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

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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 hard-coated (*Cistus monspeliensis*, *C. salviifolius* [Cistaceae], *Calicotome villosa* [Fabaceae]) or soft-coated (*Erica arborea* [Ericaceae]) seeds, which were exposed or otherwise 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 the 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 amongst 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 amongst themselves, but these differences could only be related to the provenance locality in *E. arborea* in seeds not exposed to fire cues.

Running head: Germination sensitivity to water stress across the Mediterranean Basin

Keywords: Cistaceae, *Erica arborea*, Fabaceae, heat shock, smoke, hydrotime analysis, PEG, 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% of 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 the 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 at 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 moist 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; Jöet *et al*. 2012). In these areas, fire occurs mainly during the summer dry season (Urbieta *et al*. 2015). In post-fire environments, temporal germination patterns show great variability amongst 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 properly 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 upon a larger number of species and, when they do, significant inter-specific variation is often reported. Inter-specific 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 generalizations are largely inconclusive. Similarly, research relating to the intra-specific population variation of germination patterns to habitat characteristics has not produced consistent results (Boydak *et al.* 2003; Raccuia *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 region; these include species of the Cistaceae and the woody shrubs of the Fabaceae. Other dominant species in shrublands on more mesic and acidic substrates include the Ericaceae. Seeds in this family are soft-coated and can have physiological dormancy, while the role of fire in promoting germination is less clear (Mesléard & Lepart 1991; Crosti *et al.* 2006; Moreira *et al.* 2010). While the Cistaceae shrubs are generally obligate seeders, shrubs in the Fabaceae and Ericaceae often also resprout after fire.

Knowledge about seed germination sensitivity to water stress is of the utmost importance in dry areas such as the Mediterranean region and other climate-type areas of the world with similarly alternating periods of dry and wet soils. In these environments, droughts are common and rainfall is highly variable from year to year; more so 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 a 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 is expected to 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 revision by 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 widespread woody species across the Mediterranean Basin. In so doing, we asked the following questions: Do species differ in their germination sensitivity to water stress? Is the response affected by exposing the seeds to fire cues? Do water stress response patterns vary across the Mediterranean Basin? Are germination response patterns related to the climate conditions of the locality of 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 the summer of 2010 (from July to August) from at least 20 plants per site, in order to make a single species-site sample, at sites spanning 3,237 km, in Spain, France, Tunisia, Italy, Greece and Turkey (Fig. 1; Table 1). To avoid spurious effects due to selecting sites within a close distance that might have high intraspecific variability (Moreira *et al.* 2012), the minimum distance between sites 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 has been shown to be triggered by both heat and smoke (Keeley & Fotheringham 2000). Smoke cannot stimulate the germination of seeds with an impermeable coat until this has been broken by fire or other scarifying agent (Moreira *et al.* 2010). Prior to incubation, half of the seeds were heated at 100 ºC for 10 minutes, 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 minutes, in order to simulate the effects of fire. Seeds were 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 minutes through a box containing the seeds laid out in trays. Seeds were then incubated at 20 ºC with a photoperiod of 12/12 h for 60 days in plastic Petri dishes (5.5 cm in 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 of deionized water or the appropriate polyethylene glycol solution (PEG) in order to produce four levels of water potentials: 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. Polyethylene glycol is routinely used as a water stressor agent (Baskin & Baskin 2014). The required water potential was produced with PEG 6000 and the deionized water according to the formula $\Psi=0.130[PEG]^2T-13.7[PEG]^2$, in line with Michel & Kaufmann (1973) and additional adjustments made by Hardegree and 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 them from desiccating. 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 three 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 two halves and incubated in a 1% solution of 2,3,5-triphenyl tetrazolium chloride for 48 hours in dark conditions (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 by 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 characterized 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-tinted, seeds).

Data analyses

Final germination percentage, T_0, T_{50} and seed viability data were analysed by means of generalized 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 amongst 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 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, pairwise comparisons amongst treatments were performed using the Bonferroni correction. All statistical analyses were performed using the SPSS Statistics version 19.0 (SPSS, Chicago, IL, USA).

Hydrotime 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). Hydrotime analysis quantifies the speed of germination (θ_H), the stress tolerance of germination (Ψ_b) and the uniformity of germination ($\sigma_{\Psi b}$) (Bradford & Still 2004). θ_H is the hydrotime constant (MPa h) for the population, defined as $\theta_H = (\Psi - \Psi_b(g))t_g$ where Ψ 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, Ψ_b, is the minimum water potential permitting germination and the Ψ_b of individual seeds varies as a normal distribution in the population of seeds (Gummerson 1986; Bradford 1990) with a median Ψ_b (50) and the corresponding standard deviation $\sigma_{\Psi b}$ (50).

Values of θ_H, Ψ_b (50) and $\sigma_{\Psi b}$ (50) were determined using repeated probit regression analysis in order to align the time courses to the hydrotime model as described previously by Bradford (1990). Higher θ_H indicates a longer time needed for germination (MPa h) (i.e., slower germination). Lower (i.e., more negative) values of Ψ_b mean that seeds will germinate across a wider range of water potentials. Finally, higher values of $\sigma_{\Psi b}$ (50) indicate greater germination variability within the population.

Hydrotime analysis requires appropriate germination percentages over a range of Ψs, with high germination percentages at 0MPa and in at least one other level of Ψ. In the case of species with hard-coated seeds, germination percentages without fire cues were very low, which precluded hydrotime modelling. Differences in hydrotime parameters between species were analysed by univariate general linear models and, in the case of *E. arborea*, differences between seeds exposed or otherwise to fire cues were analysed by repeated measures general linear models.

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 by θ_H and Ψ_b (50). The water-balance characteristics at each site were characterized 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 º 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 square regression. The dependent variables were θ_H and Ψ_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. *E. arborea* was the species with the highest final germination, in seeds both exposed and non-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 amongst 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 greatest in seeds exposed to fire cues. Lastly, final germination in *E. arborea* was significantly affected by fire 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 amongst populations in *E. arborea* (eight to nine days for non-water-stressed seeds), and higher and more variable amongst populations in the other three species, with *C. villosa* being the most extreme (from five 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 for *C. villosa*.

The time in reaching 50% of the final germination (T_{50}) was significantly different amongst populations in all species except for *C. villosa*. Exposing the 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 for *C. salviifolius*, in which case such increase was only observable 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) amongst populations were also found for all species (Table 2, Table S4). In the case of *C. monspeliensis*, seed viability was not affected by any of the treatments, contrary to *C. villosa* and *C. salviifolius,* which were affected by both of them (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. *E. arborea* displayed an opposite pattern, showing a decrease in viability with water stress only in seeds non-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 variability amongst populations within a species in all four species (Table 3). Species differed in θ_H and $\Psi_b(50)$ amongst them ($F_{3,14}$=3.997, *P*=0.030; $F_{3,14}$=26.553, *P*<0.001, respectively). *E. arborea* showed the highest values of θ_H and the lowest (i.e., more negative) of Ψ_b (50). Posthoc analysis showed that θ_H in *E. arborea* was significantly different from *C. monspeliensis*, with the other two neither differing from these two species nor amongst themselves. On the other hand, posthoc analysis for $\Psi_b(50)$ indicated that *E. arborea* was significantly different from the other three species. Furthermore, θ_H and $\Psi_b(50)$ in *E. arborea* did not show significant difference amongst 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±4.1 days) and intensity means (254.5±20.4 mm) of the period of vulnerability were similar amongst species (Table 1). Germination sensitivity to water stress (θ_H and $\Psi_b(50)$) was not correlated to 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 θ_H and marginally correlated with $\Psi_b(50)$ (*P*=0.060) in seeds non-exposed to fire cues; but not so in exposed seeds (Table 4). As the duration of the period of vulnerability increased, θ_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 a high tolerance to water stress (Thanos & Skordilis 1987; Boydak *et al*. 2003), whereas shrub species of the Fabaceae have shown varied 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 been previously 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 (Köchy & Tielbörger 2007; Bochet *et al*. 2007; Pérez-Fernández *et al*. 2006). Comparisons amongst 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 amongst shrubs is more variable than in the other life-forms. Clearly, more studies are needed to properly characterize fire-prone Mediterranean species, and woody ones in particular.

In our study, germination sensitivity to water stress increased markedly in the hard-coated seeded 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 to water stress, which coincides with the results from Pérez-Fernández *et al*. (2006), as commented above. Other studies have observed the opposite effect (i.e., decreased sensitivity to water stress after exposure to fire cues) (Ghebrehiwot *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 of reducing the sensitivity to increasing water stress and enlarging the range of water potentials under which germination proceeds, as well also as increasing germination speed (Bradford & Still 2004; Ghebrehiwot *et al.* 2008; Thomas *et al.* 2010). However, as we have demonstrated, the three species with physical dormancy in our study exhibited the opposite, while *E. arborea* failed to support such contention.

The Cistaceae and Fabaceae are plant families widely spread 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 case, 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 the conditions for germination and delay the process, our results are contradictory with 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, which was not affected by fire cues. Germination in this species is not cued to fire (i.e., heat and smoke effects) (Mésleard & 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 minimal and not comparable to that in the hard-coated species. Regeneration after fire in this species relies on resprouting, not on seeds (Mésleard & 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 amongst populations in all four species across the Mediterranean Basin. Hydrotime analysis supported these findings, showing a significant variability of the hydrotime parameters amongst populations of a given species. High variability amongst populations in germination responses to water stress is widespread amongst 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 seeded species, we found that the variability amongst 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 attunement 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, year after 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 both 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 amongst 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 ought to be less sensitive to water stress. The pattern found also coincides with what has been reported 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 it happened in the other three species with hard-coated seeds. Despite the locations we chose are widespread 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 more firm conclusion.

Studies correlating local characteristics and seed traits and germination responses often report contrasting results amongst species, making generalizations difficult (Cochrane *et al.* 2015b). Our study concurs with this. Only on one occasion out of five (one in each of the hard-coated seeded species and two in *E. arborea*) did we find that local water-balance conditions during the period of vulnerability explained amongst-population variability across the sites investigated. For the other occasions, variability amongst 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 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 in it due to other relevant ecological factors (e.g., fire in our case) affecting germination, as no single population may 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 in the online version of this article:

Table S1. FG mean values (± SE) for seeds non-exposed to fire cues (heat shock + smoke) or exposed to them, with each species and population germinated at different water stress treatments.

Table S2. T_0 mean values (± SE) for seeds non-exposed to fire cues (heat shock + smoke) or exposed to them, with each species and population germinated at different water stress treatments. In some cases, there was no germination under the more stressful water conditions (see Table S1); thus, no data for T_0 is available.

Table S3. T_{50} mean values (± SE) for seeds non-exposed to fire cues (heat shock + smoke) or exposed to them, with each species and population germinated at different water stress treatments. In some cases, there was no germination under the more stressful water conditions (see Table S1); thus, no data for T_{50} is available.

Table S4. V mean values (± SE) for seeds non-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.

Fig. S1. T_0 (days), T_{50} (days), and V (%) mean values (±SE) for each species and water stress treatment, as well as for seeds non-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 amongst treatments was assessed. Therefore, different letters (lowercase for no fire cues, and uppercase for fire cues) show significant differences amongst water stress treatments from pairwise comparisons with the Bonferroni correction (*P*<0.05) after GLM analysis.

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. Climatic 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.csic.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 as being less intense than the historical trend and with 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)**
C. monspeliensis										
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
C. salviifolius										
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
C. villosa										
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	0.55	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
E. arborea										
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

Table 2. *P*-values from 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 the total germination) and seed viability (V). In the case of *C. villosa* T_{50}, populations were not significantly different and, consequently, a non-nested model with three factors was fitted (Table S7). More information about GLM results can be found in tables S5-S8 in the Supplementary material. Significant *P*-values are shown in bold (*P*<0.05).

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

Table 3. Hydrotime model parameters for each of the species and populations 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 non-exposed and exposed to fire cues.

		θ_H	$\psi_b(50)$	$\sigma_{\psi b}(50)$	r^2
C. monspeliensis					
	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
C. salviifolius					
	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
C. villosa					
	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
E. arborea					
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

Table 4. Correlation (r, *P*) between θ_H and Ψ_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 non-exposed and exposed to fire cues. Significant relationships are shown in bold ($P<0.05$).

		θ_H		$\psi_b(50)$	
		r	*P*	*r*	*P*
C. monspeliensis					
	Duration	-0.272	0.658	0.545	0.342
	Intensity	-0.221	0.721	0.492	0.399
C. salviifolius					
	Duration	0.073	0.891	0.397	0.436
	Intensity	0.07	0.895	0.612	0.197
C. villosa					
	Duration	-0.275	0.656	0.554	0.333
	Intensity	-0.222	0.719	0.497	0.395
E. arborea					
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	**0.043**	-0.937	0.063
	Intensity	0.822	0.178	-0.855	0.145

Fig. 1. Geographical 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 the specific locations sampled).

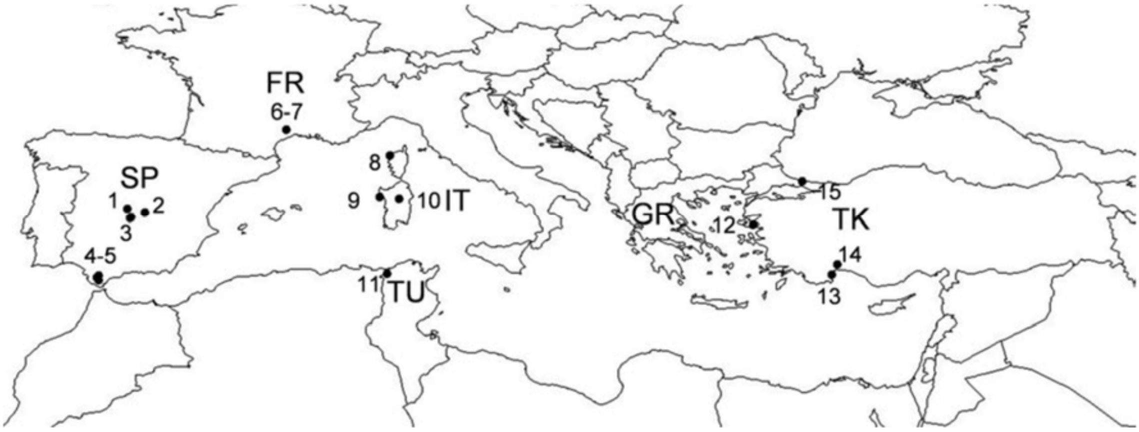

Fig. 2. Final germination percentage (FG) for each species and water stress treatment. Mean and standard errors are presented for seeds non-exposed to fire cues (heat shock + smoke) or exposed to them. When the interaction between water stress treatment and fire cues was statistically significant, differences amongst treatments was assessed. Therefore, different letters (lowercase for seeds non-exposed to fire cues, and uppercase for seeds exposed to them) show significant differences amongst water stress treatments from pairwise comparisons with the Bonferroni correction ($P<0.05$) after GLM analysis (see Table 2).

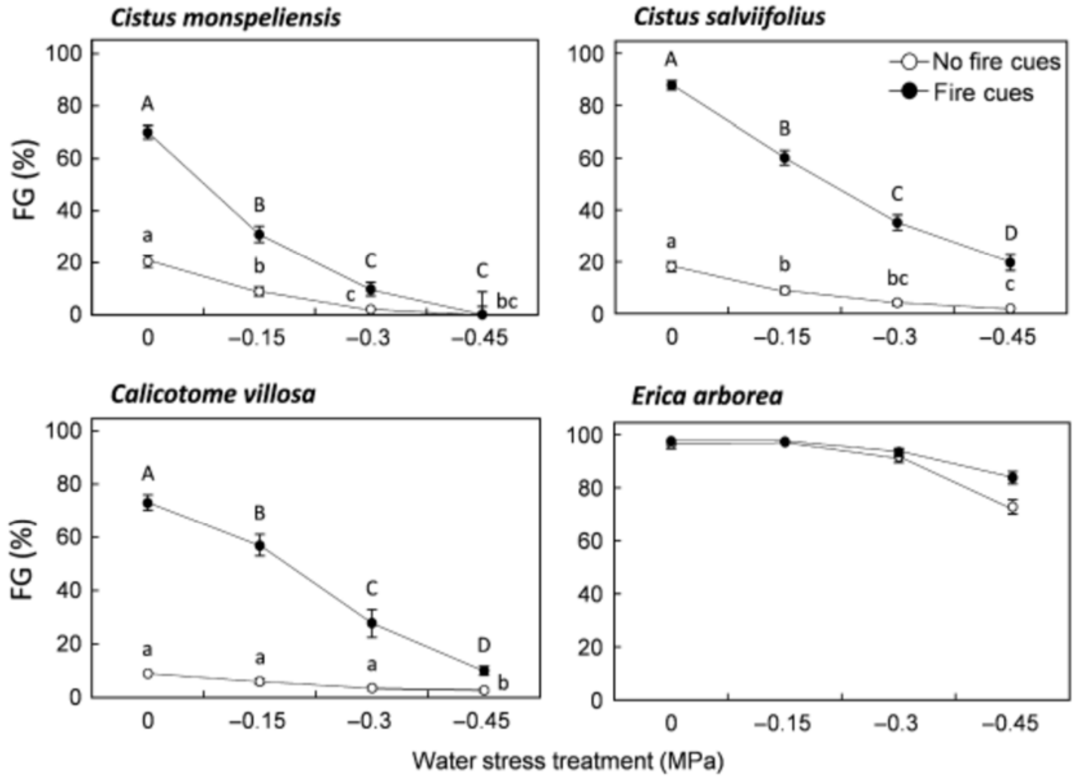

Fig. 3. Correlation between the duration of the period of vulnerability for germination and hydrotime parameters (θ_H and $\Psi_b(50)$) in non-exposed seeds of *E. arborea*.