta_H$  and  $\Psi_b(50)$  in *E. arborea* did not show significant difference among seeds exposed or otherwise to fire cues ( $F_{1,3} = 0.198$ ,  $P = 0.686$ ;  $F_{1,3} = 1.166$ ,  $P = 0.359$ , respectively).

The duration ( $111.1 \pm 4.1$  days) and intensity ( $254.5 \pm 20.4$  mm) means of the period of vulnerability were similar among species (Table 1). Germination sensitivity to water stress ( $\theta_H$  and  $\Psi_b(50)$ ) was not correlated with either the duration or intensity of the period of vulnerability for the species with hard-coated seeds (Table 4). In the case of *E. arborea*, however, the duration of the period of vulnerability was significantly correlated with  $\theta_H$  and marginally correlated with  $\Psi_b(50)$  ( $P = 0.063$ ) in seeds not exposed to fire cues, but not in exposed seeds (Table 4). As the duration of the period of vul-

**Table 4.** Correlation ( $r$ ,  $P$ ) between  $\theta_H$  and  $\Psi_b(50)$  and the period of vulnerability (duration and intensity) for four shrubs studied across the Mediterranean Basin. For species with hard-coated seeds, correlation is shown for seeds exposed to fire cues, whereas, in the case of *E. arborea*, correlations were made for seeds both not exposed and exposed to fire cues.

	$\theta_H$		$\Psi_b(50)$	
	$r$	$P$	$r$	$P$
<i>C. monspeliensis</i>				
Duration	-0.272	0.658	0.545	0.342
Intensity	-0.221	0.721	0.492	0.399
<i>C. salviifolius</i>				
Duration	0.073	0.891	0.397	0.436
Intensity	0.070	0.895	0.612	0.197
<i>C. villosa</i>				
Duration	-0.275	0.656	0.554	0.333
Intensity	-0.222	0.719	0.497	0.395
<i>E. arborea</i>				
Fire cues				
Duration	-0.862	0.138	0.486	0.514
Intensity	-0.851	0.149	0.465	0.535
No fire cues				
Duration	0.957	<b>0.043</b>	-0.937	0.063
Intensity	0.822	0.178	-0.855	0.145

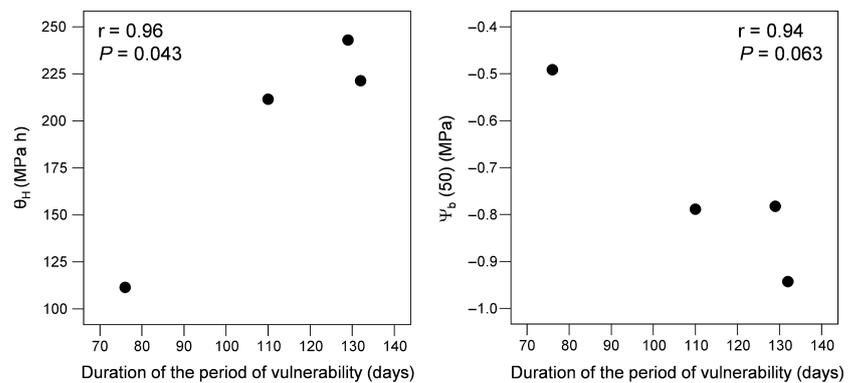
Significant relationships are shown in bold ( $P < 0.05$ ).

nerability increased,  $\theta_H$  also increased, while  $\Psi_b(50)$  became more negative (Fig. 3).

## DISCUSSION

Few studies have addressed the effects of water stress on germination in Mediterranean species. Conifers have shown high tolerance to water stress (Thanos & Skordilis 1987; Boydak *et al.* 2003), whereas shrub species of the Fabaceae have shown variable responses, from high (e.g. *Antyllis cytisoides*; Ibáñez & Passera 1997) to low tolerance (e.g. *Genista scorpius*; Bochet *et al.* 2007). In these aforementioned studies, seeds had previously been scarified although no fire cues were involved. Seeds of other *Cistus* species that were neither scarified nor exposed to fire cues showed moderate tolerance to water stress (Pérez-Fernández *et al.* 2006). Annual species, including hard-seeded Fabaceae, previously scarified but not exposed to fire cues showed high tolerance to water stress (Pérez-Fernández *et al.* 2006; Bochet *et al.* 2007; Köchy & Tielbörger 2007). Comparisons among life forms are difficult given the limited number of species studied, and the fact that seeds were not always exposed to fire cues. Nevertheless, it appears that sensitivity to water stress among shrubs is more variable than in other life forms. Clearly, more studies are needed to accurately characterise fire-prone Mediterranean species, and woody species in particular.

In our study, germination sensitivity to water stress increased markedly in the hard-coated seed species after exposing the seeds to fire cues, as demonstrated by the consistently significant interactions between fire cues and water stress treatments. The pattern of response was consistent in the three species investigated. This observation concurs with the fact that the non-dormant fraction in these species is much less sensitive



**Fig. 3.** Correlation between the duration of the period of vulnerability for germination and hydrotime parameters [ $\theta_H$  and  $\Psi_b(50)$ ] in seeds of *E. arborea* that were not exposed to fire cues.

to water stress, which coincides with results from Pérez-Fernández *et al.* (2006), as described above. Other studies have observed the opposite effect (*i.e.* decreased sensitivity to water stress after exposure to fire cues; Ghebrehwot *et al.* 2008; Thomas *et al.* 2010), but in these studies, the species investigated did not have physical dormancy. That said, lack of physical dormancy, as in *E. arborea* in our case, did not imply significant changes in sensitivity to water stress as a result of being exposed to fire cues. It has been argued that karrikins from smoke and, in general, factors that promote germination have the ability to reduce the sensitivity to increasing water stress and enlarge the range of water potentials under which germination proceeds, as well as increasing germination speed (Bradford & Still 2004; Ghebrehwot *et al.* 2008; Thomas *et al.* 2010). However, as we have demonstrated, the three species with physical dormancy in our study exhibited the opposite effect, while *E. arborea* failed to support such a contention.

The Cistaceae and Fabaceae are families widespread across the Mediterranean Basin, dominating many shrublands in the region (Tomaselli 1981). While they occupy a variety of habitats, they are abundant in dry and warm environments. From an ecological viewpoint, we expected that they would be highly tolerant to water stress, but this was not supported by our study. Additionally, contrary to our expectations, fire cues increased germination sensitivity to water stress. Hard-coated seeds in Mediterranean shrublands, notably in Cistaceae and Fabaceae, usually form persistent soil seed banks that produce a flush of seedlings once dormancy is released by the passage of fire (Trabaud 1994; De Luis *et al.* 2005). Fire temporarily produces a competitor-free environment, where success is often contingent on being the first to germinate, establish and develop. It has been argued that species adapted to fire should reduce variability in the timing of germination, such that they would germinate as early as possible in order to increase fitness (Verdú & Traveset 2005; De Luis *et al.* 2008). We found, however, that germination was tightly related to low water stress, notably after seeds were exposed to fire cues. In such cases, water stress reduced germination speed in *Cistus*; this limits the opportunities for rapid germination and early establishment after fire. Having hard seeds indicates a fire-adaptive trait that may have different origins; in some species, physical dormancy may have evolved in response to fire, whereas in other species it could have originated in response to other selective pressures that became useful in fire-prone habitats (Keeley *et al.* 2011). Provided

the mismatch between what appears advantageous after fire to ensure prompt germination and early establishment, and the fact that fire cues restrict conditions for germination and delay the process, our results contradict a fire-driven selection process behind hard-coated seeds in these shrubs.

In contrast to the other species investigated, *E. arborea* showed little sensitivity to water stress, and was not affected by fire cues. Germination in this species is not cued to fire (*i.e.* heat and smoke effects; Mesléard & Lepart 1991; Valbuena & Vera 2002; Crosti *et al.* 2006). While we found that the germination of *E. arborea* was significantly affected by fire cues, the absolute magnitude of this increment was small and not comparable to that in the hard-coated species. Regeneration after fire in this species relies on resprouting, not on seeds (Mesléard & Lepart 1991); thus, ecological implications of changes in germination for population persistence would be smaller than in seeder species.

Germination responses to water stress varied among populations in all four species examined across the Mediterranean Basin. Hydrotime analysis supported these findings, showing a significant variability of the hydrotime parameters among populations of a given species. High variability among populations in germination responses to water stress is widespread among species within the region and in other regions of the world (Raccuia *et al.* 2004; Khera & Singh 2005; Tilki & Dirik 2007; Li & Feng 2009; Cochrane *et al.* 2015a). In the case of hard-coated seed species, we found that the variability among populations in germination sensitivity to water stress of seeds exposed to fire cues was not correlated to the period of vulnerability at the sites of seed provenance. In other words, germination after fire at the various sites would have occurred without being attuned to the local environment (*i.e.* the climate of the period of vulnerability in our case). The hydrotime model could not be calculated for the non-dormant fraction. These seeds may germinate readily every year, and not in pulses as in post-fire environments, thus environmental pressures would likely be stronger on them. Given the different responses to water stress between the two fractions, we cannot extrapolate the results from one to the other. Therefore, whether population differences in the readily germinable fraction correlate to local climate conditions remains unresolved.

In the case of *E. arborea*, the variability in germination sensitivity to water stress among populations was correlated to the duration of the period of vulnerability for germination, and for the intensity of this period to some extent. Seeds from sites with longer periods of vulnerability were able to germinate

under lower water potentials and indicated higher hydrotime values (more MPa hours) than those of sites with shorter periods (*i.e.* less dry). This pattern of response would concur with expectations that populations from dry provenances should be less sensitive to water stress. The pattern found also coincides with reports for coniferous Mediterranean species (Fady 1992; Boydak *et al.* 2003). Exposing the seeds to fire cues, however, rendered these relationships non-significant. In other words, seeds of the various populations, once treated with fire cues, germinated irrespective of their local climate conditions, much as occurred in the other three species with hard-coated seeds. Despite the fact that the locations chosen are widely spaced throughout the Mediterranean region, and covered a significant range of temperature and precipitation conditions, their limited number obliges us to be cautious before reaching a firmer conclusion.

Studies correlating local characteristics and seed traits and germination responses often report contrasting results among species, making generalisations difficult (Cochrane *et al.* 2015b). Our study concurs with this. Only on one occasion out of five (one in each of the hard coat-seeded species and two in *E. arborea*) did we find that local water balance conditions during the period of vulnerability explained among-population variability across the sites investigated. For the other occasions, variability among populations was the norm, but this could not be explained by local correlates. This implies that anticipating future threats to species persistence across their distribution range as a result of a changing climate due to global warming will be complicated. Models addressing the response of a given species to a change in climate (*e.g.* Pearson *et al.* 2014) need to consider not only intraspecific variability, but also variations due to other relevant ecological factors (*e.g.* fire in our case) affecting germination, as no single population will fully capture the species response throughout its distribution range. A caveat in this work is that consideration of the relationship with the local climate only involved climate parameters. This may provide a base reference for seeds at the surface, but may not do so for seeds buried in the soil, which will affect moistening–desiccation patterns (Schütz *et al.* 2002). The

inclusion of soil seed depth and sensitivity to varying rainfall patterns was beyond the scope of this study.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

**Table S1.** FG mean values ( $\pm$  SE) for seeds not exposed to fire cues (heat shock + smoke) or exposed to them, with each species and population germinated under different water stress treatments.

**Table S2.**  $T_0$  mean values ( $\pm$  SE) for seeds not exposed to fire cues (heat shock + smoke) or exposed to them, with each species and population germinated under different water stress treatments.

**Table S3.**  $T_{50}$  mean values ( $\pm$  SE) for seeds not exposed to fire cues (heat shock + smoke) or exposed to them, with each species and population germinated under different water stress treatments.

**Table S4.** V mean values ( $\pm$  SE) for seeds not exposed to fire cues (heat shock + smoke) or exposed to them, with each species and population germinated under different water stress treatments.

**Table S5.** Complete results from GLM for effects of population, fire cues and water stress treatments on FG,  $T_0$ ,  $T_{50}$  and V.

**Figure S1.**  $T_0$  (days),  $T_{50}$  (days), and V (%) mean values ( $\pm$  SE) for each species and water stress treatment, as well as for seeds not exposed to fire cues (heat shock + smoke) or exposed to them.

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