

Multiple fire-related cues stimulate germination in *Chaenorhinum rubrifolium* (Plantaginaceae), a rare annual in the Mediterranean Basin

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Abstract

In Mediterranean fire-prone ecosystems, annual species specific to post-fire habitats should have a soil seed bank and should be able to germinate after a fire. Therefore, various fire-related cues can be expected to stimulate germination in post-fire annuals. Germination patterns of the rare annual *Chaenorhinum rubrifolium* (Plantaginaceae) were examined in response to mechanical scarification, heat shock, aqueous smoke, nitrogenous compounds, gibberellic acid, karrikinolide (KAR₁), and mandelonitrile (a cyanohydrin analogue, MAN) under dark and photoperiod conditions in the laboratory. Combinations of these treatments were also included in the experiment. Strong physiological dormancy in the seeds of *C. rubrifolium* was partially broken by several fire-related germination cues, including smoke and nitrate, under light conditions. KAR₁ and MAN also stimulated germination, and the highest improvement in germination was achieved in the KAR₁ treatment in the presence of light. Heat shock + smoke and KAR₁ + MAN combinations had positive synergetic and additive effects on germination under light conditions, respectively. The light played a crucial role in the promotion of germination. The results suggest that multiple fire-related cues operate to stimulate germination in *C. rubrifolium*, an annual species from the Mediterranean Basin. However, the species may have a broader germination niche than a fire-restricted one.

Keywords: annual plants, *Chaenorhinum rubrifolium*, fire, germination, karrikinolide, Mediterranean Basin, smoke

Introduction

Mediterranean fire-prone ecosystems harbour many plant species whose life cycle completely depends on fire. These species have developed a variety of mechanisms to persist in frequent fires, such as fire-stimulated germination, serotiny, and fire-stimulated flowering (Keeley *et al.*, 2012). For perennial species, having a fire-resistant seed bank and loss of resprouting ability are the key events in their evolutionary history of fire dependency (Pausas and Keeley, 2014). For annual species, the co-existence of three abilities is crucial for the population to survive a fire: the presence of a permanent seed bank in the soil (Traba *et al.*, 2004), the resistance of seeds to the temperatures in the seed bank caused by fire (Tavşanoğlu *et al.*, 2015), and post-fire stimulation of germination (Keeley and Bond, 1997; Keeley and Fotheringham, 1998b).

Post-fire annuals are a significant component of early post-fire communities in Mediterranean-type ecosystems of California and the Mediterranean Basin (Kazanis and Arianoutsou, 2004; Keeley *et al.*, 2005; Kavgacı *et al.*, 2010; Keeley *et al.*, 2012; Tormo *et al.*, 2014), but exhibited relatively less importance in south-western Australia and the Cape region (Cowling *et al.*, 1996; Keeley *et al.*, 2012). Physiological studies showed that high temperatures and the chemicals found in smoke produced from burning plant material during a fire are responsible cues for post-fire stimulation of germination in annual plants (Keeley and Bond, 1997; Keeley and Fotheringham, 1998b; Downes *et al.*, 2010). Typically, the species belonging to plant families in which seeds express physical dormancy (e.g. Fabaceae, Cistaceae, Rhamnaceae) use higher temperatures in the soil seed bank during a fire as a cue for subsequent germination (Moreira *et al.*, 2010) because the heat shock causes structural changes in the hard seed coat (Baskin and Baskin, 2014). Chemicals produced by burning plant material, on the other hand, stimulate

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germination in species in which seeds do not express physical dormancy. This is observed in a variety of families across the phylogenetic spectrum worldwide (Pausas and Keeley, 2009), but so far documented in only a few specific families in Mediterranean-type ecosystems, such as Lamiaceae, Ericaceae and Poaceae (Brown, 1993; Moreira *et al.*, 2010; Keeley *et al.*, 2012; Çatav *et al.*, 2014). Field observations suggest that smoke enhances the establishment of seedlings of annual species to a greater extent than those of perennials (Tormo *et al.*, 2014). In addition to smoke, however, germination stimulation in annual species may be caused by nitrogenous compounds (Thanos and Rundel, 1995; Luna and Moreno, 2009; Çatav *et al.*, 2015), which are considered another cue in early post-fire environments (Thanos and Rundel, 1995).

In the past, fire-related germination capacity of the Mediterranean Basin species was underestimated in comparison with other Mediterranean-type ecosystems (e.g. Keeley and Fotheringham, 2000), until supporting evidence on germination stimulation by smoke was reported in recent years (e.g. Pérez-Fernández and Rodríguez-Echeverría, 2003; Moreira *et al.*, 2010; Çatav *et al.*, 2014). However, these studies primarily include woody and herbaceous perennial species, and there is a lack of information regarding the germination behaviour of annuals in relation to fire in the Mediterranean Basin (Paula *et al.*, 2009; Moreira and Pausas, 2016). To our best knowledge, moreover, the role of chemicals in smoke (such as karrikins and cyanohydrins) in promoting seed germination of native plants has only been studied in Mediterranean-type ecosystems of California, the Cape region, and south-western Australia (Merritt *et al.*, 2006; Dixon *et al.*, 2009; Flematti *et al.*, 2011; Long *et al.*, 2011; Downes *et al.*, 2013; Downes *et al.*, 2014); however, the effect of specific smoke chemicals on germination has not yet been tested for any plant species in the Mediterranean Basin. Furthermore, there remains a need for research on the physiological and ecological roles of such compounds in smoke (Keeley *et al.*, 2012).

In this study, our goal was to increase our knowledge regarding the germination niche of annual species in relation to fire in the Mediterranean Basin. To accomplish this goal, we examined the effects of various fire-related cues including more general signals in post-fire environments (heat shock, smoke, nitrogenous compounds, light, and specific smoke chemicals) on the germination of a rare annual in the Mediterranean Basin. Because we observed that the studied species was restricted to a burned site (see Materials and methods), we expected to detect stimulation of germination in some of the treatments we applied. We also hypothesized that combinations of these cues might have a synergistic or antagonistic effect on the germination of our study species because many fire-related germination cues co-occur simultaneously in a natural wildfire.

Materials and methods

Species

Chaenorhinum rubrifolium (Robill. & Cast. ex Lam. & DC.) Fourr. (Plantaginaceae) is an annual species distributed primarily in the western and central Mediterranean Basin, becoming rare, with a discrete distribution, through the eastern part of the Basin (Fig. 1A). Although there were a few observations of the post-fire emergence of *C. rubrifolium* (Céspedes *et al.*, 2014; G. Ergan *et al.*, Hacettepe University, Ankara, Turkey, unpublished observations), nothing is known about the mechanism of population regeneration by germination following fire. The rareness of the species and the existence of the records of post-fire regeneration by seedling emergence make *C. rubrifolium* an ideal model organism to test the response of rare annual Mediterranean species to fire-related germination cues.

Seed collection and storage

We located a population of *C. rubrifolium* in an 8-month-old burned area (in the first spring following a fire that affected a 160-ha area) in Ören, Muğla, south-western Turkey (37.054° N, 27.953° E, 285 m asl). The pre-fire vegetation of the area was mature Turkish red pine (*Pinus brutia*) forest with shrubby vegetation in the understory. The study area was on calcareous bedrock and had a Mediterranean climate with 716.6 mm of annual precipitation, 18.0°C annual mean temperature, and a substantial 5-month-long dry period from June to October (data from Turkish State Meteorological Service).

The range of the *C. rubrifolium* population was restricted to a ca 50 m² site within the burned area. Extensive surveys showed no evidence of the presence of the species at any site within the burned area or in any of the various habitats (including a 15-year-old burned area, shrubland, pine forest, and roadside habitat) around the burned area, although many other species unique to the burned area were found in numerous burned sites (G. Ergan *et al.*, Hacettepe University, Ankara, Turkey, unpublished observations). This observation confirmed the rareness of the species in the region.

We collected ripe fruits of *C. rubrifolium* from ca 10 individuals in the field in May 2014 and the seeds (Fig. 1B) were separated from fruit parts using sieves of various mesh sizes in the laboratory. We stored the seeds in paper envelopes under dark conditions at ca 20°C and ca 50% RH for 4 months until the experiments were performed. Mean (\pm SE) seed mass of the population was 0.033 \pm 0.0006 mg as determined by weighing four replicates of 50 seeds. A water absorption

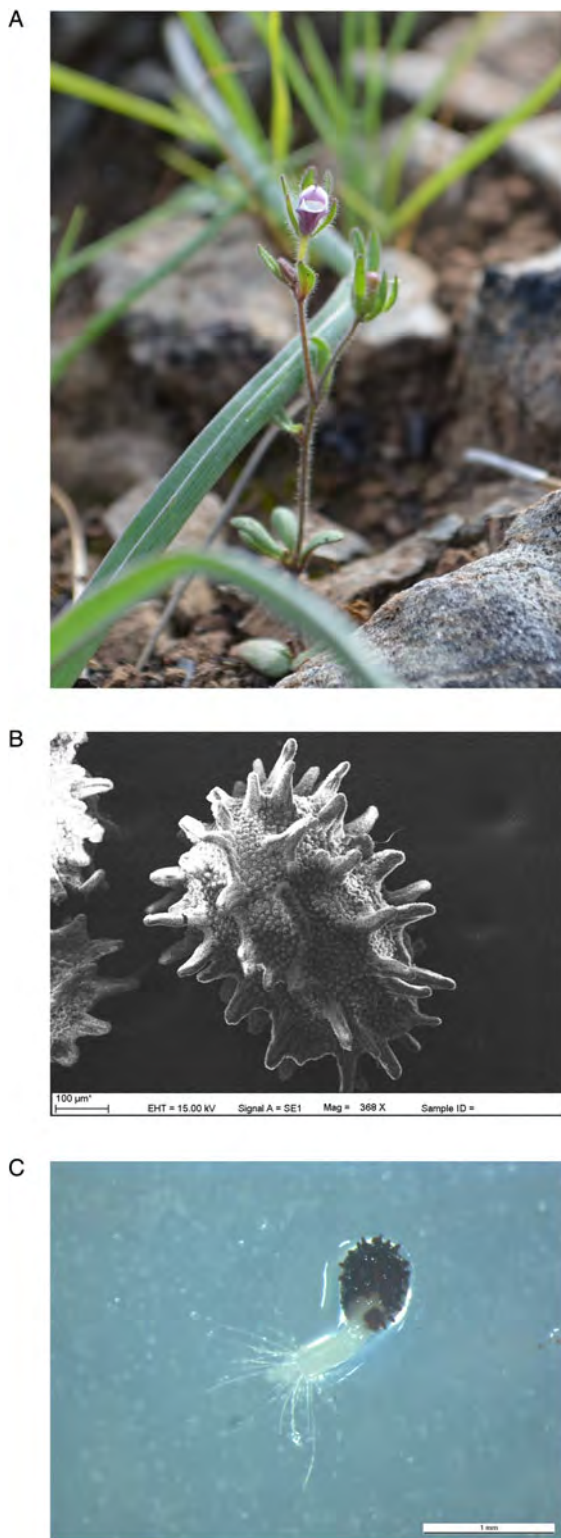


Figure 1. (A) A *Chaenorhinum rubrifolium* individual in its natural habitat (burned area). The seed of *C. rubrifolium*: (B) scanning electron microscope (368 \times), and (C) just after germination (light microscope).

test was conducted to determine if seeds were water permeable by weighting three replicates of 20 seeds before and after 24 h incubation in distilled water.

Before the second measure, seeds were dried off with a filter paper to not overestimate the increase in seed mass due to water particles on the seed surface.

Experimental design

We performed four germination experiments to elucidate post-fire germination behaviour of *C. rubrifolium* (see below). Because of the large size of the experimental design, we divided the experiments into four periods and conducted them in two different laboratories. The first experiment started in September 2014, and the last experiment was finalized in February 2015. In this manner, we conducted all the experiments within 6 months (Table 1).

Four independent replicates of 25-seed batches were used for each treatment and control in each experiment. Each experiment was conducted under both dark and photoperiod (12 h:12 h) conditions, except the combinations of heat shock and smoke, which were conducted under photoperiod conditions only (Table 1). During the regular monitoring of germination in darkness and photoperiod treatments, seeds were exposed to daylight for short durations (*ca* 5–10 min).

Experiment 1: Effects of heat shocks and mechanical scarification. We applied heat shock treatments with different intensities (60, 80, 100, 120 and 140°C for 5 min) to the seeds in aluminium pockets in a temperature-controlled oven. For the mechanical scarification treatment, the seeds were rubbed between two pieces of 500- μ m thick sandpaper. Heat shock treatments were applied to test both fire response and physical dormancy of seeds, while mechanical scarification treatment was for testing physical dormancy only. A group of seeds placed in aluminium pockets was not subjected to heat shock or scarification and served as the control (dry control).

Experiment 2: Effect of aqueous smoke solutions. We applied aqueous smoke treatments in various concentrations (100% = 1:1, 10% = 1:10, and 1% = 1:100) to the seeds in Eppendorf tubes for 24 h in the Ankara laboratory. To obtain 1:1, 1:10 and 1:100 concentrations, we used the methodology of Jäger *et al.* (1996) to prepare and apply aqueous smoke solutions (for more details, see Çağatay *et al.* 2014) from *Quercus coccifera* leaves. The obtained solutions were stored at 4°C until their use in the experiments. We also applied distilled water to a group of seeds in Eppendorf tubes for 24 h to serve as the control for smoke and smoke+heat shock experiments (wet control).

We prepared another aqueous smoke solution at a 1:20 (5%) concentration using the methodology described in Downes *et al.* (2013) in the Muğla laboratory. Eighty grams of wheat hay was burnt in a bee

Table 1. The properties of the experiments conducted in different time periods

No.	Place	Start date	Final date	Tdays	Treatments applied				
					Light	H	S	H + S	C
1	Ankara	02.09.2014	21.10.2014	49	D	+	+		
2	Ankara	20.11.2014	07.01.2015	48	P	+	+		
3	Ankara	17.12.2014	04.02.2015	49	P			+	
4	Muğla	05.01.2015	23.02.2015	49	D + P		+		+

Place is the laboratory in which the experiment was conducted, Start date and Final date indicate the starting and finalized dates of the experiment, Tdays is the duration of the experiment in days, Light indicates the light treatment (D: dark, P: photoperiod) applied during the experiment, and H, S and C are heat shock treatments, smoke treatments and chemical treatments, respectively. D + P indicates dark and photoperiod treatments were both applied in the experiment, whereas H + S indicates heat shock + smoke treatments. '+' means that a particular treatment was applied in a particular experiment.

smoker, and the smoke was bubbled through 500 ml of distilled water in a glass bottle for 12 min. We applied this treatment to the seeds in the Muğla laboratory to determine if aqueous smoke solutions produced using this methodology had a stimulative effect on germination. However, we only compared the results of this treatment with the wet control conducted in the Muğla laboratory, and we did not compare them with those from the smoke experiment in Ankara laboratory, in which the smoke-producing methodology was based on a different approach.

Experiment 3: Effects of smoke chemicals, nitrogenous compounds and gibberellic acid. We applied several chemical compounds, which have previously been reported (or suggested) to stimulate germination after fires in Mediterranean-type ecosystems, to the seeds in aqueous or gaseous form. These chemical compounds primarily included smoke chemicals, such as karrikinolide (KAR₁; 0.1 µM) (Van Staden *et al.*, 2004) and mandelonitrile (MAN; 50 µM) as a cyanohydrin analogue (Flematti *et al.*, 2011), and nitrogenous compounds, such as nitric oxide (NO), nitrite (NO₂), and nitrate (NO₃) (Thanos and Rundel, 1995; Keeley and Fotheringham, 1998a; Pérez-Fernández and Rodríguez-Echeverría, 2003; Luna and Moreno, 2009). Although we directly applied KAR₁ and MAN, non-ionic molecules of nitrogenous compounds were used to apply the target compounds to the seeds. Consequently, sodium nitroprusside (300 µM; Kępczyński and Sznigir, 2014), sodium nitrite (1 mM; Bethke *et al.*, 2006), and potassium nitrate (10 mM; Thanos and Rundel, 1995; Çatav *et al.*, 2015) were used to create the target treatments of NO, NO₂ and NO₃, respectively. In addition to these chemicals, we also applied gibberellic acid (GA₃, 100 µM; Daws *et al.*, 2007) to the seeds because there is evidence that KAR₁ and GA₃ similarly stimulate the germination process in some species (Merritt *et al.*, 2006; Cembrowska-Lech and Kępczyński, 2016). All chemical treatments were applied to seeds in Eppendorf tubes for 24 h, except MAN and NO. Because of the slow release

of free cyanide from cyanohydrin solutions (Flematti *et al.*, 2011), seeds were first incubated in Eppendorf tubes containing distilled water for 24 h for the MAN treatment. Next, a germination medium was prepared using 0.8% agar and 50 µM MAN, and finally, seeds were placed in Petri dishes containing this medium. For the NO treatment, seeds were exposed to sodium nitroprusside in gaseous form for 24 h using the methodology described in Bethke *et al.* (2006) and Kępczyński and Sznigir (2014). KAR₁ and GA₃ were initially dissolved in ethanol (95%) to make the primary stock solutions, which were stored at -20°C until further use. All the chemical compound solutions used in the experiment were purchased from commercial suppliers (Sigma-Aldrich, Merck and Carbosynth). We applied distilled water to a group of seeds in Eppendorf tubes for 24 h to serve as the control for the chemical compound experiments (wet control).

Experiment 4: Effects of treatment combinations. We applied the combinations of heat shock + smoke treatments and KAR₁ + MAN treatments to the seeds to elucidate the impact of combinations of fire-related cues on germination. In the former combination, first heat shock and then aqueous smoke treatment were applied using the procedures given above. For this combination treatment, only one aqueous smoke solution (1:10), but three different intensities of heat shock (80, 100 and 120°C for 5 min) were used. For the latter combination, because of the slow release rate of free cyanide, as explained above, KAR₁ and MAN solutions were not applied simultaneously. Seeds were first incubated in 0.1 µM of KAR₁ solution for 24 h and then transferred to an agar medium containing 50 µM of MAN. We also considered the combined effects of light and other treatments as another combination when an experiment was conducted under both dark and photoperiod (12 h:12 h) conditions.

Additional experiment: Effects of laboratory storage, incubation temperature and short exposures to

daylight. An additional experiment was also included in the study to show if 2 years of laboratory storage of seeds affects dormancy state and germination in *C. rubrifolium*. We also tested the effect of different incubation temperatures (15 and 20°C) on germination in this experiment. This experiment was conducted in August and September 2016 for 49 days in the Ankara laboratory. Due to the limited remaining number of seeds, we only established 'wet control', '1:10 aqueous smoke solution' and KAR₁ in both dark and 12 h:12 h photoperiod conditions. We also included an absolute dark control in this experiment to understand if there is any effect of exposing daylight for short durations during germination checks in darkness and photoperiod treatments. In this case, we checked germination only once at the end of the experiment, so that we protected seeds from the short duration of sunlight. Again, due to lack of seeds, we performed this part of the experiment only for KAR₁ treatment at 20°C incubation temperature, for which we had obtained the highest germination in the original experiment. Consequently, we were able to compare the absolute dark control, dark and 12 h:12 h photoperiod conditions in KAR₁ treatment.

Monitoring germination

After the treatments, seeds were sown in Petri dishes including agar as the substrate material. These were later placed in an incubator at 20°C (±0.5°C) constant temperature (an exception was the additional experiment in which seeds were incubated at both 15 and 20°C constant temperatures), favourable conditions for the germination of many Mediterranean species (Thanos, 1993; Luna *et al.*, 2012), and under constant dark and photoperiod (12 h:12 h, at a light intensity of 100 μmol m⁻² s⁻¹) conditions. The seeds were monitored for germination under a stereomicroscope every 2 days for the first 2 weeks of incubation (Fig. 1C), and then once a week until the end of the experiments (the 48th or 49th day of the incubation). Radicle emergence defined germination. At the end of the experiments, the viability of non-germinated seeds was determined by the cut test, and the seeds with an intact embryo were considered viable.

Statistical analyses

Before any statistical analysis, empty seeds (*ca* 1.5% of whole seeds sown) were removed from the data set to correct the total number of seeds in each Petri dish. For each control and treatment, seeds were classified as germinated or non-germinated in the final germination data. Final germination of each treatment was compared with the corresponding control using

generalized linear mixed-effects models (GLMM) with a binomial error distribution, and differences were tested by an analysis of deviance. In the analysis, we considered treatments as fixed factors and the Petri dish replicates as the random factor. The controls used in the analyses were (1) the dry control for the heat shock treatments, (2) the wet control for the aqueous smoke and chemical solution treatments, (3) the 1:10 aqueous smoke treatment for the combined effect of aqueous smoke+heat shock treatments, and (4) both KAR₁ and MAN treatments separately for testing the synergetic effect of KAR₁+MAN treatment.

We performed additional GLMM analyses on the data for the treatments with significant effects to explore the impact of treatment combinations on germination. In these analyses, comparisons of germination under dark *vs* photoperiod conditions in each treatment were conducted to elucidate the effect of light on 1:1 and 1:10 aqueous smoke, nitrate, MAN and KAR₁ treatments. The effect of light on KAR₁+MAN treatment combination was also explored. Comparisons of germination of each aqueous smoke+heat shock treatment combinations (*i.e.* 1:10 *vs* treatment combination) were also made separately to obtain test statistics and significance values for each combination. A significant improvement achieved by the combined treatment was assumed to show the presence of a positive synergetic effect when the germination percentage in the treatment combinations was considerably higher (>10% difference) than the sum of germination percentage of the two individual treatments. With the same approach, the presence of a positive additive effect was assumed when the germination percentage in the treatment combinations was approximately equal (<10% difference) to the sum of germination percentage of the two individual treatments.

Mean germination time (hereafter, MGT) was determined using the formula $\sum(nD)/\sum n$, where n is the number of seeds germinated on day D , and D is the number of days from the beginning of the incubation period (Çatav *et al.*, 2015). Differences in MGT between treatments and control were analysed by one-way ANOVAs followed by Tukey's HSD tests. Before the analysis, Shapiro-Wilk and Bartlett's tests were applied to check data normality and homogeneity of variance, respectively. As no germination was obtained in controls, MGT comparisons were only made between 1:10 aqueous smoke treatment and aqueous smoke+heat shock treatment combinations; and also among chemical treatments in which germination was observed (KAR₁, MAN, NO₃ and the combined KAR₁+MAN treatment under photoperiod conditions).

GLMMs were performed with the *lme4* package in R (Bates *et al.*, 2015).

Results

The seeds of *C. rubrifolium* expressed strong dormancy because zero germination was recorded in control (untreated) groups. Heat shock treatments and the mechanical scarification treatment resulted in zero germination as well, and therefore failed to break the dormancy. Furthermore, mechanical scarification of the seeds resulted in approximately 50% mortality as detected by rotted embryos during the cut test (mean ratio of rotten seeds in other treatments and controls was 8.3%) and mean (\pm SE) increase in seed mass was $10.3 \pm 2.9\%$ in the water absorption test. These observations indicated there was no physical dormancy in *C. rubrifolium* seeds, and the presence of fully developed embryos in untreated seeds suggests that seeds probably exhibited physiological dormancy.

Aqueous smoke solutions at 1:1 and 1:10 concentrations significantly increased germination in comparison with the control under the photoperiod condition (7.9%, $P=0.01$ and 18.7%, $P=0.0006$, respectively; Fig. 2, Table 2), whereas the most diluted smoke solution (1:100) had no effect on germination (0.0%, $P>0.05$; Fig. 2). The aqueous smoke solution at 1:20

Table 2. Summary of the generalized linear mixed-effects model for predicting germination response of *C. rubrifolium* to aqueous smoke treatments

Fixed factors	Deviance	d.f.	χ^2	P
Intercept	123.2			
Treatment	89.7	3	33.6	<0.0001
Light	105.6	1	17.6	<0.0001
Treatment \times Light	63.0	3	8.2	0.041

Comparison for treatments performed with the wet control. Akaike information criterion (AIC) of the model is 81.0.

concentration produced using a different methodology also significantly increased germination in comparison with the control (8.0%, $P=0.005$ and 32.9%, $P<0.0001$ under dark and photoperiod conditions, respectively). Among the chemicals tested in this study, NO (0.0% in dark, and 1.0% in photoperiod conditions, $P>0.05$), NO₂ (0.0% in both dark and photoperiod conditions), and GA₃ (1.2% in dark, and 2.5% in photoperiod conditions, $P>0.05$) had no effect on germination in comparison with the control (Fig. 3). On the other hand, NO₃ (3.0% in dark, and 16.1% in photoperiod conditions, $P=0.0009$), MAN (0.0% in dark, and 16.7% in photoperiod conditions, $P=0.002$), and KAR₁ (3.9% in dark, and 46.6% in photoperiod conditions,

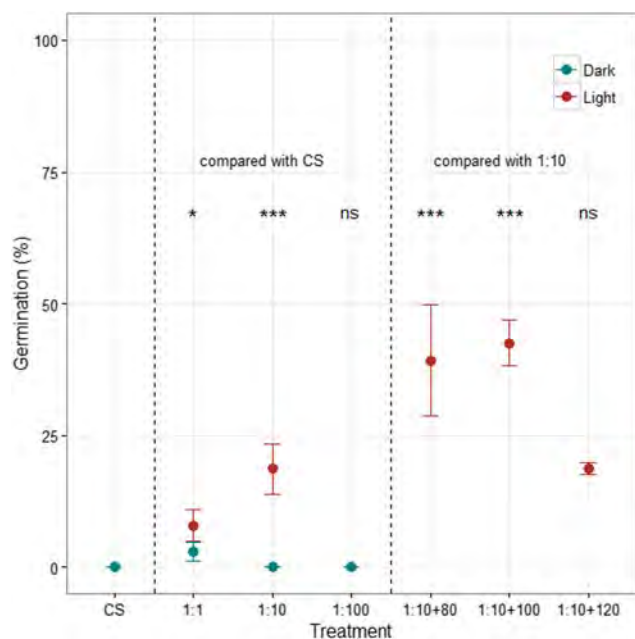


Figure 2. Mean (\pm SE) germination percentage in the control (CS), smoke, and smoke + heat shock combination treatments under dark ('Dark') and photoperiod ('Light') conditions. The concentrations of different aqueous smoke treatments are shown as 1:1, 1:10 and 1:100. Only the smoke treatment at 1:10 concentration under photoperiod conditions was used for smoke + heat shock combination treatments (+80, +100, and +120 are 80, 100 and 120°C for 5 min each, respectively). Results of the pairwise statistical comparison of each treatment with the corresponding control (GLMM, see Materials and methods for details) are given (ns, not significant; * $P<0.05$; *** $P<0.001$).

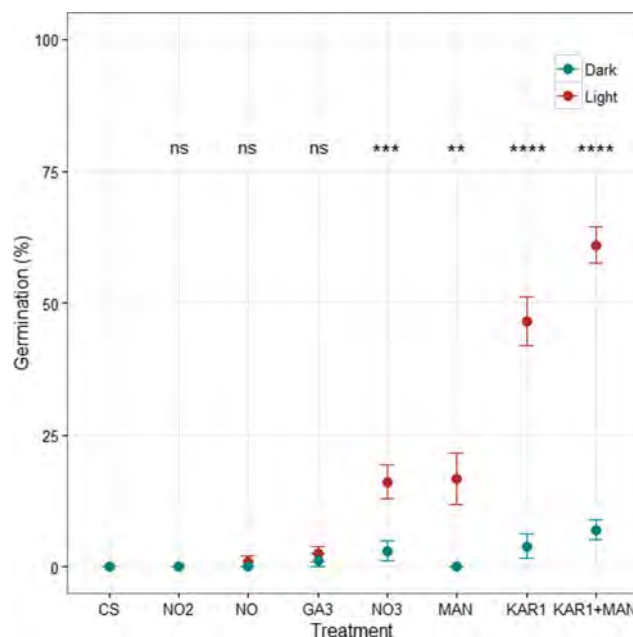


Figure 3. Mean (\pm SE) germination percentage in the control (CS) and various chemical solution treatments under dark ('Dark') and photoperiod ('Light') conditions. NO is nitric oxide, NO₂ is nitrite, NO₃ is nitrate, GA₃ is gibberellic acid, MAN is mandelonitrile, KAR₁ is karrikinolide, and KAR₁ + MAN is the combination treatment including KAR₁ and MAN. Results of the pairwise statistical comparison of each treatment with the control (GLMM, see Materials and methods for details) are given (ns, not significant; ** $P<0.01$; *** $P<0.001$; **** $P<0.0001$).

Table 3. Summary of the generalized linear mixed-effects model for predicting germination response of *C. rubrifolium* to all chemical solution treatments

Fixed factors	Deviance	d.f.	χ^2	<i>P</i>
Intercept	525.1			
Treatment	301.1	7	224.0	<0.0001
Light	407.2	1	117.9	<0.0001
Treatment × Light	147.2	7	14.8	0.038

Comparison for treatments performed with the wet control. Akaike information criterion (AIC) of the model is 181.2.

Table 4. Summary of the generalized linear mixed-effects model for predicting germination response of *C. rubrifolium* to heat shock treatments

Fixed factors	Deviance	d.f.	χ^2	<i>P</i>
Intercept	32.9			
Treatment	32.8	5	0.2	>0.05
Light	32.7	1	0.6	>0.05
Treatment × Light	32.8	5	0	>0.05

Comparison for treatments performed with the dry control. Akaike information criterion (AIC) of the model is 58.8.

$P < 0.0001$) significantly increased germination under photoperiod conditions (Fig. 3, Table 3). However, none of these treatments significantly increased germination in comparison with the control under dark conditions ($P > 0.05$).

Although none of the heat shock treatments stimulated germination (Table 4), the combination of smoke solution (1:10) and heat shock (80 and 100°C) treatments significantly increased the germination percentage under the photoperiod conditions in comparison with 1:10 smoke treatment only (39.2%, $P = 0.0006$, and 42.6%, $P = 0.0002$, respectively; Fig. 2, Table 5). Consequently, these treatment combinations resulted in a positive synergetic effect on germination (Table 6). On the other hand, the combination of the 120°C heat shock treatment and 1:10 smoke treatment did not increase germination in comparison with 1:10 smoke treatment only (18.6%, $P > 0.05$; Fig. 2). The combination of KAR₁ and MAN resulted in the maximum increase in germination in comparison with the wet control (7.0% in the dark, and 61.0% in photoperiod

Table 5. Summary of the generalized linear mixed-effects model for predicting germination response of *C. rubrifolium* to heat shock + aqueous smoke treatments

Fixed factors	Deviance	d.f.	χ^2	<i>P</i>
Intercept	104.2			
Treatment	77.7	3	26.5	<0.0001

Comparison for treatments performed with the 1:10 aqueous smoke treatment (only in light conditions). Akaike information criterion (AIC) of the model is 87.7.

conditions, $P = 0.041$ and $P < 0.0001$, respectively; Fig. 3). Clearly, the KAR₁+MAN combination had a positive additive effect on germination under photoperiod conditions (Table 6), i.e. the germination percentage obtained from this combination treatment (61.0%) was almost the same as that obtained from the sum of the two separate treatments of KAR₁ and MAN (46.6% + 16.7% = 63.3%).

Although zero germination occurred in the controls under the dark and photoperiod conditions, when a treatment was combined with photoperiod treatment significant improvement in germination was obtained in many cases (Figs 2 and 3). In these cases, the interaction effects of mixed model analyses yielded significant results as well, suggesting that the combination of the light and many other treatments had a positive synergetic effect on germination (Table 6).

Results of the additional experiment showed that seeds were viable even after 2 years of laboratory storage. KAR₁ and 1:10 aqueous smoke treatments significantly increased germination in dark and photoperiod conditions in comparison with the control at both 15°C and 20°C incubation temperatures (Fig. 4A and B, $P < 0.0001$). Under photoperiod conditions, germinations reached ca 97% in 1:10 smoke and KAR₁ treatments at 15°C (Fig. 4A), and 90 and 95% in smoke and KAR₁ treatments in 20°C (Fig. 4B), respectively. These were higher values in comparison with the original experiment results. Control germinations were still lower; controls had zero germination (both in the dark and photoperiod) at 20°C (Fig. 4B), and 2 and 19% in dark and photoperiod conditions in 15°C (Fig. 4A). However, although we obtained 19% control germination (at 15°C under photoperiod) after laboratory storage of an extra 1.5 years, the difference between 15 and 20°C incubation temperatures was only critically significant ($P = 0.055$). Moreover, the effect of light on germination was still significant (dark *vs* photoperiod; $P = 0.002$). On the other hand, the absolute dark control had 3% germination after 2 years of laboratory storage of seeds, while dark control and photoperiod had 64 and 95%, respectively; the difference between absolute dark control *vs* dark and photoperiod conditions was significant in the presence of KAR₁ ($P < 0.0001$; Fig. 4B).

Difference in MGT was not significant between 1:10 aqueous smoke treatment and heat shock + smoke combined treatments ($P > 0.05$, Fig. 5A), but MGT in MAN and KAR₁+MAN combined treatments were significantly higher (i.e. indicating slower germination) than KAR₁ and NO₃ treatments ($P < 0.0001$, Fig. 5B).

Discussion

Our study showed that physiological dormancy in the seeds of *C. rubrifolium* was partially broken by several

Table 6. Summary of GLMMs regarding the combination effects of the treatments on germination in *C. rubrifolium*

Treatment combinations	χ^2	<i>P</i>	Effect
Smoke (1:1) + light	1.9	>0.05	No
Smoke (1:10) + light	24.8	<0.0001	Positive synergetic
Smoke (1:10) + heat shock (80°C)	12.3	<0.001	Positive synergetic
Smoke (1:10) + heat shock (100°C)	14.5	<0.001	Positive synergetic
Smoke (1:10) + heat shock (120°C)	~0	>0.05	No
Nitrate + light	9.6	<0.01	Positive synergetic
MAN + light	19.8	<0.0001	Positive synergetic
KAR ₁ + light	53.4	<0.0001	Positive synergetic
(KAR ₁ + MAN) + light	69.7	<0.0001	Positive synergetic
(KAR ₁ + MAN) under light (<i>vs</i> KAR ₁)	4.3	0.04	
(<i>vs</i> MAN)	42.7	<0.0001	Positive additive

Only treatments with significant effects were considered for the analysis of combination effect. Smoke + heat shock treatments were tested only under photoperiod conditions. The effect of KAR₁ + MAN combination under light conditions was tested in comparison with both KAR₁ and MAN treatments separately. The significance of the combination of any treatment with light was evaluated by considering the interaction in the two-way model.

fire-related germination cues, including smoke and nitrate, in the presence of light. KAR₁ and MAN also stimulated germination under light conditions. Many of the cues had positive synergetic or additive effects on germination when they combined. These results suggest that multiple fire-related cues operate to break dormancy and to enhance germination in *C. rubrifolium*, a Mediterranean annual species found in fire-prone environments. Because plant species with annual life cycles completely depend on seed germination for regeneration, their seeds should be adapted to local environmental conditions (Venable and Brown, 1988; Kos and Poschod, 2010). Consequently, a strong selection pressure might be present for seed traits in annual species in comparison with the species with perennial life cycles (Keeley *et al.*, 1985; Venable and Brown, 1993). In fire-prone ecosystems, local fire regime is a candidate for being one of the strongest pressures on seed traits (Thomas *et al.*, 2003; Moreira *et al.*, 2012; Tavşanoğlu *et al.*, 2015; Fichino *et al.*, 2016). Therefore, annual species specific to post-fire habitats should be adapted to germination after a fire, as shown in the Mediterranean-type ecosystems of California, South Africa, and Western Australia (Keeley *et al.*, 1985; Keeley and Bond, 1997; Keeley and Fotheringham, 1998b; Downes *et al.*, 2010). However, more complex interactions among several germination cues can also be present in post-fire annuals (Preston and Baldwin, 1999). In the Mediterranean Basin, annual species comprise an important proportion of the flora of post-fire habitats (Kazanis and Arianoutsou, 2004; Kavgacı *et al.*, 2010; Tormo *et al.*, 2014). Our results on *C. rubrifolium* constitute a step towards explaining the physiology of the well-known post-fire establishment behaviour of annual plants in the Mediterranean Basin. Indeed, the stimulation of germination with several fire-related cues in our laboratory experiments is in accordance with the field observations on the seedling emergence of *C. rubrifolium* in the first

year after a fire (Céspedes *et al.*, 2014; G. Ergan *et al.*, Hacettepe University, Ankara, Turkey, unpublished observations). More research is promising for a comprehensive understanding of the adaptations of annual species to local fire regimes, and further research should involve more annuals to draw more general conclusions on the post-fire germination behavior of annual species in the Mediterranean Basin.

Species-specific germination response to aqueous smoke solutions, karrikins and glyceronitrile has been demonstrated. Many species from the Mediterranean fire-prone habitats of Western Australia respond to aqueous smoke, but not to KAR₁ or *vice versa* (Downes *et al.*, 2010; Downes *et al.*, 2013; Downes *et al.*, 2014). Glyceronitrile promotes germination in some of the species that positively respond to aqueous smoke (Downes *et al.*, 2013), or all three treatments stimulate germination in some species (Downes *et al.*, 2015). Our results on the stimulation of germination in *C. rubrifolium* by aqueous smoke, KAR₁, and MAN support the latter observation. Moreover, the observed additive positive effect of KAR₁ and MAN on the germination in our study gives experimental support to molecular evidence that these compounds stimulate germination by different mechanisms (Flematti *et al.*, 2013). In some cases, however, the interaction of two smoke compounds may have an opposing effect on germination (Light *et al.*, 2010). In our study, MAN decreased the germination rate in comparison to KAR₁ and NO₃ treatments, while KAR₁ + MAN combination resulted in an intermediate germination rate. This finding also supports these compounds act in different ways on seed germination. To understand the interactions of smoke chemicals and their role in nature, more studies on various taxa from different fire-prone ecosystems are needed.

In general, observing a positive germination response to heat shocks or smoke is dependent on the water permeability of the seed coat of a given

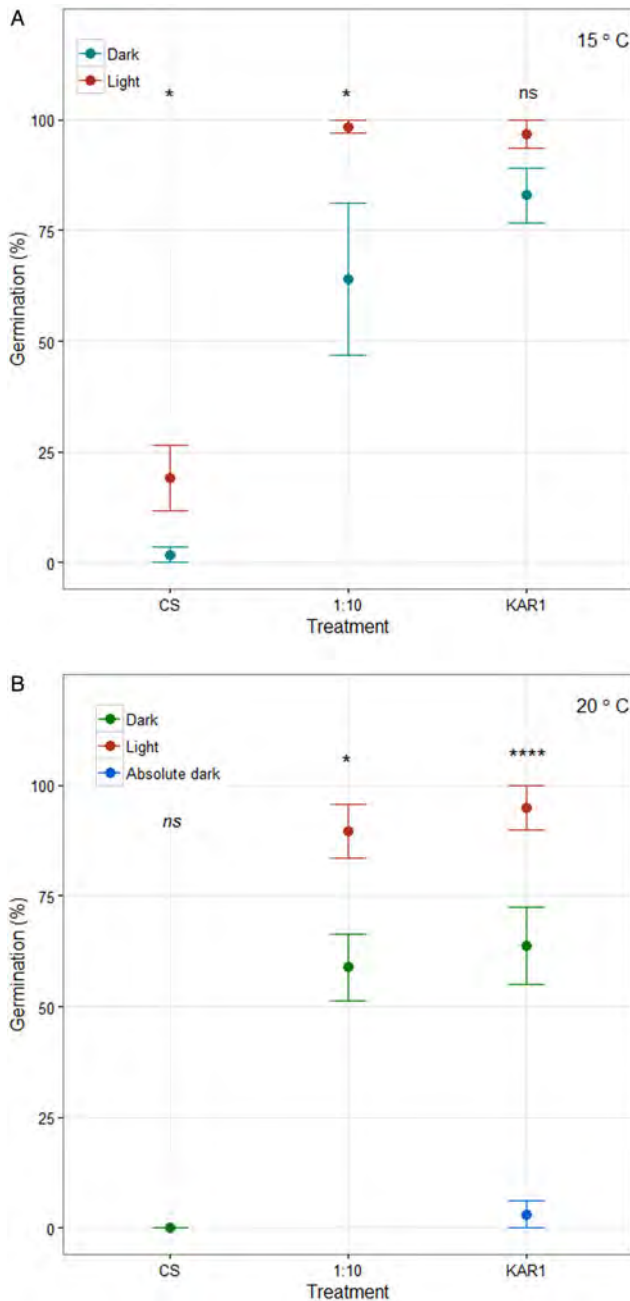


Figure 4. Mean (\pm SE) germination percentage in the control (CS), 1:10 smoke ('1:10'), and karrikinolide ('KAR₁') treatments under dark ('Dark'), photoperiod ('Light'), and absolute dark conditions at (A) 15°C and (B) 20°C incubation temperatures. Results of the pairwise statistical comparison of each treatment with the corresponding control (GLMM, see Materials and methods for details) are given (ns, not significant; * $P < 0.05$; **** $P < 0.0001$).

species (Moreira *et al.*, 2010), and therefore the combination effect of heat shock and smoke follows one of these cues (Williams *et al.*, 2003; Moreira *et al.*, 2012; Fichino *et al.*, 2016). However, the combined effect of smoke and heat shock resulted in more germination than could be obtained from only smoke or heat shock separately in *C. rubrifolium* under light

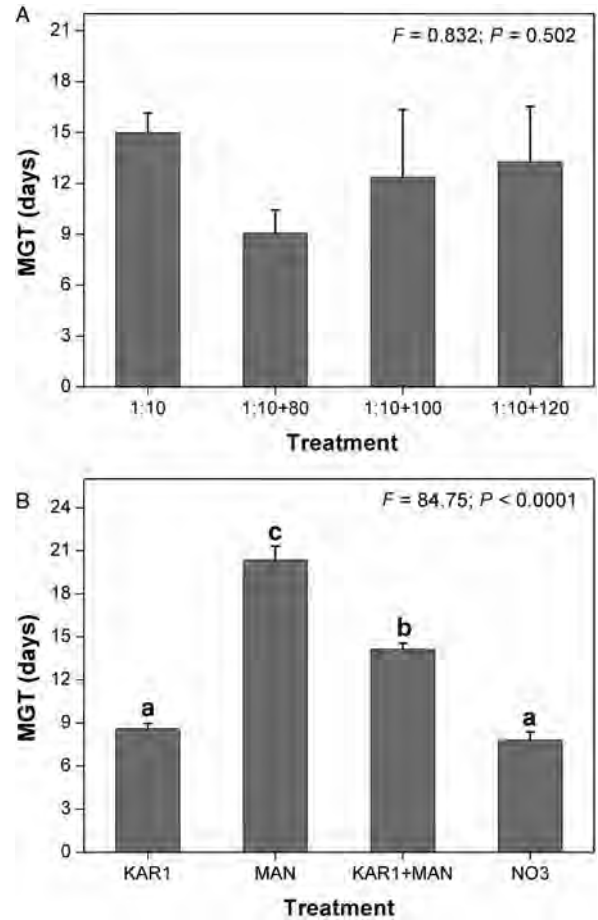


Figure 5. Mean (\pm SE) mean germination time (MGT, in days) values for (A) the combined heat shock + smoke treatments (including 1:10 aqueous smoke treatment as control), and (B) chemical treatments. Results of ANOVAs are presented in the panels. Statistical significance ($P < 0.05$) between chemical treatments is shown by lower case letters above the bars (panel B).

conditions. This finding is also novel for the Mediterranean Basin, and supports the previous observations on the stimulative effect of the combination of heat shock and smoke on germination in fire-prone ecosystems of Australia (Keith, 1997; Tieu *et al.*, 2001; Thomas *et al.*, 2003) and South Africa (Ghebrehiwot *et al.*, 2012).

Light was a prerequisite for stimulation of germination by other fire-related cues. In other words, smoke and other fire-related cues in the environment become an important factor in the germination of *C. rubrifolium* in the presence of light. This result suggests that although fire has a prominent place in the germination niche of *C. rubrifolium*, light is a key element of post-fire germination in this annual species. On the other hand, there was a clear effect of laboratory storage period on germination in the presence of smoke-related germination cues, but overall germination response to light seems not to be affected by

the after-ripening period. Moreover, the amount of light energy to which seeds were subjected during germination checks was enough to break dormancy imposed by dark conditions partially in the presence of KAR₁ after laboratory storage. This result does not affect our conclusion for the original experiment, because we already had very little germination in the darkness treatment. Consequently, this result also supports the dependence of *C. rubrifolium* seeds on light for germination. Light is an important cue for germination in many plant species in Mediterranean-type ecosystems, especially those with small seeds (Thanos, 1993; Bell *et al.*, 1999; Koutsovoulou *et al.*, 2014), and may play a role in post-fire germination (Roy and Arianoutsou-Faraggitaki, 1985). However, no general trend was found in studies that tested the role of the presence of light in post-fire germination of plants in Mediterranean-type ecosystems (Bell *et al.*, 1999; Thanos and Rundel, 1995; Luna and Moreno, 2009). Similarly, there is no general pattern in the case of nitrates (Bell *et al.*, 1999; Çatav *et al.*, 2015). The result obtained in our study is a good example of how light and the cues created by burning vegetation such as smoke and smoke chemicals interact to stimulate germination in a Mediterranean species immediately after fire. Considering the fact that the removal of vegetation after a fire opens the window of germination for light-dependent species, such an interaction could be expected to be found in more Mediterranean species. Our results on the positive synergetic effect of KAR₁ and light contradict those of Long *et al.* (2011), which show the influence of light on the germination of eight Brassicaceae species independent of KAR₁. In fact, the effect of the interaction of environmental cues such as light, smoke and nitrates can be complex, and may depend on a species' habitat requirements (Bell *et al.*, 1999; Merritt *et al.*, 2006) and establishment behaviour (Todorović *et al.*, 2010).

Our results also show that seeds of the study species become more sensitive to dormancy-breaking cues (i.e. light and smoke) in dry storage under ambient conditions. This finding might indicate the existence of a risk-reducing strategy for *C. rubrifolium* by avoiding germination of all fresh seeds immediately after they are transferred to the soil seed bank (i.e. in the second post-fire year), and by spreading germination possibility through time until the dormancy-breaking cues appear again. Indeed, this species continued to exist in the second post-fire year in the study site, but disappeared in the third year after fire (G. Ergan *et al.*, Hacettepe University, Ankara, Turkey, unpublished observations), possibly due to the presence of the smoke chemicals in the soil for a while after the fire event (Ghebrehiwot *et al.*, 2011). This observation is consistent with our germination results after 2 years of laboratory storage and supports the conclusion above.

The *C. rubrifolium* population in our study site showed a pyroendemic behaviour (Keeley and

Pausas, 2016) as the seeds give positive germination response to several fire-related cues, and the individuals exist just for 2 years in a post-fire habitat in the region. On the other hand, there are observations on the existence of *C. rubrifolium* in nitrogen-rich disturbed habitats in the western part of the Mediterranean Basin (Peinado *et al.*, 1985; Herranz *et al.*, 2003), and our results confirmed the role of more general agents (i.e. nitrate and light) on the germination in this species. It is clear that fire has an important place in the germination in *C. rubrifolium* seeds, but it should also be noted that the species may have a broader germination niche than a fire-restricted one.

Conclusions

The Mediterranean Basin has been underestimated with respect to the presence of the species with fire-related germination in comparison with other Mediterranean-type ecosystems (Moreira and Pausas, 2016). Our study on the fire-related germination niche of an annual species suggests that much evidence has been overlooked by focusing on the germination of perennial, especially woody, species. Furthermore, our results on KAR₁ and MAN under light conditions are the first records of the stimulation of germination by smoke chemicals in a plant species in the Mediterranean Basin. Because annual species comprise an important part of the plant community in post-fire environments in the Mediterranean Basin, and because of the gap in our knowledge regarding their post-fire germination properties, particular focus on the germination ecology of Mediterranean Basin annuals, with respect to fire, is required.

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Conflicts of interest

None.

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