

Effects of aqueous smoke and nitrate treatments on germination of 12 eastern Mediterranean Basin plants

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Determining the relative role of various cues on seed germination in Mediterranean plants is important to understanding their response to variable conditions. We studied germination responses of 12 eastern Mediterranean Basin species (*Alyssum caricum*, *A. minus*, *Carthamus dentatus*, *Daucus broteri*, *D. carota*, *Hypericum aviculariifolium*, *Muscari comosum*, *Onopordum caricum*, *Rumex crispus*, *Sarcopoterium spinosum*, *Silene vulgaris*, *Smyrniium rotundifolium*) to different smoke and nitrate concentrations in laboratory. Smoke treatments resulted in significant increase in germination of *D. carota*, *O. caricum*, and *S. spinosum*, but had a negative effect on germination of *Hypericum aviculariifolium*. Of the 12 studied species, five showed significant improvement in germination after at least one of the nitrate treatments compared with the control. In total, smoke and nitrate treatments significantly increased the germination percentage in six of the 12 studied species. All the species that have smoke- and/or nitrate-stimulated germination were propagule-persisters (P+), able to recruit after fire. The results reveal that both smoke and nitrate improve germination of Mediterranean species. Our results also suggest the presence of species-specific germination response to smoke and nitrate in Mediterranean plants.

Introduction

Fire is a common disturbance that has an significant impact on the structure and function of plant communities (Verdú & Pausas 2007, Keeley *et al.* 2012). Many plant species have evolved traits or mechanisms allowing their persistence after fire. Examples of such mechanisms include resprouting, serotiny and fire-stimulated germination (Keeley *et al.* 2011). The latter is the result

of dormancy-breaking effects of heat (Thanos *et al.* 1992, Herranz *et al.* 1998, Moreira & Pausas 2012) and chemical compounds found in charred wood (Keeley *et al.* 1985, Keeley & Bond 1997) and smoke (De Lange & Boucher 1990, Dixon *et al.* 1995, Moreira *et al.* 2010). In addition to its important role in stimulating seed germination, it has been demonstrated that chemical compounds in smoke also enhance seedling growth (Van Staden *et al.* 2006, Moreira *et al.* 2010),

flowering (Keeley 1993), somatic embryogenesis (Senaratna *et al.* 1999) and pollen germination as well as tube elongation (Papenfus *et al.* 2014). Consequently, stimulation of germination and seedling growth by fire-produced smoke plays a key role in plant recruitment (Tormo *et al.* 2014).

Soils of Mediterranean-type ecosystems are usually characterized by their low nutrient content (Rundel 1982, Cowling *et al.* 1996, Wisheu *et al.* 2000). In these ecosystems, concentrations of nitrogenous compounds in the soil (e.g., ammonium and nitrate) increase after fires (Arianoutsou-Faraggitaki & Margaritis 1982, Stock & Lewis 1986, Christensen 1994). The increase of these compounds in nutrient-poor Mediterranean habitats may play an important role in regulation of post-fire germination (Thanos & Rundel 1995, Bell *et al.* 1999, Henig-Sever *et al.* 2000).

Effects of smoke on seed germination of Mediterranean Basin plants have received more attention in recent years (Crosti *et al.* 2006, Reyes & Trabaud 2009, Moreira *et al.* 2010, Çatav *et al.* 2014, Tormo *et al.* 2014). However, there is limited information about the effect of nitrate on seed germination, with the exception of few studies conducted in the Mediterranean Basin (Doussi & Thanos 1997, Henig-Sever *et al.* 2000, Pérez-Fernández & Rodríguez-Echeverría 2003, Pérez-Fernández *et al.* 2006, Luna & Moreno 2009). Positive, negative, and null effects of nitrates on germination of Mediterranean plants were found in these studies. This suggests a species-specific germination response to nitrates. However, the role of nitrates in the germination ecology of Mediterranean plants is poorly understood due to the contrasting results obtained. Therefore, more studies are still needed to better understand the role of nitrates in germination of plant species in the Mediterranean Basin.

In this study, we examined the germination responses of 12 eastern Mediterranean Basin species to different smoke and nitrate concentrations in laboratory conditions. We hypothesized that smoke and nitrate improve germination of the species studied. To test this hypothesis, seeds of each species were subjected to different concentrations of aqueous smoke solution, potassium nitrate (KNO₃) and ammonium nitrate (NH₄NO₃). Germination percentages and mean

germination times of each species were assessed, and the results were compared with those for the control conditions. By carrying out this experiment, we aimed to take a step towards filling the gaps in our knowledge on the role of nitrates and smoke as germination stimulants.

Material and methods

Study species, study area and seed collection

Seeds of 12 plant species were collected from fire-prone areas of Muğla Province, southwestern Turkey, eastern Mediterranean Basin (Table 1). The study region has a Mediterranean climate with mild, wet winters and hot, dry summers. Seeds were collected between July and September 2012, coinciding with the dispersal period of each species. Seeds were separated from fruit parts by hand and stored in paper envelopes at room temperature until the start of the experiment in January 2013. Mean seed mass was determined by weighing four samples of 100 seeds of each species.

Preparation of smoke solutions

Dry needles of *Pinus brutia*, a common tree species in the region, were used in preparation of smoke solutions. Four 5-g samples of this plant material were heated separately in glass flasks in the oven for 30 min at 195 °C. The mouth of each flask was tightly covered with an aluminium foil to capture the smoke generated from the heated needles. Subsequently, 50 ml of distilled water was poured over the heated material and left for 10 minutes (Çatav *et al.* 2012). By this procedure, the active chemicals found in smoke are dissolved in water (Jäger *et al.* 1996). The solutions were filtrated from the flasks into a bottle to obtain a concentrated smoke solution (pH 4.50).

The experiment

The germination-medium compositions are given in Table 2. Twenty-five seeds of each

species were sown directly in Petri dishes containing 20 ml of the media. Each treatment consisted of four replicates. Petri dishes were then placed in an incubator at 20 ± 1 °C in darkness. Previous studies showed that these conditions are favourable for germination of many Mediterranean-climate species (Bellairs & Bell 1990, Luna *et al.* 2011). The seeds were checked for germination under a stereomicroscope every 2 days during the first 2 weeks, and then weekly until the end of the experiment. It should be noted that the seeds were exposed to daylight for short durations (10–20 minutes) during the germination checks. The criterion of germination was visible radicle protrusion. Germinated seeds were counted and removed from the Petri dishes

at every check. The experiment was ended on the 35th day of the incubation period, i.e. one week after last germination event had been recorded. Viability of non-germinated seeds was determined by a cutting test, and the seeds with and intact internal content were considered viable.

Data analysis

After removing empty seeds, the remaining seeds were then scored as germinated or non-germinated. For each species, the final proportion of germinated seeds in each treatment was compared with the control using the analysis of deviance with a binomial error distribution (Moreira

Table 1. Taxonomic status and ecological characteristics of the study species. GF is growth form (Ah = annual herb, Bh = biennial herb, Ph = perennial herb, S = shrub), and SM is mean seed mass in mg. RM is the post-fire regeneration mechanism and follows the Pausas *et al.* (2004) 'P & R' system; P+ and P– indicate presence and absence, respectively, of propagule persistence after fire, while R+ and R– refer to presence and absence, respectively, of post-fire resprouting ability. RM is based on Paula *et al.* (2009), Tavşanoğlu (2008), and field observations. Nomenclature follows Davis (1965–1985), and <http://www.mobot.org/MOBOT/research/APweb/> for the current family names.

Code	Family	Species	RM	GF	SM
DBR	Apiaceae	<i>Daucus broteri</i>	P+ & R–	Ah	0.74
DCA	Apiaceae	<i>Daucus carota</i>	P+ & R+	Bh	1.03
SRO	Apiaceae	<i>Smyrniun rotundifolium</i>	unknown	Bh	7.76
CDE	Asteraceae	<i>Carthamus dentatus</i>	P+ & R–	Ah	30.60
OCA	Asteraceae	<i>Onopordum caricum</i> *	P+ & R–	Bh	7.23
ACA	Brassicaceae	<i>Alyssum caricum</i> *	P+ & R–	S	0.43
AMI	Brassicaceae	<i>Alyssum minus</i> var. <i>minus</i>	P– & R–	Ah	1.11
SVU	Caryophyllaceae	<i>Silene vulgaris</i> var. <i>vulgaris</i>	P– & R–	Ph	1.88
HAV	Clusiaceae	<i>Hypericum aviculariifolium</i> subsp. <i>aviculariifolium</i> var. <i>aviculariifolium</i> *	P– & R–	Ph	0.28
MCO	Liliaceae	<i>Muscari comosum</i>	P– & R+	Ph	4.52
RCR	Polygonaceae	<i>Rumex crispus</i>	P– & R–	Ph	2.63
SSP	Rosaceae	<i>Sarcopoterium spinosum</i>	P+ & R+	S	3.15

* endemic to Turkey.

Table 2. Media formulations. CSS = concentrated smoke solution. pH of germination media were adjusted using 0.1 M HCl or 0.1 M NaOH.

Treatment	Agar (g l ⁻¹)	CSS (ml l ⁻¹)	KNO ₃ (g l ⁻¹)	NH ₄ NO ₃ (g l ⁻¹)	pH
Control	10	–	–	–	5.8
Smoke 1:10	10	100	–	–	5.8
Smoke 1:100	10	10	–	–	5.8
10 mM KNO ₃	10	–	1.01	–	5.8
25 mM KNO ₃	10	–	2.53	–	5.8
10 mM NH ₄ NO ₃	10	–	–	0.8	5.8
25 mM NH ₄ NO ₃	10	–	–	2.0	5.8

et al. 2010). Mean germination time (MGT, days) was determined using the following equation based on Tompsett & Pritchard (1998): $\Sigma(n \times D)/k$, where n is the number of seeds that germinated on day D , D is the number of days from the beginning of the incubation period, and k is the total number of seeds that germinated during the incubation period. For each species, the differences between the mean germination time in the control and treatment groups were analyzed using one-way ANOVA followed by Dunnett's test for multiple comparisons. Homogeneity of variance was verified with Bartlett's test before each analysis. Because of the large number of pairwise comparisons, the significance level for all analyses was set at $p = 0.01$. This approach is less conservative than the Bonferroni correction (Moran 2003), and has been effectively used in many studies in recent years (e.g. Moreira et al. 2010, Tavşanoğlu & Gürkan 2014).

Results

The germination percentages in the controls varied among the study species, ranging from 0% to 100% (Table 3). In four species (*Carthamus dentatus*, *Muscari comosum*, *Onopordum caricum*, *Smyrniium rotundifolium*) the dormancy level was very high (< 6% germination in the

control), whereas another four species (*Alyssum caricum*, *A. minus*, *Rumex crispus*, *Silene vulgaris*) had very low levels of dormancy (> 90% germination in the control). Smoke and nitrate treatments failed to break high-level dormancy (zero germination in the control) in *Muscari comosum* and *Smyrniium rotundifolium*. Moreover, germination in the three species with no dormancy (*Alyssum minus*, *Rumex crispus*, *Silene vulgaris*) was not affected by any of the treatments (Table 3).

Smoke treatments resulted in a significant increase in germination of three species, but affected negatively germination of *Hypericum aviculariifolium* (Table 3). Of the 12 species studied, five showed significant improvement in germination after at least one of the nitrate treatments as compared with the control (Table 3). Regarding the effect of nitrates on germination, 10 mM KNO_3 was the most effective treatment in which germination of five species was stimulated. The remaining nitrate treatments were the least effective ones, as germination of only two species was stimulated in each. However, none of the nitrate treatments affected germination of the study species negatively (Table 3).

Germination response of the species to smoke and nitrate treatments varied. Only in two species (*Onopordum caricum* and *Sarcopoterium spinosum*) germination was improved both by

Table 3. Mean germination percentages (\pm SE) in the control, and in the smoke and nitrate treatments. For species codes see Table 1. In parentheses are p values for pairwise comparisons of treatments with the control (analysis of deviance). Values that are significantly different from the control (at $p < 0.01$) are given boldface. “–” indicates that the treatment was not performed due to limited number of seeds.

Species	Control	Smoke solution		KNO_3		NH_4NO_3	
		1:10	1:100	10 mM	25 mM	10 mM	25 mM
DBR	28 \pm 6	20 \pm 6 (n.s.)	34 \pm 4 (n.s.)	48 \pm 5 (0.005)	41 \pm 8 (n.s.)	24 \pm 4 (n.s.)	25 \pm 6 (n.s.)
DCA	78 \pm 5	93 \pm 3 (0.003)	80 \pm 5 (n.s.)	83 \pm 2 (n.s.)	83 \pm 5 (n.s.)	84 \pm 5 (n.s.)	85 \pm 3 (n.s.)
SRO	0	0	0	0	0	0	0
CDE	5 \pm 2	10 \pm 7 (n.s.)	–	50 \pm 7 (< 0.001)	–	35 \pm 7 (< 0.001)	–
OCA	3 \pm 1	37 \pm 2 (< 0.001)	18 \pm 3 (< 0.001)	18 \pm 5 (< 0.001)	25 \pm 4 (< 0.001)	28 \pm 8 (< 0.001)	35 \pm 6 (< 0.001)
ACA	92 \pm 3	98 \pm 2 (n.s.)	98 \pm 1 (n.s.)	100 \pm 0 (< 0.001)	–	94 \pm 2 (n.s.)	–
AMI	100 \pm 0	100 \pm 0 (n.s.)	100 \pm 0 (n.s.)	100 \pm 0 (n.s.)	100 \pm 0 (n.s.)	100 \pm 0 (n.s.)	100 \pm 0 (n.s.)
SVU	100 \pm 0	99 \pm 1 (n.s.)	100 \pm 0 (n.s.)	98 \pm 2 (n.s.)	100 \pm 0 (n.s.)	100 \pm 0 (n.s.)	100 \pm 0 (n.s.)
HAV	48 \pm 7	23 \pm 7 (< 0.001)	23 \pm 14 (< 0.001)	55 \pm 6 (n.s.)	47 \pm 4 (n.s.)	53 \pm 5 (n.s.)	60 \pm 1 (n.s.)
MCO	0	0	0	0	0	0	0
RCR	99 \pm 1	99 \pm 1 (n.s.)	99 \pm 1 (n.s.)	99 \pm 1 (n.s.)	100 \pm 0 (n.s.)	99 \pm 1 (n.s.)	100 \pm 0 (n.s.)
SSP	15 \pm 3	68 \pm 5 (< 0.001)	62 \pm 4 (< 0.001)	39 \pm 6 (< 0.001)	34 \pm 3 (0.002)	23 \pm 8 (n.s.)	32 \pm 3 (0.005)

smoke and nitrate. The remaining species had different responses to smoke and nitrate treatments. For example, germination of *Carthamus dentatus* and *Daucus broteri* was enhanced by nitrate but not by smoke; inversely, germination percentage in *Daucus carota* was increased only by smoke. Nitrate applications did not decrease the germination percentage in comparison with that in the control in *Hypericum aviculariifolium*, the only species in which germination was negatively affected by smoke. An association between regeneration mechanism of species and their germination response to smoke and/or nitrate treatments was found. That is, the species with germination enhanced by smoke and/or nitrate were all propagule-persisters (P+), and non-stimulated species were all propagule non-persisters (P-) except one in which regeneration mechanism is unknown (Table 3).

Apart from *Daucus broteri* in which MGT increased after a smoke treatment (1:100), smoke treatments did not significantly change MGT in the remaining species (Table 4). *Sarcopoterium spinosum* germinated significantly faster in the lower concentrations of nitrate than in the control. On the other hand, nitrate treatments resulted in slower germinations in *Onopordum caricum* and *Daucus broteri*. No significant changes in MGT were found in other species after the nitrate treatments.

Discussion

The smoke and nitrate treatments significantly increased the germination percentages in six of the 12 studied species (*Alyssum caricum*, *Carthamus dentatus*, *Daucus broteri*, *D. carota*, *Onopordum caricum* and *Sarcopoterium spinosum*). Of the study species, only germination of *Hypericum aviculariifolium* was negatively affected by smoke. As far as we know, our results provide the first evidence of smoke- and nitrate-stimulated germination in *Onopordum caricum*, which is endemic to Turkey. This result may have implications to *in-situ* and/or *ex-situ* conservation of endemic plants in Mediterranean vegetation in Turkey.

Availability of nitrogenous compounds can increase in the soil during the first rainy season following fire (Thanos & Rundell 1995) and, in Mediterranean regions, prolific germination of many plants generally occurs during this period (Thanos & Georghiou 1988, Bell *et al.* 1995). Consequently, many researchers focused on the role of nitrogenous compounds in post-fire germination (Thanos & Rundel 1995, Bell *et al.* 1999, Henig-Sever *et al.* 2000). The results of those studies indicate that nitrate and ammonium can play an important role in germination of several plants (e.g., *Emmenanthe penduliflora*, *Phacelia grandiflora*). However, Keeley

Table 4. Mean (\pm SE) mean germination times (days) in the control, and in the smoke and nitrate treatments. For species codes see Table 1. In parentheses are *p* values for pairwise comparisons of treatments with the control (Dunnett's test). Values that are significantly different from the control (at $p < 0.01$) are given boldface. "-" indicates that MGT was not obtained due to either low germination percentage or the lack of treatment for the germination experiment.

Species	Control	Smoke solution		KNO ₃		NH ₄ NO ₃	
		1:10	1:100	10 mM	25 mM	10 mM	25 mM
DBR	4.5 \pm 0.3	6.0 \pm 0.6 (n.s.)	9.3 \pm 2.0 (0.0067)	6.1 \pm 0.6 (n.s.)	11.7 \pm 1.6 (0.0002)	6.3 \pm 0.4 (n.s.)	10.3 \pm 1.5 (0.0010)
DCA	5.6 \pm 0.1	4.7 \pm 0.2 (n.s.)	4.9 \pm 0.4 (n.s.)	6.3 \pm 0.3 (n.s.)	6.1 \pm 0.2 (n.s.)	5.4 \pm 0.3 (n.s.)	5.5 \pm 0.2 (n.s.)
SRO	-	-	-	-	-	-	-
CDE	5.3 \pm 0.7	4.0 \pm 0 (n.s.)	-	4.4 \pm 0.2 (n.s.)	-	4.3 \pm 0.1 (n.s.)	-
OCA	2.0 \pm 0	2.6 \pm 0.2 (n.s.)	3.2 \pm 0.4 (n.s.)	3.4 \pm 0.4 (n.s.)	3.4 \pm 0.2 (n.s.)	3.8 \pm 0.2 (0.0062)	4.0 \pm 0.1 (0.0017)
ACA	2.1 \pm 0	2.0 \pm 0 (n.s.)	2.0 \pm 0 (n.s.)	2.0 \pm 0 (n.s.)	-	2.1 \pm 0 (n.s.)	-
AMI	2.0 \pm 0	2.0 \pm 0 (n.s.)	2.0 \pm 0 (n.s.)	2.0 \pm 0 (n.s.)	2.0 \pm 0 (n.s.)	2.0 \pm 0 (n.s.)	2.0 \pm 0 (n.s.)
SVU	2.1 \pm 0.1	2.1 \pm 0.1 (n.s.)	2.1 \pm 0.1 (n.s.)	2.0 \pm 0 (n.s.)	2.0 \pm 0 (n.s.)	2.0 \pm 0 (n.s.)	2.0 \pm 0 (n.s.)
HAV	8.5 \pm 0.2	8.7 \pm 0.3 (n.s.)	8.4 \pm 0.9 (n.s.)	9.2 \pm 0.4 (n.s.)	9.5 \pm 0.3 (n.s.)	8.2 \pm 0.4 (n.s.)	8.4 \pm 0.2 (n.s.)
MCO	-	-	-	-	-	-	-
RCR	2.4 \pm 0.1	2.5 \pm 0.1 (n.s.)	2.4 \pm 0.1 (n.s.)	2.6 \pm 0.2 (n.s.)	3.0 \pm 0.3 (n.s.)	2.4 \pm 0.1 (n.s.)	2.5 \pm 0.1 (n.s.)
SSP	16.1 \pm 1.5	11.9 \pm 0.5 (n.s.)	13.3 \pm 0.8 (n.s.)	9.6 \pm 0.8 (0.0039)	11.9 \pm 2.0 (n.s.)	9.9 \pm 0.7 (0.0067)	10.7 \pm 1.1 (0.0235)

and Fotheringham (1998) reported that KNO_3 solutions indeed stimulate germination in *E. penduliflora* and *P. grandiflora*, but only at low pH. Accordingly, they proposed that both oxidizing gases in smoke and acids are potential germination triggers for these post-fire annuals. On the other hand, Pérez-Fernández *et al.* (2006) showed that germination of their study species (mainly herbaceous plants) was not affected by the concentration of H^+ with or without the nitrate ion. Although our study cannot be compared with the above ones since our experimental design did not include a pH gradient, our results indicate that germination success of species is variable in smoke and nitrate treatments at the same pH level (in our case $\text{pH} = 5.8$).

The mechanism of improving seed germination by nitrogenous compounds was investigated by Henig-Sever *et al.* (2000). The results of their study suggest that nitrate increases the seed sensitivity to GA_3 (gibberellic acid), whereas ammonium directly affects lipase activity. Germination induction by nitrates is achieved in the concentration range up to 50 mM, while higher concentrations have a negative effect (Thanos & Rundel 1995, Bell *et al.* 1999, Pérez-Fernández & Rodríguez-Echeverría 2003). The concentrations we applied in our study were within this range, so this could explain why we found no negative effects of any nitrate treatment on the germination of the studied species.

The association between the capacity to recruit after fire (P+, P-) and germination response of species suggests that smoke- and nitrate-stimulated germination are important determinants of the regeneration strategy in Mediterranean plants. In the Mediterranean Basin, fire-prone plant communities are phylogenetically and phenotypically clustered as a result of the filtering processes of fire proceeding on the species that have fire-persistent traits (Pausas & Verdú 2008). It has been shown that flammability-enhancing traits in post-fire seeders (P+) vary adaptively in response to fire (Moreira *et al.* 2014) by producing the necessary cues for triggering germination from the seed bank and opening spaces for the regeneration of the offspring (Pausas & Moreira 2012). Moreover, propagule persistence (P+) is an evolutionary-conserved trait at the genus and family levels

since the appearance of Mediterranean climate in the region (Verdú & Pausas 2007). Therefore, many families in which P+ trait is observed include species with enhanced post-fire germination (Çatav *et al.* 2014) and post-fire establishment (Tavşanoğlu & Gürkan 2014). Paula and Pausas (2008) also demonstrated the higher capacity of fire stimulated germination in non-resprouters (R-) than in resprouters (R+). However, since our study included just a dozen species, the results on regeneration mechanism should be considered carefully.

In summary, our results reveal that both smoke and nitrate play a role in germination stimulation of Mediterranean species, at least for the subset of the studied species. Our results also suggest the presence of species-specific germination response to smoke and nitrates in Mediterranean plants.

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