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

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1   "Research Article" (post-print version)

2   **Germination sensitivity to water stress in four shrubby species across**

3   **the Mediterranean Basin**

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23   **ABSTRACT**

24   Mediterranean shrublands are generally water-limited and fire-driven ecosystems. Seed-

25   based post-fire regeneration may be affected by varying rainfall patterns, depending on

26   species' sensitivity to germinate under water stress. In our study, we considered the

27   germination response to water stress in four species from several sites across the

28   Mediterranean Basin. Seeds of species with hard-coated (*Cistus monspeliensis*, *C.

29   salviifolius* [Cistaceae], *Calicotome villosa* [Fabaceae]) or soft-coated (*Erica arborea*

30   [Ericaceae]) seeds, which were exposed or otherwise to a heat shock and smoke (fire

31   cues), were made to germinate under water stress. Final germination percentage,

32   germination speed and viability of seeds were recorded. Germination was modelled using

33   hydrotime analysis and correlated to the water-balance characteristics of seed

34   provenance. Water stress was found to decrease final germination in the three hard-seeded

35   species, as well as reduce germination speed. Moreover, an interaction between fire cues

36   and water stress was found, whereby fire cues increased the sensitivity to water stress.

37   Seed viability after germination under water stress also declined in two hard-seeded

38   species. Conversely, *E. arborea* showed little sensitivity to water stress, independent of

39   fire cues. Germination responses varied amongst populations of all species, and

40   hydrotime parameters were not correlated to site water-balance, except in *E. arborea*,

41   when not exposed to fire cues. In conclusion, the species studied differed in germination

42   sensitivity to water stress; furthermore, fire cues increased this sensitivity in the three

43   hard-seeded species, but not in *E. arborea*. Moreover, populations within species

44   consistently differed amongst themselves, but these differences could only be related to

45   the provenance locality in *E. arborea* in seeds not exposed to fire cues.

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

49

INTRODUCTION

51 The germination process is the beginning of the autonomous life of a plant, and is

52 controlled by water availability provided that suitable temperatures are present.

53 Germination starts with the imbibition of the seed, which prompts the initiation of

54 metabolic processes that will result in radicle elongation. The uptake of water is triphasic

55 (Finch-Savage & Leubner-Metzger 2006), while the length of each phase depends on

56 species and environmental conditions, although seed germination occurs only when seeds

57 hold 30-35% of water (Roberts & Ellis 1989). If water in the seed environment is limiting

58 water uptake may start, but the germination process cannot be concluded if all three

59 phases of imbibition are not completed. Water availability is, therefore, an important

60 limiting factor for germination, affecting the final germination percentage as well as the

61 rate and uniformity of emergence (Bewley & Black 1994).

62 Seeds in the field will be exposed to various water potentials depending on the

63 position in the soil profile, soil characteristics and weather conditions following a rainfall

64 event. Seeds in the soil may hydrate once a rain event occurs, after which seeds start to

65 dehydrate as the soil dries out until a new rain event eventually allows re-imbibition of

66 the seeds (Batlla & Benech-Arnold 2006). As the soil dries out, its water potential

67 declines, thereby potentially limiting germination (Koller & Hadas 1982; Downs &

68 Cavers 2000). Seeds at the soil surface or at the very upper soil layers are subjected to

69 significant fluctuations in water content, with more rapid wetting after the rain, but also

70 faster drying. In contrast, seeds buried deeper in the soil will not be moist until larger

71   amounts of rain have fallen. Once moistened, they will remain wet for a longer time due

72   to the exponential decay of the evaporation rate in relation to soil depth (Allen *et al*. 1998;

73   Xiao *et al*. 2011).

74   In Mediterranean areas, germination occurs after autumn rains once soils are

75   wetted (Espigares & Peco 1993; Céspedes *et al*. 2012). Duration and timing of the wet

76   season have important consequences for seed germination and recruitment, with delayed

77   and shorter wet seasons leading to lower final germination and lower richness and

78   diversity of species (Miranda *et al*. 2009; Céspedes *et al*. 2012; Jöet *et al*. 2012). In these

79   areas, fire occurs mainly during the summer dry season (Urbieta *et al*. 2015). In post-fire

80   environments, temporal germination patterns show great variability amongst species, and

81   are closely related to variations in rainfall (Quintana *et al.* 2004; Moreno *et al*. 2011).

82   Germination is highest during wet years, only occurring during the first post-fire year;

83   during dry years, however, germination is lower and extends over several years (Moreno

84   *et al*. 2011). Seeds that germinate in the second or later years post-fire have little chance

85   of establishing (Quintana *et al*. 2004; Moreno *et al*. 2011).

86   Germination response to gradients of water stress has been studied under

87   laboratory conditions by exposing seeds to polyethylene glycol (PEG), an inert, water-

88   binding polymer with a non-ionic impermeable long chain that properly simulates

89   drought stress under dry soil conditions. Many of these studies typically address the

90   response of a single species, with greater focus on species of agricultural interest, such as

91   crop species or weeds (Almansouri *et al*. 2001; Zhang *et al*. 2010). Few studies have

92   focused upon a larger number of species and, when they do, significant inter-specific

93   variation is often reported. Inter-specific variation to water stress has been related to

94   habitat characteristics (Evans & Etherington 1990; Sy *et al*. 2001; Schütz *et al*. 2002),

95   climate characteristics (Köchy & Tielbörger 2007) and life-history traits (Kos & Poschlod

96   2008), although generalizations are largely inconclusive. Similarly, research relating to

97   the intra-specific population variation of germination patterns to habitat characteristics

98   has not produced consistent results (Boydak *et al.* 2003; Raccuia *et al.* 2004; Tilki & Dirik

99   2007; Petrů & Tielbörger 2008; Atia *et al.* 2011; Cochrane *et al.* 2015a).

100   Fire plays a major role in Mediterranean ecosystems. After a fire event, many

101 species regenerate solely from seeds, which are most often stored in the soil seed bank

102 and resistant to high temperatures (Luna *et al.* 2007). Species with hard-coated seeds (i.e.,

103 with physical dormancy) are common, with dormancy-breaking being cued to fire (heat

104 with or without smoke) (Ne'eman *et al.* 2012). Many of these species dominate the

105 various types of shrubland in the region; these include species of the Cistaceae and the

106 woody shrubs of the Fabaceae. Other dominant species in shrublands on more mesic and

107 acidic substrates include the Ericaceae. Seeds in this family are soft-coated and can have

108 physiological dormancy, while the role of fire in promoting germination is less clear

109 (Mesléard & Lepart 1991; Crosti *et al.* 2006; Moreira *et al.* 2010). While the Cistaceae

110 shrubs are generally obligate seeders, shrubs in the Fabaceae and Ericaceae often also

111 resprout after fire.

112   Knowledge about seed germination sensitivity to water stress is of the utmost

113 importance in dry areas such as the Mediterranean region and other climate-type areas of

114 the world with similarly alternating periods of dry and wet soils. In these environments,

115 droughts are common and rainfall is highly variable from year to year; more so the lower

116 the rainfall (Lionello *et al.* 2006). This implies that after a fire event, when recruitment

117 of obligate seeders is most vulnerable, germination might proceed under reduced rainfall.

118 Additionally, in a context of changing climate, this situation can be even more critical. In

119 the Mediterranean region, global warming is projected to increase mean surface

120 temperatures more than the mean global average, and modify the precipitation regime

121 with a lengthened and more intense drought period during the year (Ruffault *et al.* 2014).

122 Rainfall is projected to be concentrated in the autumn and winter months with fewer, but

123 more intense, precipitation events (Giorgi & Lionello 2008), which is consistent with

124 recent observations (Bindoff *et al.* 2013). Plant establishment is expected to be affected

125 by limited water availability in addition to high temperatures. Notwithstanding, a small

126 number of studies have anticipated the likely impact of changes in climate, rainfall and

127 drought, in particular, upon germination (see revision by Walck *et al.* 2011).

128 In this study, we analysed the effects of water stress and fire-related cues (i.e.,

129 exposure of seeds to heat shock and smoke) on seed germination and viability of four

130 widespread woody species across the Mediterranean Basin. In so doing, we asked the

131 following questions: Do species differ in their germination sensitivity to water stress? Is

132 the response affected by exposing the seeds to fire cues? Do water stress response patterns

133 vary across the Mediterranean Basin? Are germination response patterns related to the

134 climate conditions of the locality of seed provenance?

135

136 **MATERIAL AND METHODS**

137 **Study species and seed collection**

138 Seeds of four typical Mediterranean shrubland woody species were collected from across

139 the Mediterranean Basin. These were *Cistus monspeliensis* L., *C. salviifolius* L.

140 (Cistaceae), *Calicotome villosa* (Poir.) Link. (Fabaceae) and *Erica arborea* L.

141 (Ericaceae). The first three species have hard-coated seeds (i.e., the seeds have physical

142 dormancy), while *E. arborea* has soft seeds (i.e., the seeds do not have physical

143 dormancy) (Baskin & Baskin 2014). Seeds were collected on ripening in the summer of

144 2010 (from July to August) from at least 20 plants per site, in order to make a single

145   species-site sample, at sites spanning 3,237 km, in Spain, France, Tunisia, Italy, Greece

146   and Turkey (Fig. 1; Table 1). To avoid spurious effects due to selecting sites within a

147   close distance that might have high intraspecific variability (Moreira *et al.* 2012), the

148   minimum distance between sites was 387 km, such that climate- and other fire-related

149   pressures would be unique to each site. Seeds were stored in paper bags, at room

150   temperature, until the germination experiments began in January of the following year.

151

152   **Germination experiments**

153   In fire-prone environments, germination has been shown to be triggered by both

154   heat and smoke (Keeley & Fotheringham 2000). Smoke cannot stimulate the germination

155   of seeds with an impermeable coat until this has been broken by fire or other scarifying

156   agent (Moreira *et al.* 2010). Prior to incubation, half of the seeds were heated at 100 ºC

157   for 10 minutes, which is a common temperature and timeframe in shrubland fires (Moreno

158   *et al.* 2011; Céspedes *et al.* 2012), and then exposed to smoke for 20 minutes, in order to

159   simulate the effects of fire. Seeds were heated in an electric oven. Smoke was produced

160   by burning a mixture of fine fuel from several species, including *Cistus* spp. and *E.*

161   *arborea*. Smoke was continuously funnelled for 20 minutes through a box containing the

162   seeds laid out in trays. Seeds were then incubated at 20 ºC with a photoperiod of 12/12 h

163   for 60 days in plastic Petri dishes (5.5 cm in diameter) over two filter papers (Whatman

164   no. 1). Seeds were germinated under different levels of water stress by moistening the

165   Petri dishes with either 1.2 ml of deionized water or the appropriate polyethylene glycol

166   solution (PEG) in order to produce four levels of water potentials: 0, -0.15, -0.30 and -

167   0.45 MPa. Filter papers were replaced weekly and the corresponding PEG solution added

168   to avoid changes in the germinating solution. Polyethylene glycol is routinely used as a

169   water stressor agent (Baskin & Baskin 2014). The required water potential was produced

170   with PEG 6000 and the deionized water according to the formula $\Psi=0.130[PEG]^2T-$

171    $13.7[PEG]^2$, in line with Michel & Kaufmann (1973) and additional adjustments made by

172   Hardegree and Emmerich (1990). Six replicates of 25 seeds per species and site were used

173   in each of the treatments. All Petri dishes were sealed with Parafilm in order to prevent

174   them from desiccating. Petri dishes were placed at random on the plate of a temperature-

175   and humidity-controlled chamber (Model G-21, Ibercex). Germination was recorded

176   every day for the first 30 days and every three days until the end of the experiment, with

177   radicle emergence used as the criterion for scoring a seed as germinated. When the

178   experiment ended, the viability of each non-germinated seed was checked using the

179   tetrazolium test for Cistaceae and Fabaceae. The tetrazolium test was undertaken after the

180   seeds were cut into two halves and incubated in a 1% solution of 2,3,5-triphenyl

181   tetrazolium chloride for 48 hours in dark conditions (Moore 1985). Given their small size,

182   the tetrazolium test could not be conducted with *E. arborea* seeds. Instead, in the case of

183   *E. arborea*, a 1% solution of gibberellic acid (GA_3) was added to non-germinated seeds,

184   until germination was completed. Seeds that were infected by fungi were considered non-

185   viable.

186   Four variables were obtained: final germination percentage at the end of the

187   experiment corrected by viability (FG) (i.e., germination percentages were estimated in

188   relation to viable seeds and not in relation to the total number of seeds), germination speed

189   characterized by the time to initiate germination (T_0) (i.e., the time until the first seed

190   germinated) and the time to produce 50% of the total germination obtained (T_{50}), and,

191   finally, seed viability (V) (viability percentages were assessed by considering germinated

192   seeds plus non-germinated, but tetrazolium-tinted, seeds).

193

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

219   distribution in the population of seeds (Gummerson 1986; Bradford 1990) with a median

220   Ψ_b (50) and the corresponding standard deviation $\sigma_{\Psi b}$ (50).

221   Values of θ_H, Ψ_b (50) and $\sigma_{\Psi b}$ (50) were determined using repeated probit

222   regression analysis in order to align the time courses to the hydrotime model as described

223   previously by Bradford (1990). Higher θ_H indicates a longer time needed for germination

224   (MPa h) (i.e., slower germination). Lower (i.e., more negative) values of Ψ_b mean that

225   seeds will germinate across a wider range of water potentials. Finally, higher values of

226    $\sigma_{\Psi b}$ (50) indicate greater germination variability within the population.

227   Hydrotime analysis requires appropriate germination percentages over a range of

228   Ψs, with high germination percentages at 0MPa and in at least one other level of Ψ. In the

229   case of species with hard-coated seeds, germination percentages without fire cues were

230   very low, which precluded hydrotime modelling. Differences in hydrotime parameters

231   between species were analysed by univariate general linear models and, in the case of *E.*

232   *arborea*, differences between seeds exposed or otherwise to fire cues were analysed by

233   repeated measures general linear models.

234   We were interested in determining the relationship between germination

235   sensitivity to water stress and the local water-balance characteristics at the sites where

236   seeds were harvested. The germination sensitivity to water stress was described by θ_H and

237   Ψ_b (50). The water-balance characteristics at each site were characterized by what we

238   called the "period of vulnerability" for germination. This period of vulnerability was

239   determined from a daily water-balance, which was calculated as the difference between

240   precipitation and potential evapotranspiration (P-PET). The daily potential

241   evapotranspiration was calculated in line with the FAO-56 Hargreaves equation (Allen *et*

242   *al.* 1998), and the daily temperature and precipitation data were obtained for the climate

243   reference period 1961-1990 from the WATCH climate dataset at 0.5 º latitude/longitude

244   resolution (http://www.eu-watch.org/). Mean water-balance was calculated for each day

245   and then applied to a quadratic model. The period of vulnerability was defined as the

246   period between the time when the water-balance was at its minimum (i.e., maximum

247   drought) and when it became continuously positive, and described with the following two

248   variables: duration (number of days between the height of drought and continuous

249   wetness; i.e., positive water-balance) and intensity (cumulative water-balance throughout

250   the days of the period of vulnerability). Overall, the period of vulnerability is expected to

251   occur following seed dispersal during the dry season (i.e., from mid-summer), when water

252   stress is maximum, until early autumn, depending on patterns of seasonal rain. The

253   relationship between germination sensitivity to water stress and both variables of the

254   period of vulnerability was determined by least square regression. The dependent

255   variables were θ_H and Ψ_b, while the duration and intensity of the period of vulnerability

256   were the independent variables.

257

RESULTS

259   The four species differed in their germination responses to the treatments. *E. arborea* was

260   the species with the highest final germination, in seeds both exposed and non-exposed to

261   fire cues (Table S1). Germination of the other species was increased considerably by fire

262   cues, with *C. monspeliensis* being the species with the lowest final germination values.

263   Final germination was significantly different amongst populations in all species (Table

264   2). Species with hard-coated seeds were significantly affected by both fire cues and water

265   stress (Table 2). Final germination increased with fire cues and decreased with water

266   stress. Moreover, a significant interaction between these two factors emerged (Table 2;

267   Fig. 2), whereby the negative effect of water stress was greatest in seeds exposed to fire

268   cues. Lastly, final germination in *E. arborea* was significantly affected by fire cues, albeit

269   with a minor positive effect, and not significantly affected by water stress. No interaction

270   between the two treatments was ascertained (Table 2).

271   Time to initiate germination (T_0) in seeds not treated with fire cues was lowest

272   and significantly homogeneous amongst populations in *E. arborea* (eight to nine days for

273   non-water-stressed seeds), and higher and more variable amongst populations in the other

274   three species, with *C. villosa* being the most extreme (from five to 25 days in the non-

275   water-stressed seeds) (Table S2). Population was a significant factor in all four species.

276   T_0 generally decreased in seeds exposed to fire cues, with significant effects observed in

277   *C. salviifolius* and *C. villosa*. Water stress significantly increased T_0 in all species except

278   for *C. villosa*.

279   The time in reaching 50% of the final germination (T_{50}) was significantly different

280   amongst populations in all species except for *C. villosa*. Exposing the seeds to fire cues

281   significantly increased T_{50} in hard-coated species, but was unchanged in *E. arborea*.

282   Water stress increased T_{50} in all species except for *C. salviifolius*, in which case such

283   increase was only observable in seeds that had previously been exposed to fire cues (Table

284   2, Fig. S1). Fire cues and water stress interaction was also significant in *C. monspeliensis*

285   (Table 2).

286   Significant differences in seed viability (V) amongst populations were also found

287   for all species (Table 2, Table S4). In the case of *C. monspeliensis*, seed viability was not

288   affected by any of the treatments, contrary to *C. villosa* and *C. salviifolius,* which were

289   affected by both of them (Table 2). Viability of *E. arborea* seeds was only affected by

290   fire cues treatment (Table 2). Additionally, an interaction between fire cues and water

291   stress treatments emerged for *C. salviifolius* and *E. arborea*. Contrary to *E. arborea*, seed

292   viability of *C. salviifolius* was not affected by water stress in the absence of fire cues,

293   although seed viability decreased with increased water stress after exposure to fire cues.

294   *E. arborea* displayed an opposite pattern, showing a decrease in viability with water stress

295   only in seeds non-exposed to fire cues (Fig. S1).

296   The hydrotime model was generally compatible with the timeline of germination

297   (with r^2 values ranging from 0.69 to 0.95) (Table 3). Overall, hydrotime parameters

298   showed great variability amongst populations within a species in all four species (Table

299   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,

300   *P*<0.001, respectively). *E. arborea* showed the highest values of θ_H and the lowest (i.e.,

301   more negative) of Ψ_b (50). Posthoc analysis showed that θ_H in *E. arborea* was

302   significantly different from *C. monspeliensis*, with the other two neither differing from

303   these two species nor amongst themselves. On the other hand, posthoc analysis for $\Psi_b(50)$

304   indicated that *E. arborea* was significantly different from the other three species.

305   Furthermore, θ_H and $\Psi_b(50)$ in *E. arborea* did not show significant difference amongst

306   seeds exposed or otherwise to fire cues ($F_{1,3}$=0.198, *P*=0.686; $F_{1,3}$=1.166, *P*=0.359,

307   respectively).

308   The duration (111.1±4.1 days) and intensity means (254.5±20.4 mm) of the period

309   of vulnerability were similar amongst species (Table 1). Germination sensitivity to water

310   stress (θ_H and $\Psi_b(50)$) was not correlated to either the duration or intensity of the period

311   of vulnerability for the species with hard-coated seeds (Table 4). In the case of *E. arborea*,

312   however, the duration of the period of vulnerability was significantly correlated with θ_H

313   and marginally correlated with $\Psi_b(50)$ (*P*=0.060) in seeds non-exposed to fire cues; but

314   not so in exposed seeds (Table 4). As the duration of the period of vulnerability increased,

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

316

317   **DISCUSSION**

318   Few studies have addressed the effects of water stress on germination in

319   Mediterranean species. Conifers have shown a high tolerance to water stress (Thanos &

320   Skordilis 1987; Boydak *et al*. 2003), whereas shrub species of the Fabaceae have shown

321   varied responses, from high (e.g., *Antyllis cytisoides*) (Ibáñez & Passera 1997) to low

322   tolerance (e.g., *Genista scorpius*) (Bochet *et al*. 2007). In these aforementioned studies,

323   seeds had been previously scarified although no fire cues were involved. Seeds of other

324   *Cistus* species that were neither scarified nor exposed to fire cues showed moderate

325   tolerance to water stress (Pérez-Fernández *et al*. 2006). Annual species, including hard-

326   seeded Fabaceae, previously scarified but not exposed to fire cues showed high tolerance

327   to water stress (Köchy & Tielbörger 2007; Bochet *et al*. 2007; Pérez-Fernández *et al*.

328   2006). Comparisons amongst life-forms are difficult given the limited number of species

329   studied, and the fact that seeds were not always exposed to fire cues. Nevertheless, it

330   appears that sensitivity to water stress amongst shrubs is more variable than in the other

331   life-forms. Clearly, more studies are needed to properly characterize fire-prone

332   Mediterranean species, and woody ones in particular.

333   In our study, germination sensitivity to water stress increased markedly in the

334   hard-coated seeded species after exposing the seeds to fire cues, as demonstrated by the

335   consistently significant interactions between fire cues and water stress treatments. The

336   pattern of response was consistent in the three species investigated. This observation

337   concurs with the fact that the non-dormant fraction in these species is much less sensitive

338   to water stress, which coincides with the results from Pérez-Fernández *et al*. (2006), as

339 commented above. Other studies have observed the opposite effect (i.e., decreased

340 sensitivity to water stress after exposure to fire cues) (Ghebrehiwot *et al.* 2008; Thomas

341 *et al.* 2010); but, in these studies, the species investigated did not have physical dormancy.

342 That said, lack of physical dormancy, as in *E. arborea* in our case, did not imply

343 significant changes in sensitivity to water stress as a result of being exposed to fire cues.

344 It has been argued that karrikins from smoke and, in general, factors that promote

345 germination have the ability of reducing the sensitivity to increasing water stress and

346 enlarging the range of water potentials under which germination proceeds, as well also as

347 increasing germination speed (Bradford & Still 2004; Ghebrehiwot *et al*. 2008; Thomas

348 *et al.* 2010). However, as we have demonstrated, the three species with physical dormancy

349 in our study exhibited the opposite, while *E. arborea* failed to support such contention.

350   The Cistaceae and Fabaceae are plant families widely spread across the

351 Mediterranean Basin, dominating many shrublands in the region (Tomaselli 1981). While

352 they occupy a variety of habitats, they are abundant in dry and warm environments. From

353 an ecological viewpoint, we expected that they would be highly tolerant to water stress,

354 but this was not supported by our study. Additionally, contrary to our expectations, fire

355 cues increased germination sensitivity to water stress. Hard-coated seeds in

356 Mediterranean shrublands, notably in Cistaceae and Fabaceae, usually form persistent

357 soil seed banks that produce a flush of seedlings once dormancy is released by the passage

358 of fire (Trabaud 1994; De Luis *et al*. 2005). Fire temporarily produces a competitor-free

359 environment where success is often contingent on being the first to germinate, establish

360 and develop. It has been argued that species adapted to fire should reduce variability in

361 the timing of germination, such that they would germinate as early as possible in order to

362 increase fitness (Verdú & Traveset 2005; De Luis *et al.* 2008). We found, however, that

363 germination was tightly related to low water stress, notably after seeds were exposed to

364   fire cues. In such case, water stress reduced germination speed in *Cistus*. This limits the

365   opportunities for rapid germination and early establishment after fire. Having hard seeds

366   indicates a fire-adaptive trait that may have different origins; in some species, physical

367   dormancy may have evolved in response to fire, whereas in other species, it could have

368   originated in response to other selective pressures that became useful in fire-prone

369   habitats (Keeley *et al.* 2011). Provided the mismatch between what appears advantageous

370   after fire to ensure prompt germination and early establishment, and the fact that fire cues

371   restrict the conditions for germination and delay the process, our results are contradictory

372   with a fire-driven selection process behind hard-coated seeds in these shrubs.

373   In contrast to the other species investigated, *E. arborea* showed little sensitivity

374   to water stress, which was not affected by fire cues. Germination in this species is not

375   cued to fire (i.e., heat and smoke effects) (Mésleard & Lepart 1991; Valbuena & Vera

376   2002; Crosti *et al.* 2006). While we found that the germination of *E. arborea* was

377   significantly affected by fire cues, the absolute magnitude of this increment was minimal

378   and not comparable to that in the hard-coated species. Regeneration after fire in this

379   species relies on resprouting, not on seeds (Mésleard & Lepart 1991); thus, ecological

380   implications of changes in germination for population persistence would be smaller than

381   in seeder species.

382   Germination responses to water stress varied amongst populations in all four

383   species across the Mediterranean Basin. Hydrotime analysis supported these findings,

384   showing a significant variability of the hydrotime parameters amongst populations of a

385   given species. High variability amongst populations in germination responses to water

386   stress is widespread amongst species within the region and in other regions of the world

387   (Raccuia *et al.* 2004; Khera & Singh 2005; Tilki & Dirik 2007; Li & Feng 2009; Cochrane

388   *et al.* 2015a). In the case of hard-coated seeded species, we found that the variability

389   amongst populations in germination sensitivity to water stress of seeds exposed to fire

390   cues was not correlated to the period of vulnerability at the sites of seed provenance. In

391   other words, germination after fire at the various sites would have occurred without

392   attunement to the local environment (i.e., the climate of the period of vulnerability in our

393   case). The hydrotime model could not be calculated for the non-dormant fraction. These

394   seeds may germinate readily, year after year, and not in pulses as in post-fire

395   environments, thus environmental pressures would likely be stronger on them. Given the

396   different responses to water stress between both fractions, we cannot extrapolate the

397   results from one to the other. Therefore, whether population differences in the readily

398   germinable fraction correlate to local climate conditions remains unresolved.

399   In the case of *E. arborea,* the variability in germination sensitivity to water stress

400   amongst populations was correlated to the duration of the period of vulnerability for

401   germination, and for the intensity of this period to some extent. Seeds from sites with

402   longer periods of vulnerability were able to germinate under lower water potentials and

403   indicated higher hydrotime values (more MPa hours) than those of sites with shorter

404   periods (i.e., less dry). This pattern of response would concur with expectations that

405   populations from dry provenances ought to be less sensitive to water stress. The pattern

406   found also coincides with what has been reported for coniferous Mediterranean species

407   (Fady 1992; Boydak *et al*. 2003). Exposing the seeds to fire cues, however, rendered these

408   relationships non-significant. In other words, seeds of the various populations once

409   treated with fire cues germinated irrespective of their local climate conditions, much as it

410   happened in the other three species with hard-coated seeds. Despite the locations we

411   chose are widespread throughout the Mediterranean region, and covered a significant

412   range of temperature and precipitation conditions, their limited number obliges us to be

413   cautious before reaching a more firm conclusion.

414   Studies correlating local characteristics and seed traits and germination responses

415   often report contrasting results amongst species, making generalizations difficult

416   (Cochrane *et al.* 2015b). Our study concurs with this. Only on one occasion out of five

417   (one in each of the hard-coated seeded species and two in *E. arborea*) did we find that

418   local water-balance conditions during the period of vulnerability explained amongst-

419   population variability across the sites investigated. For the other occasions, variability

420   amongst populations was the norm, but this could not be explained by local correlates.

421   This implies that anticipating future threats to species' persistence across their

422   distribution range, as a result of changing climate due to global warming, will be

423   complicated. Models addressing the response of a given species to a change in climate

424   (e.g., Pearson *et al*. 2014) need to consider not only intraspecific variability, but also

425   variations in it due to other relevant ecological factors (e.g., fire in our case) affecting

426   germination, as no single population may fully capture the species' response throughout

427   its distribution range. A caveat in this work is that consideration of the relationship with

428   the local climate only involved climate parameters. This may provide a base-reference

429   for seeds at the surface, but may not do so for seeds buried in the soil, which will affect

430   moistening-desiccation patterns (Schütz *et al*. 2002). The inclusion of soil seed depth and

431   sensitivity to varying rainfall patterns was beyond the scope of this study.

432

439

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629   regulator. *Annals of Botany*, **106**, 1027-1035.

630

631 **SUPPORTING INFORMATION**

632 Additional supporting information may be found in the online version of this article:

633   **Table S1.** FG mean values ($\pm$ SE) for seeds non-exposed to fire cues (heat shock +

634   smoke) or exposed to them, with each species and population germinated at different

635   water stress treatments.

636   **Table S2.** T_0 mean values ($\pm$ SE) for seeds non-exposed to fire cues (heat shock +

637   smoke) or exposed to them, with each species and population germinated at different

638   water stress treatments. In some cases, there was no germination under the more

639   stressful water conditions (see Table S1); thus, no data for T_0 is available.

640   **Table S3.** T_{50} mean values ($\pm$ SE) for seeds non-exposed to fire cues (heat shock +

641   smoke) or exposed to them, with each species and population germinated at different

642   water stress treatments. In some cases, there was no germination under the more

643   stressful water conditions (see Table S1); thus, no data for T_{50} is available.

644   **Table S4.** V mean values ($\pm$ SE) for seeds non-exposed to fire cues (heat shock +

645   smoke) or exposed to them, with each species and population germinated under

646   different water stress treatments.

647   **Table S5.** Complete results from GLM for effects of population, fire cues and water

648   stress treatments on FG, T_0, T_{50} and V.

649   **Fig. S1.** T_0 (days), T_{50} (days), and V (%) mean values ($\pm$SE) for each species and water

650   stress treatment, as well as for seeds non-exposed to fire cues (heat shock + smoke) or

651   exposed to them. When the interaction between water stress treatment and fire cues was

652   statistically significant, differences amongst treatments was assessed. Therefore,

653   different letters (lowercase for no fire cues, and uppercase for fire cues) show

654   significant differences amongst water stress treatments from pairwise comparisons with

655   the Bonferroni correction ($P<0.05$) after GLM analysis.

656

657 **Table 1.** Characteristics of locations where seeds were harvested. Population number

658 (Pop) refers to Fig. 1. Code refers to the first initials of each country and, in the case of

659 Spain, we used additional coding related to the geographic position (C: centre refers to

660 populations 1-3; S: south refers to populations 4-5) since several populations were

661 studied. Climatic data was obtained for the climate reference period 1961-1990 from the

662 WATCH climate dataset (http://www.eu-watch.org). Duration (days) and intensity

663 (mm) of the period of vulnerability (i.e., the period between maximum drought until

664 continuous positive water balance) for germination are indicated for each population.

665 Additionally, the Standardised Precipitation-Evapotranspiration Index (SPEI 10)

666 (http://sac.csic.es/spei/index.html) is provided for 2010 when seeds were harvested.

667 This is a drought index based on the climatic water balance (P-PET), with positive

668 values indicating drought as being less intense than the historical trend and with

669 negative values being the opposite (Beguería *et al.* 2014).

670

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

671

672 **Table 2.** *P*-values from GLM for effects of population (P), fire cues (Fc) and water stress

673 (Ws) nested within population on final germination (FG), T_0 (time to initiate

674 germination), T_{50} (time to reach 50% of the total germination) and seed viability (V). In

675 the case of *C. villosa* T_{50}, populations were not significantly different and, consequently,

676 a non-nested model with three factors was fitted (Table S7). More information about

677 GLM results can be found in tables S5-S8 in the Supplementary material. Significant *P*-

678 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**

679

680　**Table 3.** Hydrotime model parameters for each of the species and populations studied. In

681　the case of species with hard-coated seeds, hydrotime analyses were only possible for

682　seeds exposed to fire cues (heat shock + smoke). In the case of *E. arborea*, hydrotime

683　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

684

685 **Table 4.** Correlation (r, *P*) between θ_H and Ψ_b (50) and the period of vulnerability

686 (duration and intensity) for four shrubs studied across the Mediterranean Basin. For

687 species with hard-coated seeds, correlation is shown for seeds exposed to fire cues,

688 whereas, in the case of *E. arborea*, correlations were made for seeds both non-exposed

689 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

690

691

692 **Fig. 1.** Geographical locations from which seeds were collected. Seeds were harvested

693 from six countries (Spain, France, Italy, Tunisia, Greece and Turkey) and 15 populations

694 across the Mediterranean Basin (See Table 1 for further details on the specific locations

695 sampled).
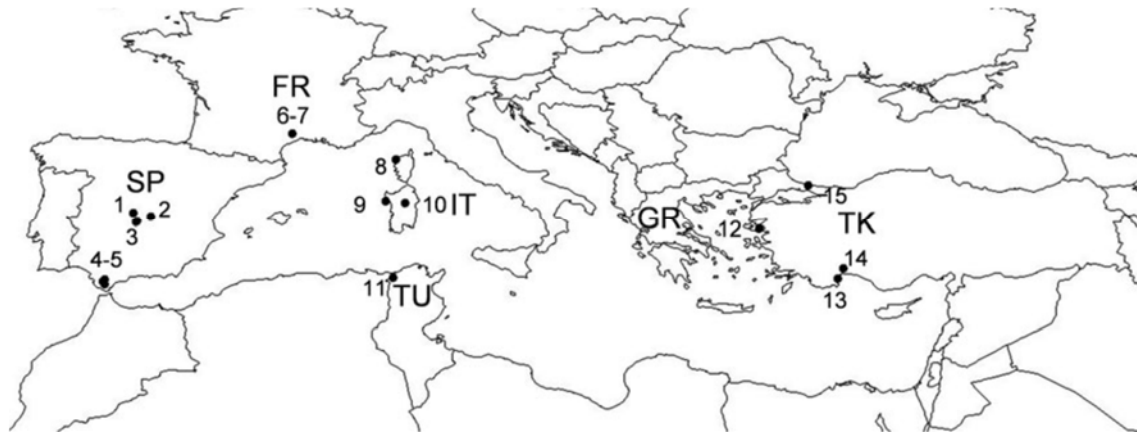
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700 **Fig. 2.** Final germination percentage (FG) for each species and water stress treatment.

701 Mean and standard errors are presented for seeds non-exposed to fire cues (heat shock +

702 smoke) or exposed to them. When the interaction between water stress treatment and fire

703 cues was statistically significant, differences amongst treatments was assessed. Therefore,

704 different letters (lowercase for seeds non-exposed to fire cues, and uppercase for seeds

705 exposed to them) show significant differences amongst water stress treatments from

706 pairwise comparisons with the Bonferroni correction ($P<0.05$) after GLM analysis (see
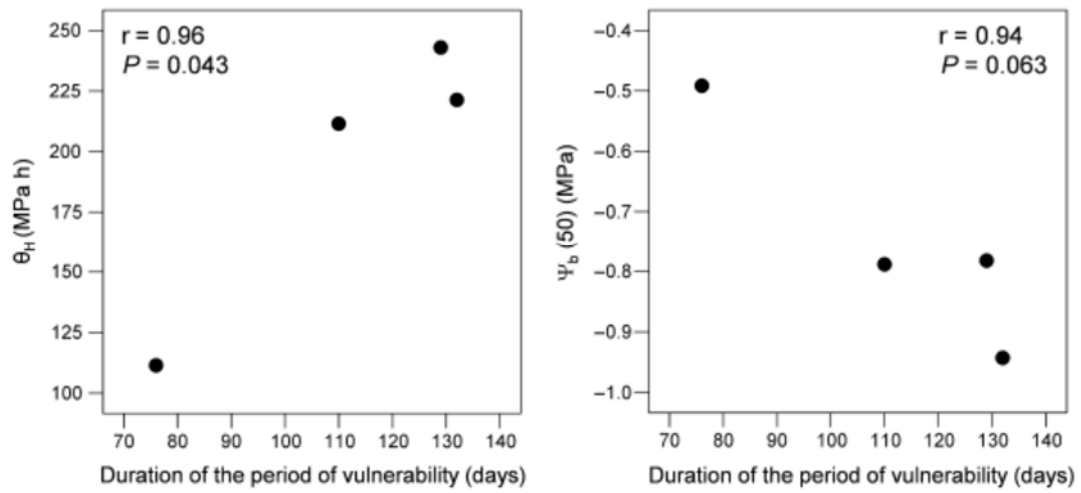
707 Table 2).
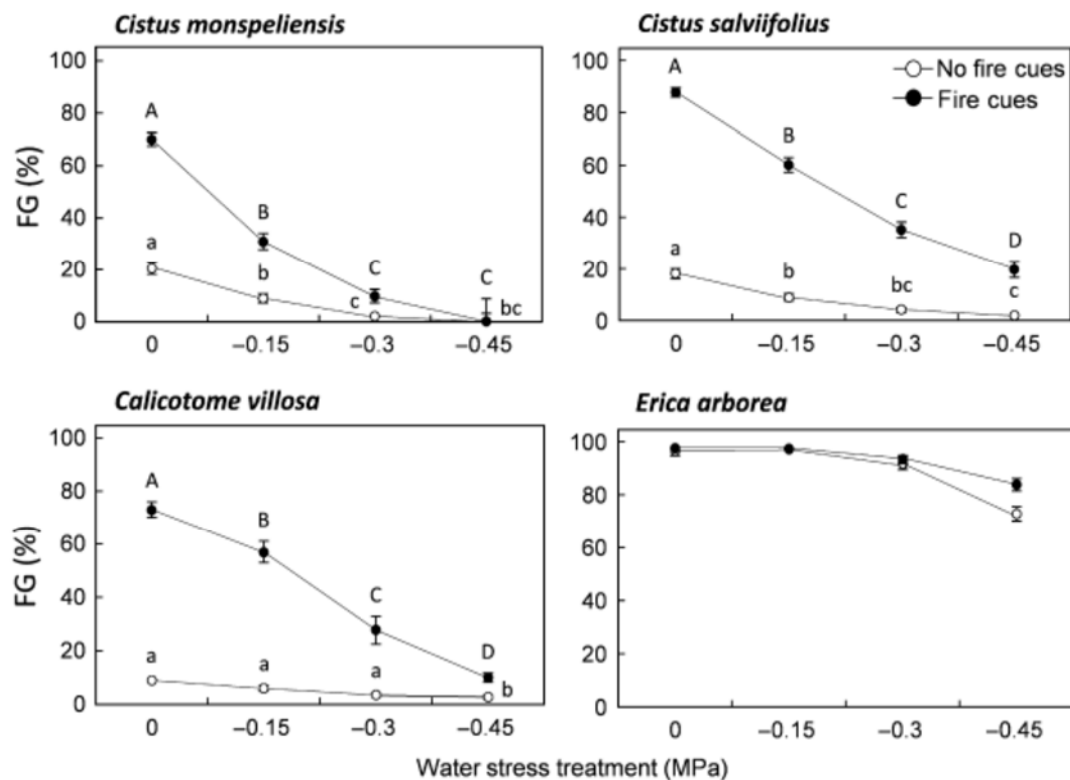
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710

711 **Fig. 3.** Correlation between the duration of the period of vulnerability for germination

712 and hydrotime parameters (θ_H and Ψ_b (50)) in non-exposed seeds of *E. arborea*.

713

714