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Mediterranean woody plant specialized metabolites affect germination of *Linum perenne* at its dry and upper thermal limits

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Abstract

Aims: Soil temperature and moisture impact plants not only during growth and survival but also during seed germination and interaction of seeds with the chemical environment. The quantitative impacts of either temperature and moisture or plant specialized metabolites (PSM) on germination are widely studied. However, the combined effect of PSM and moisture or temperature on germination remains poorly understood.

Methods: We addressed this issue by studying the effect of PSM extracted from four Mediterranean woody plants on germination speed and final percentages of a subordinate herbaceous plant, *Linum perenne*.

Results: By using hydro- and thermal time threshold models, we show how PSM interact with temperature and moisture levels to limit germination at dry and upper thermal limits, with the magnitude of effects depending on the source plant. PSM effects on germination, also observed on natural soils, persisted after their removal from the seed environment.

Conclusions: We conclude that the impact of climate change on reproduction of herbaceous plants can be modulated by effects of PSM from woody plants, which might exacerbate the negative impacts of global changes on biodiversity.

Keywords: Plant Specialized Metabolites, allelopathy, allelochemicals, climate change

Introduction

Climate change induces unpreceded warming and drought in many parts of the world (Giorgi and Lionello 2008; Hoerling et al. 2012) and modify ecosystems not only by altering adult plant growth and survival but also by modifying plant regeneration and plant-plant interactions (Klanderud 2005). Plant-plant interactions are sensitive to climatic changes and include many more chemical interactions involving plant specialized metabolites (PSM) than previously thought (Fernandez *et al.,* 2015, Rasman and Agrawal, 2011). Germination is particularly sensitive to PSM (Fernandez et al. 2013) and also shows a fine-tuned sensibility to soil water potentials and temperatures that decide on its success (Bradford *et al.,* 2008). Whereas the effects of PSM on germination have been largely studied for a large range of source and target species (Fernandez et al. 2006; Flematti et al. 2004; Herranz et al. 2006; Jefferson and Pennacchio 2003; Ruprecht et al. 2008; Souto et al. 2001), we lack knowledge on how chemical cues affect the seed environment and seed sensibility under different warming and drought conditions.

A comprehensive way to study the effects of warming and drought on the seed environment consists of using population-based threshold models (Bradford 2002; Huang et al. 2016), since they can capture the effect of chemicals in the seed environment (Bradford et al. 2008; Liptay and Schopfer 1983; Ni and Bradford 1992). Population-based models capture the limiting effects of exceeding cold, warm or dry conditions by using base temperature (T_b) as minimal temperature threshold, ceiling temperature (T_c) as maximum temperature threshold and base water potential (Ψ_b) as minimum moisture threshold parameter (Bradford 2002). Together with a time and dispersion parameter, germination speed and final percentages can be modeled for any temperature or water potential (Bradford 2002). Since threshold parameters encapsulate the response to the entire range of temperature and water conditions for germination, they are emerging functional traits for regeneration processes (Arène et al. 2017; Saatkamp et al. 2019) and give a deep insight on how

germination is affected by both, climatic conditions of the environment and the chemical environment (Bradford et al. 2008). Until now, there has been no attempt to use germination threshold models to study the effects of PSM and to reveal how climatic changes might interact with PSM during plant regeneration.

PSM importantly explain how plants interact with their biotic and abiotic environment (Rasmann and Agrawal 2011; Williams et al. 1989). However, the difficulties to study the seed environment below ground and at the soil surface during germination (Saatkamp et al. 2011; Saatkamp et al. 2019) limit our understanding on the role of chemical cues in nature until now. Release of PSM exert toxic or stimulatory effects on other plants (Rice 1984) and in this way changes the outcome of competition and the reproduction of competing plants by increasing the fitness of source plants (Rasmann and Agrawal 2011; Williams et al. 1989). However, recent works also show that chemical interactions vary between ecosystems and according to subtle changes in soil moisture and temperature (Blanco 2007). Temperature and water stresses may increase the production of PSM in the environment (Allemann et al. 2016; Briggs et al. 2009; Einhellig 1996; Lobón et al. 2002; Ma et al. 2015; Melkania 1992; Wang et al. 2011) and increase the toxicity of PSM (Allemann et al. 2016; Einhellig and Eckrich 1984; Gatti et al. 2014; Oueslati et al. 2005; Wang et al. 2011). Interestingly, also a plant's sensitivity to PSM can be modified by environmental factors (Einhellig 1996; Lobón et al. 2002; Wang et al. 2011). At the level of seed physiology, PSM can act as plant growth inhibitors (Fernandez et al. 2015; Hashoum et al. 2017; Scognamiglio et al. 2013), in an analog way to abscisic acid (ABA). ABA is a well-known signal substance in plants that inhibits germination and has opposite roles compared to gibberellic acid (GA3) in regulating germination (Holdsworth et al. 2008; Vesty et al. 2016). Moreover, ABA has been shown to play a role in limiting germination at high temperatures (Benech-Arnold et al. 2006) by decreasing germination speed and final percentages

72 (Bradford et al 2008). Studying PSM together with exposure to GA3 may hence increase our
73 understanding of the mechanisms behind the interactions of PSM with climatic extremes.
74 In Mediterranean forests, PSM released into the environment play a prominent role at the ecosystem
75 level, by inhibiting germination and growth of neighboring plants, and modifying soil communities
76 (Fernandez et al. 2015; Hashoum et al. 2017). Emission of volatile organic compounds and leaching
77 rate of PSM are particularly high and respond strongly to climatic changes in the Mediterranean
78 (Chomel et al. 2016; Fernandez et al. 2015; Gavinet et al. 2018). While PSM attain target plants by
79 volatile emissions, root exudates, leaching from green leaves or during decomposition of senescent
80 leaves (Herranz et al. 2006; Rice 1984; Ruprecht et al. 2008), some authors stress that the main PSM
81 involved in chemical plant-plant interactions are water-soluble (Vyvyan 2002). Mediterranean
82 woody plants, such as *Quercus pubescens* Willd. (Fagaceae), *Pinus halepensis* Mill. (Pinaceae), *Acer*
83 *monspessunalum* L. (Aceraceae) and *Cotinus coggygria* Scop. (Anacardiaceae) are known for the
84 inhibitory effect of their PSM on both, germination and growth (Fernandez et al 2013; Fernandez et
85 al. 2016; Hashoum et al 2017). Climate in the Mediterranean is characterized by rapid transitions
86 between dry summers and moist winters and a high inter-annual variability in rainfall. Climate
87 models also agree on increasing drought conditions for the next decades in this area (Hoerling et al.
88 2012). Mediterranean forest ecosystems are hence a prominent model to study how PSM interact
89 with temperature and drought and at which level PSM inhibit seed germination, interacting with
90 hormone levels inside seeds or with substrate characteristics.
91 The main objective of this study is hence to unravel the mechanisms that link the climatic niche of
92 seed germination to different types of PSM in Mediterranean ecosystems. More specifically, we
93 address whether (1) PSM from the leaves of Mediterranean woody plants interact with temperature
94 and water in their effect on germination speed and percentages of a subordinate herbaceous plant,
95 *Linum perenne* L., (2) natural soils reduce the effect of aqueous PSM leaf extracts on germination,

(3) the duration and timing of seed exposure to PSM impact their effects on germination niche and, (4) PSM may counteract the effects of GA3 and inhibit germination in a similar way to ABA.

Material and Methods

Seed material

We used seed material from *Linum perenne* variety *'Lin vivace bleu'* purchased from Vilmorin SA (La Ménitré, Limagrain, France). *L. perenne* is distributed in temperate and Mediterranean Europe and overlaps substantially with the distribution areas of *Q. pubescens, A. monspessulanum, C. coggygria* and *P. halepensis*. Moreover, this species occurs naturally in forest margins and semi-open habitats associated with these woody plants.

Plant specialized metabolites

Green leaves from the three dominant woody plants naturally present in Downy oak forests were collected at the Oak Observatory at the *Observatoire de Haute Provence* (OHP) located 60 km north of Marseille, South of France (43°56'115" N, 05°42'642" E). The site is 680m above sea level and presents a mean annual temperature of 11.9 °C and a mean annual precipitation of 830 mm (1967-2000). This forest was managed for centuries by coppicing, dominated by Downy oak (*Q. pubescens*; 75% coverage) and Montpellier maple (*A. monspessulanum*; 25% coverage), with understory vegetation dominated by smoke tree (*C. coggygria*; 30% coverage); Aleppo pine (*P. halepensis*) occurs in initial successional stages on drier and warmer habitats around the study site and might increase due to climate change.

In order to study changes in PSM during senescence we also sampled senescent leaves from *C. coggygria*. A previous study identified senescent leaves in *C. coggygria* to have a markedly different chemical composition and a significantly stronger allelopathic effect compared to the other species

120 (Gavinet et al. 2019; Hashoum et al. 2017). Leaves and needles were harvested in summer 2013 at
121 the OHP and then kept in the freezer at - 20°C until the beginning of the experiment.
122 Since PSM are mainly released into the environment through rain and dew leachates transferring leaf
123 compounds to the soil (Herranz et al. 2006; Rice 1984; Ruprecht et al. 2008), we chose to mimic
124 natural leachates in this study through foliar aqueous extracts from fresh and senescent leaves.
125 Leaves of the four sources species (*Q. pubescens*, *A. monspessulanum*, *C. coggygria* and *P.*
126 *halepensis*) were soaked in deionized water to obtain 1 % aqueous extracts, we standardized by dry
127 weight of leaves after drying parts of the sample for 72h in an oven at 60°C. Extracts were left at
128 room temperature in darkness for 24 hours. This protocol enables to extract a maximum of PSM by
129 limiting their degradation (Souto et al. 1994). They were then filtered through a filter paper
130 (Whatman No 1). Extracts were stored at 4 °C until the start of the experiments.
131 We measured the osmolality (mOsmol/kg H_2O) of all extracts using a micro-osmometer from
132 Roebling (Type13/13DR – Autocal) to ensure that the inhibitory effects observed on germination
133 rate and growth were not due to osmotic pressures but to PSM (Anderson and Loucks 1966). The
134 osmometer was recalibrated every 10 samples with deionized water and certified solution of 300
135 mOsmol/kg H_2O.
136
137 *Preparation of Polyethylene glycol (PEG) solutions*
138 Water potentials used in our experiments were simulated by the use of PEG8000 according to the
139 equations provided by Michel (Michel 1983) taking into account the temperatures of incubation
140 chambers and setting the PEG concentrations in order to obtain water potentials of -0.5, and -0.2
141 MPa and 0 MPa (pure water). PEG solutions were then added into Petri dishes containing two
142 Whatman No 2.
143

144

145 *Bioessays*

146 Five different germination experiments (summarized in Table 1) were carried out to meet the
147 objectives of our study. All germination experiments were done with Petri dishes, which were
148 regularly controlled and watered. Petri dishes were sealed when water potentials were controlled by
149 PEG solutions. Growth chambers were used to perform germination experiments under controlled
150 conditions of constant temperature and 12h of light (cool white fluorescent tubes, ± 10 000 lux; ±
151 250 µmol $m^{-2}.s^{-1}$). Germinations were controlled daily during the first week, then once per week until
152 the end of the experiment. Seeds with extruding white radicles were counted and removed on every
153 control. Germination experiments lasted 3-4 weeks according to speed of germination in the different
154 conditions and were stopped when 90% of germination was reached. We did not remove seeds that
155 just split their seed coats open, as in *L. perenne* there can be 1-2 days delay between seed coat
156 splitting and appearance of the radicle. For all experiments and for each treatment, four replicates of
157 25 seeds were used, extracts were applied once at the first watering and, as a control, seeds were
158 watered with deionised water. All seeds sown without PEG solutions were regularly watered with
159 deionised water. Petri dishes with PEG-solutions were filmed. The experiments lasted between 21
160 and 42 days in order to reach the maximum possible germination for each tested condition.

161 *Interaction of PSM, temperature and water potential*

162 In order to test the interaction of PSM with temperature and water potential conditions on
163 germination, we varied PSM concentration, temperature and water potentials experimentally in
164 germination tests (Experiment 1 and 2 in Table 1). First, we studied the effects aqueous extracts of
165 green leaves from all source species (*A. monspessulanum, P. halepensis, Q. pubescens,* and *C.*
166 *coggygria)* on germination using temperatures of 10, 15, 20 and 25 °C. We also included
167 germination trials at 5, 18, 22 and 30 °C using extracts of *A. monspessulanum* to complement our

168 data. The range of temperatures covered temperatures during the germination season in the habitat of
169 source and target species. Second, we studied the interactions of reduced water potentials using PEG
170 concentrations equivalent to -0.2 and -0.5 MPa at temperatures of 10, 15, 20, and 25 °C, in order to
171 know if PSM, temperature and reduced water potential interact on limiting seed germination. We
172 also aimed to quantify the differences between green and senescent leaves by adding senescent
173 leaves of *C. coggygria* using temperatures of 5, 10, 15, 18, 20, 22, 25 and 30 °C with reduced water
174 potential equivalent to -0.2 and -0.5 MPa at 15, 20, 22, 25 and 30 °C.
175
176 *Interaction of exposition time and substrate with PSM effects*
177 One of our objectives was to test whether a temporary exposition to PSM still had a limiting effect
178 on germination. To this end, we temporarily exposed 25 seeds in four replicate Petri dishes per
179 treatment to green leaf extracts of *A. monspessulanum*. *A. monspessulanum* was one of the source
180 species with the strongest inhibiting effect on germination (Experiment 3 in Table 1). In this setting,
181 seeds were exposed to water in the beginning, then from day 4 to day 10 to leaf extracts of *A.*
182 *monspessulanum* and then again to water. For a second treatment, we first exposed seeds to leaf
183 extracts of *A. monspessulanum* and then from day 4 to day 10 to water and then again to leaf
184 extracts. We also used two control treatments: one where seeds where only watered with deionised
185 water and another with seeds being permanently exposed to leaf extracts of *A. monspessulanum.* All
186 treatments were kept at 25 °C constant temperature.
187 Moreover, we also wanted to know if PSM interact with natural soil. Therefore, we compared
188 germination of *L. perenne* on filter paper with germination on natural soil from the study site as
189 substrate (Experiment 4 in Table 1). Natural soil from the study site is a clay rich, mollic leptosol
190 (IUSS Working Group 2006), with pH 7.5 and low active lime content. We performed this
191 experiment at 25 °C. In this experiment, we used 25 seeds in four replicate Petri dishes per treatment.

192

193 *Interaction of gibberellic acid (GA3) and PSM on germination at high temperature*

194 In order to test the interaction of PSM and gibberellic acid on germination, we performed bioassays
195 using seeds that had been in contact with GA3 at 50 ppm during 24 h and, as a control, seeds without
196 GA3 treatment (Experiment 5 in Table 1). After that, seeds were divided and sown either with leaf
197 extracts of *A. monspessulanum* or with water as a control during 3 weeks at 25 °C.

198

199 **Table 1:** Description of the five germination experiments and their respective objectives carried out
200 in this study. PSM of four source species were extracted: *Quercus pubescens, Pinus halepensis, Acer*
201 *monspessulanum* and *Cotinus cogyggria.* Green leaves were used for the four species and scenescent
202 leaves were also studied for *Cotinus cogyggria*. For experiment 1, we additionally included data
203 from 5, 18, 22 and 30°C for the treatment with extracts from *Acer monspessulanum* leaves; all
204 experiments were performed with 25 seeds in each of four replicate Petri dishes per treatment.

No	Objectives	Methods
1	Quantify interaction between plant specialized metabolites (PSM) and temperature for germination	Germination trials at 10, 15, 20 and 25°C with PSM of four woody plants* and a control treatment
2	Quantify interaction between PSM and moisture	Germination trials at 10, 15, 20, 22 and 25°C PEG concentrations equivalent to 0, -0.2 and -0.5 MPa
3	Test reversibility and resilience of PSM effects during germination	Germination trial at 25°C exposition to *Acer monspessulanum* extracts continuously for 21 days, from day 4 to day 10, until day 4 and after day 10 and no exposition control
4	Test if soil vs filter paper modify PSM effects	Germination trials at 25°C on filter paper and on natural soil, with and without extracts from *Acer monspessulanum*
5	Test interaction between GA3 and PSM	Germination trials at 25°C with and without *Acer monspessulanum* extracts combined with and without exposition to 50 ppm GA3 during 24h

205

206

207 *Germination modelling*

208 We used thermal time and hydrotime modelling to study the effects of different leachates on the
209 germination behavior of *L. perenne* seed populations and more specifically how the relationship
210 between seed environment – seed germination is influenced by PSM. Thermal time and hydrotime
211 models of germination use the parameters of temperature and water potential threshold values, as
212 well as parameters for speed and dispersion in order to predict the germination timing over the entire
213 range of temperature and water potentials used in the experiments. These models also synthesize
214 information from many different germination trials into few ecological meaningful parameters. This
215 enabled us to compare effects of PSM on germination in a comprehensive way. Since our data
216 showed non-linear trends of base water potentials to temperature (Fig. 4A), we decided not to use
217 hydro-thermal time models that require a linear relation. Hence, we used three separate germination
218 models: (i) suboptimal thermal time models, (ii) hydrotime models, (iii) and supraoptimal thermal
219 time models. For these three models, we compared parameters fitted for germination data from pure
220 water and from each PSM treatment.

221

222 (i) Suboptimal thermal time models were used for all germination data from 5° to 18°C. We decided
223 to use 18 °C as cut-off value since previous analysis of temperature effects on germination speed
224 (measured as the inverse of time needed for 50 % of seeds to germinate) showed a linear increase
225 until 18 °C. The cumulative germination proportions (G) were modelled as a function of temperature
226 and time to germination (t_g) according to equation 1, we linearized germination proportions using
227 probit-transformation.

228

$$\text{probit}(G) = \frac{\log10\left((T_{env} - T_b)t_g\right) - \log10\left(\theta_{TT}\right)}{\sigma_{\theta_{TT}}} \qquad \text{(eq. 1)}$$

229

230

231 This model has three free parameters: base temperature T_b [°C], thermal time constant θ_{TT} [°C x
232 days] and a dispersion parameter σ_{TT} capturing the dispersion around θ_{TT} measured on the scale of
233 thermal time. We estimated T_b [°C], thermal time constant θ_{TT} [°C x days] and σ_{TT} using maximum-
234 likelihood by minimizing sum log-likelihood of differences between observed and estimated probit-
235 transformed germination proportions (probit(G)). We used the log-transformed thermal time to
236 linearize the relationship between germination proportions and thermal time. We also retrieved 95%
237 confidence intervals using the quadratic method implemented in *confint* and *mle* functions in the
238 bbmle package in R (Bolker 2017).
239 (ii) Supra-optimal thermal time models were fitted to germination data using the regression of
240 probit-transform of germination proportions against supra-optimal thermal time.
241

$$\text{probit}(G) = \frac{T_{c(g)} - \theta_{TT}/t_g + T_{env}}{\sigma_{T_c}}$$

242 (eq. 2)

243

244 We estimated thermal time θ_{TT}, ceiling temperature T_c and its dispersion parameter σ_{Tc} using
245 maximum-likelihood by minimizing sum log-likelihood of differences between observed and
246 estimated probit-transformed germination proportions (probit(G)), note that the dispersion parameter
247 takes negative values because the slope of the relation between germination proportions and
248 temperatures above the optimum is negative. We used all experimental data from temperatures above
249 20 °C to model supra-optimal of germination of *L. perenne* seeds.

250 (iii) Hydrotime models were fitted for germination data at water potentials of 0, -0.2 and -0.5 MPa
251 simulated by different concentrations of PEG (see above) at temperatures of 15, 20 and 25°. We
252 discarded data from an experiment with PEG at 10°C because of too few germinations.
253 In this case, the probit transform of cumulative germination was modelled as a function of
254 environmental water potential (Ψ_{env}), time to germination (t_g) using the following equation:
255

$$\text{probit}(G) = \frac{\Psi_{b(g)} - \theta_H / t_g - \Psi_{env}}{\sigma_{\Psi_b}}$$

256 (eq. 3)
257
258 We estimated the parameters using maximum-likelihood by minimizing sum log-likelihood of
259 differences between observed and estimated probit-transformed germination proportions (probit(G)),
260 the estimated parameters were three: base water potential Ψ_b its dispersion parameter $\sigma_{\psi b}$ and
261 hydrotime constant θ_H [MPa x days].
262 We compared PSM effects on final germination percentages within specific temperature and water
263 potential treatments (Fig. 1C, 2C and 3C) using one-way analysis of variance followed by Tukey's
264 honest significant difference test, results of which we plotted as letters into figures.
265 Finally, we tested the interactions of PSM and GA3 using a two-way ANOVA on germination
266 percentages.
267

Results

Germination and thermal time models at sub-optimal temperatures

PSM significantly reduced germination speed in terms of thermal time needed for germination compared to the control experiment (Fig. 1A and B). Thus, the 95% confidence intervals of the thermal time constant θ_{T50} for *L. perenne* germination under influence of PSM of *A. monspessulanum* and senescent *C. cogyggria* leaves did not overlap with the θ_{T50} of the control treatment without PSM (Tab. 2). Contrastingly, the 95% confidence intervals of base temperature for germination T_b and dispersion $\sigma_{\theta T50}$ (slope in Fig. 1A) overlapped between control and all metabolite treatments, suggesting that germination speed and not temperature requirements are affected by PSM at sub-optimal temperatures. However, at suboptimal temperatures, PSM did not impact final germination percentages (Fig. 1C), albeit a slight increase could be detected for *P. halepensis* compared to extracts from senescent leaves of *Cotinus cogyggria*.

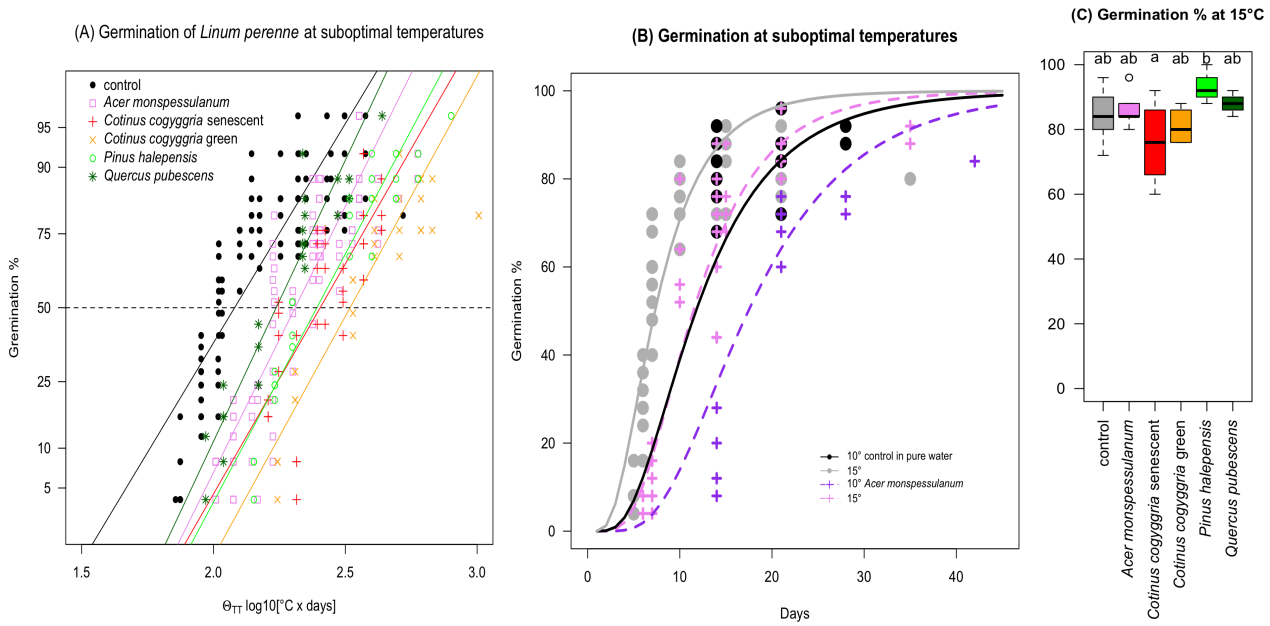


Fig. 1 (A) Effect of plant specialized metabolites (PSM) on germination speed of *L. perenne* in suboptimal temperatures in water; germination observations are standardized to log-thermal time, $\theta_{T(g)}$, using base temperature T_b according to Table 1, germination proportions are on probit-scale; coloured lines according to thermal time parameters in Table 1; **(B)** Example of observed germination dynamics and thermal time model for germination of *L. perenne* seeds in water and in *A. monspessulanum* green leaf extracts at 10 and 15°C; **(C)** Final germination percentages of *L. perenne*

at 15°C for control and five PSM treatments, letters indicate significant differences ($p < 0.05$) in a Tukey-test, PSM effect was significant in a one-way ANOVA ($p < 0.001$, $F = 3.54$).

Table 2 Maximum-likelihood estimation of thermal time model parameters for germination in the suboptimal temperature range (<20°C) for seeds of *L. perenne*. Plant specialized metabolites (PSM) treatments: Germination in pure water (N), with 1% aqueous extract of green leaves of *A. monspessulanum* (A), *C. cogyggria* (Cg), *Q. pubescens* (Q), *P. halepensis* (P) and 1% aqueous extract of senescent leaves of *C. cogyggria* (Cs); values in brackets are 95% confidence intervals.

PSM	Base temperature T_b [°C]	Thermal time constant θ_{T50} [°C x d]	Dispersion parameter $\sigma_{\theta T50}$ log10[°C x d]	R^2	N
control	0.04 (-1.90 - 1.97)	120.26 (102.62 - 137.90)	0.25 (0.22 - 0.28)	0.72	135
A	-2.00 (-4.76 - 0.76)	204.05 (165.93 - 242.17)	0.21 (0.18 - 0.23)	0.79	79
Cs	-2.68 (-7.45 - 2.10)	253.95 (172.55 - 335.36)	0.24 (0.16 - 0.32)	0.57	36
Cg	-14.15 (-31.54 - 3.24)	329.64 (118.91 - 540.37)	0.23 (0.19 - 0.26)	0.88	24
P	-13.46 (-33.49 - 6.56)	246.58 (63.21 - 429.95)	0.22 (0.19 - 0.25)	0.88	26
Q	-0.58 (-4.79 - 3.62)	172.94 (116.84 - 229.03)	0.19 (0.16 - 0.23)	0.88	21

Supra-optimal thermal time and ceiling temperature

Table 3 Thermal time model parameters for germination in the supra-optimal temperature range (>20°C) for seeds of *L. perenne*; Plant specialized metabolites (PSM) : Germination in pure water (N), with 1% aqueous extract of green leaves of *A. monspessulanum* (A), *C. cogyggria* (Cg), *Q. pubescens* (Q), *P. halepensis* (P) and 1% aqueous extract of senescent leaves of *C. cogyggria* (Cs).

PSM	Ceiling temperature T_{c50} [°C]	Thermal time constant θ_{Tc} [°C x d]	Dispersion parameter σ_{Tc} [°C]	R^2	N
control	37.92 (34.05 - 41.80)	102.98 (77.05 - 128.90)	-10.75 (-13.24 - -8.26)	0.78	143
A	24.59 (23.31 - 25.86)	45.53 (32.02 - 59.03)	-3.42 (-4.37 - -2.46)	0.56	56
Cs	23.34 (21.49 - 25.20)	53.66 (28.94 - 78.38)	-4.69 (-6.99 - -2.38)	0.51	30
Cg	28.33 (26.21 - 30.45)	70.62 (49.83 - 91.42)	-4.70 (-6.13 - -3.28)	0.80	30
P	56.10 (40.22 - 71.97)	249.36 (131.77 - 366.94)	-22.84 (-33.59 - -12.08)	0.93	46
Q	31.46 (28.33 - 34.58)	126.92 (86.52 - 167.32)	-6.12 (-8.10 - -4.14)	0.89	20

In contrast to suboptimal temperatures, at supra-optimal temperatures, PSM significantly affected not only germination speed but also final germination percentages (Fig. 2B and 2C). More precisely, final germination percentages at 25 °C were lowered compared to the control for compounds of *A. monspessulanum, C. cogyggria* senescent and green leaves as well as *Q. pubescens* but not for *P. halepensis*. Germination models showed that these lower final percentages were related to lower ceiling temperature (T_c) for germination (Table 3). This lowering of T_c for germination was accompanied by faster germination as indicated by the lower thermal time constants. Limitation of germination at high temperatures was much stronger with extracts of *A. monspessulanum* green leaves and senescent ones of *C. coggygria* compared to green leaves of *Q. pubescens* and *C. coggygria* aqueous extracts (Fig. 2A). Interestingly the application of PSM from *P. halepensis* increased Tc, enabling *Linum perenne* to germinate faster (Fig. 2A).

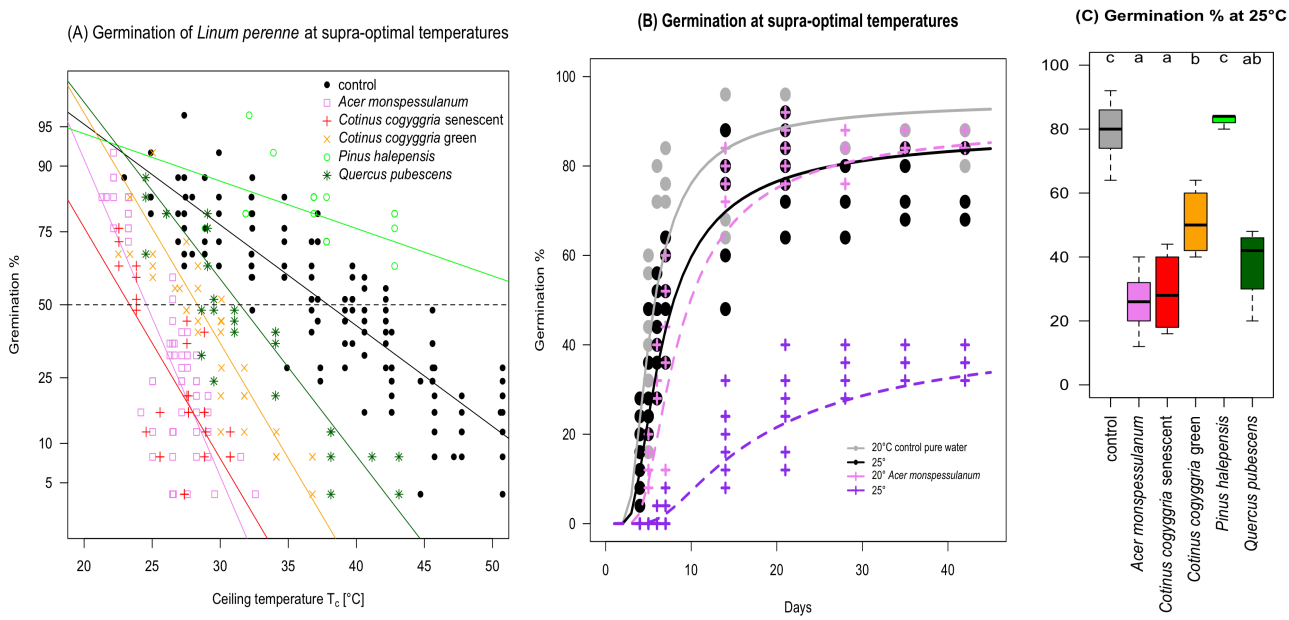


Fig. 2 (A) Effect of plant specialized metabolites (PSM) on high temperature limits for germination of *L. perenne* seeds in pure water; germination time courses are standardized to ceiling temperatures, T_c, using supra-optimal thermal time, note that the ceiling temperature for 50% germination, $T_{c(50)}$, corresponds to the intersection with the dashed line, slopes of regression lines equal $1/\sigma_{Tc50}$, percentages are on probit-scale; intersections of temperatures with the T_c indicate lower final germination percentages at that temperature; **(B)** Supra-optimal thermal time models and observed germination for *L. perenne* seeds under control and *A. monspessulanum* treatments; **(C)** Final germination percentages of *L. perenne* at 25°C under control and five PSM treatments, letters

indicate significant differences ($p < 0.05$, a<b<c) in a Tukey-test, PSM effect was significant according to a one-way ANOVA ($p < 0.001$, $F = 37.88$).

Hydrotime model

The hydrotime models using germination data obtained at different PEG solutions (Table 3 and Fig. 3) show that aqueous extracts from leaves of *C. coggygria, A. monspessulanum* and *Q. pubescens* increased base water potentials for germination (Table 4). This increase led to decreased final germination percentages when high PEG-concentrations were combined with extracts from *C. coggygria, A. monspessulanum* or *Q. pubescens* (Fig. 3B and C). This effect was accompanied by a decrease in the hydrotime constant θ_H which is negatively correlated to base water potential $\Psi_{b(50)}$ (Table 4). Extracts from needles of *P. halepensis* had the opposite effect, they decreased base water potential especially at 15 °C leading to faster and more complete germination.

Table 4 Hydrotime model parameters ($\Psi_{b(50)}$, $\sigma_{\Psi 50}$, θ_H) for germination at 15 °C for seeds of *L. perenne*; PSM: Germination in pure water (control), with 1% aqueous extract of green leaves of *A. monspessulanum* (A), *C. cogyggria* (Cg), *Q. pubescens* (Q), *P. halepensis* (P) and 1% aqueous extract of senescent leaves of *C. cogyggria* (Cs).

PSM	Base water potential $\Psi_{b(50)}$ [MPa]	Hydrotime θ_H [MPa x d]	$\sigma_{\Psi 50}$ [MPa]	R2	N
15°C					
control	-1.5 (-1.81 - -1.19)	11.22 (8.68 - 13.76)	0.63 (0.48 - 0.77)	0.83	130
A	-0.67 (-0.75 - -0.59)	6.68 (5.72 - 7.65)	0.27 (0.23 - 0.31)	0.84	81
Cs	-0.33 (-0.41 - -0.26)	3.52 (2.45 - 4.6)	0.22 (0.19 - 0.25)	0.86	34
Cg	-0.99 (-1.19 - -0.79)	9.72 (7.4 - 12.04)	0.43 (0.32 - 0.55)	0.88	35
P	-1.83 (-2.4 - -1.27)	14.14 (9.27 - 19.02)	0.65 (0.41 - 0.88)	0.89	44
Q	-0.87 (-1.05 - -0.7)	8.3 (6.26 - 10.34)	0.31 (0.23 - 0.39)	0.82	30
20°C					
control	-0.73 (-0.81 - -0.64)	4.24 (3.62 - 4.86)	0.36 (0.31 - 0.41)	0.81	116
A	-0.37 (-0.45 - -0.29)	3.26 (2.47 - 4.04)	0.26 (0.2 - 0.32)	0.72	62
Cs	-0.19 (-0.24 - -0.14)	2.36 (1.86 - 2.87)	0.2 (0.16 - 0.23)	0.86	25
Cg	-0.64 (-0.76 - -0.52)	5.11 (3.94 - 6.28)	0.28 (0.21 - 0.35)	0.76	38

P	-0.74 (-0.85 - -0.63)	4.85 (3.98 - 5.72)	0.39 (0.32 - 0.47)	0.87	54
Q	-0.62 (-0.72 - -0.51)	6.53 (5.13 - 7.93)	0.27 (0.21 - 0.33)	0.82	28
25°C					
control	-0.42 (-0.48 - -0.36)	3.01 (2.55 - 3.47)	0.34 (0.3 - 0.38)	0.81	97
A	0.13 (-0.29 - 0.02)	3.72 (1.78 - 5.66)	0.47 (0.29 - 0.64)	0.61	27
Cs	-0.1 (-0.36 - 0.16)	4.06 (-0.42 - 8.54)	0.21 (0.04 - 0.38)	0.36	8
Cg	-0.13 (-0.22 - -0.04)	3.59 (2.25 - 4.92)	0.3 (0.2 - 0.4)	0.72	19
P	-0.33 (-0.39 - -0.28)	2.7 (2.22 - 3.17)	0.26 (0.21 - 0.3)	0.83	40
Q	-0.03 (-0.21 - 0.15)	3.15 (0.73 - 5.58)	0.29 (0.15 - 0.43)	0.54	15

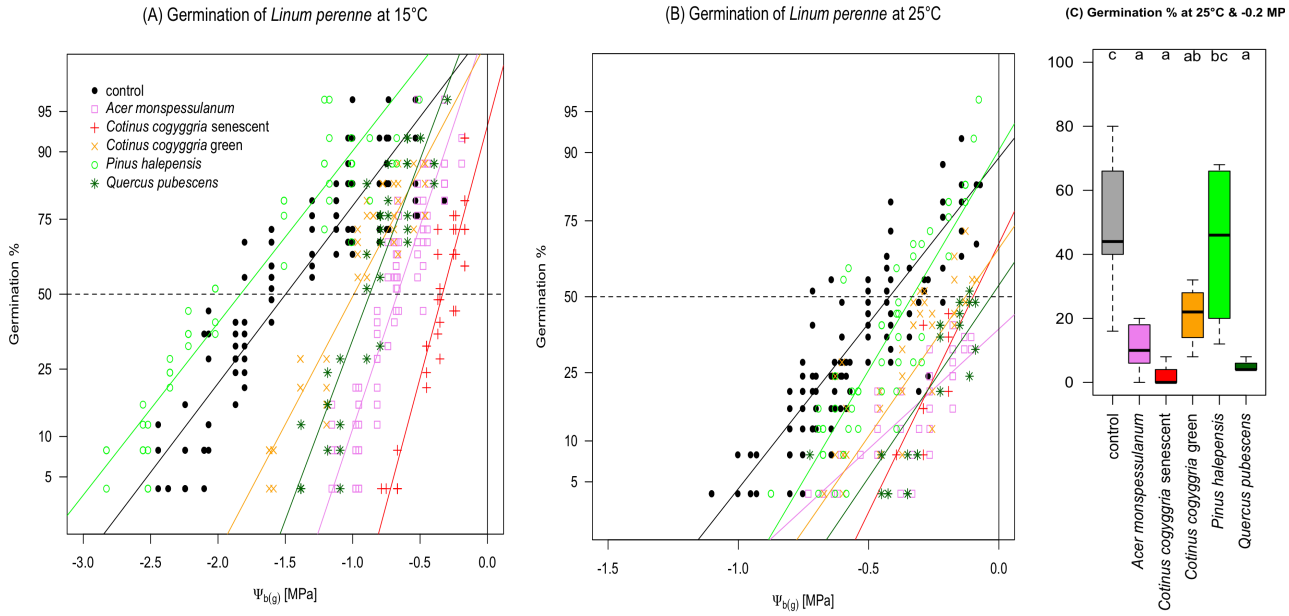


Fig. 3 Effect of plant specialized metabolites (PSM) on water requirements for germination of *L. perenne* seeds; (A) at 15°C (B) at 25°C; germination time courses are standardized to base water potentials, $\Psi_{b(g)}$, using hydrotime, note that the base water potential for 50% germination, $\Psi_{b(50)}$, corresponds to the intersection with the dashed line, slopes of regression lines equal $1/\sigma_{\psi 50}$, percentages are on probit-scale; intersections with the $\Psi_{b(g)} = 0$ line in (B) indicate lower final germination percentages in pure water; (C) Final germination percentages of *L. perenne* at 25°C and water-potential of -0.2 Mpa for control and five plant specialized metabolites (PSM) treatments, letters indicate significant differences ($p < 0.05$) in a Tukey-test, PSM effect was significant in a one-way ANOVA ($p < 0.001$, $F = 10.01$).

The impact of PSM on water requirements for germination depended on the temperature (Fig. 3A and 3B). At 15°C PSM differed clearly among species in their effect on water requirements for germination, increasing water potentials necessary for germination, except for pine needles (Fig.

3A). Contrastingly, at 25°C, only aqueous extracts of green leaves of *C. coggygria*, *A. monspessulanum* and *Q. pubescens* had a clear effect (Table 4). Particularly interesting was the effect of *Q. pubescens* leaves, which tended to a stronger effect on water requirements at high compared to low temperatures (Fig. 3). Final germination percentages decreased heavily when high temperatures were combined with the effects of PSM (Fig. 3C).

Moreover, temperature also affected base water potentials for germination (Fig. 4A) by increasing base water potentials for germination at 22° and 25°C compared to 15°C. However, we did not combine hydrotime with thermal time models since our data from water potential gradients at 10°C suggest a non-linear relationship between $\Psi_{b(50)}$ and temperature (Fig. 4).

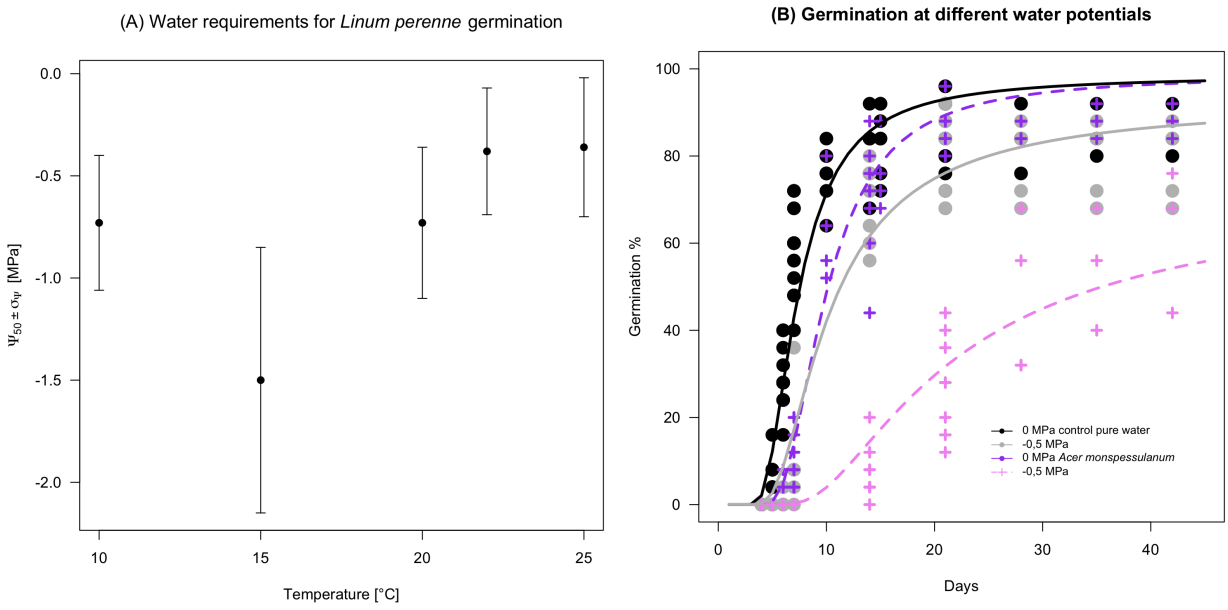
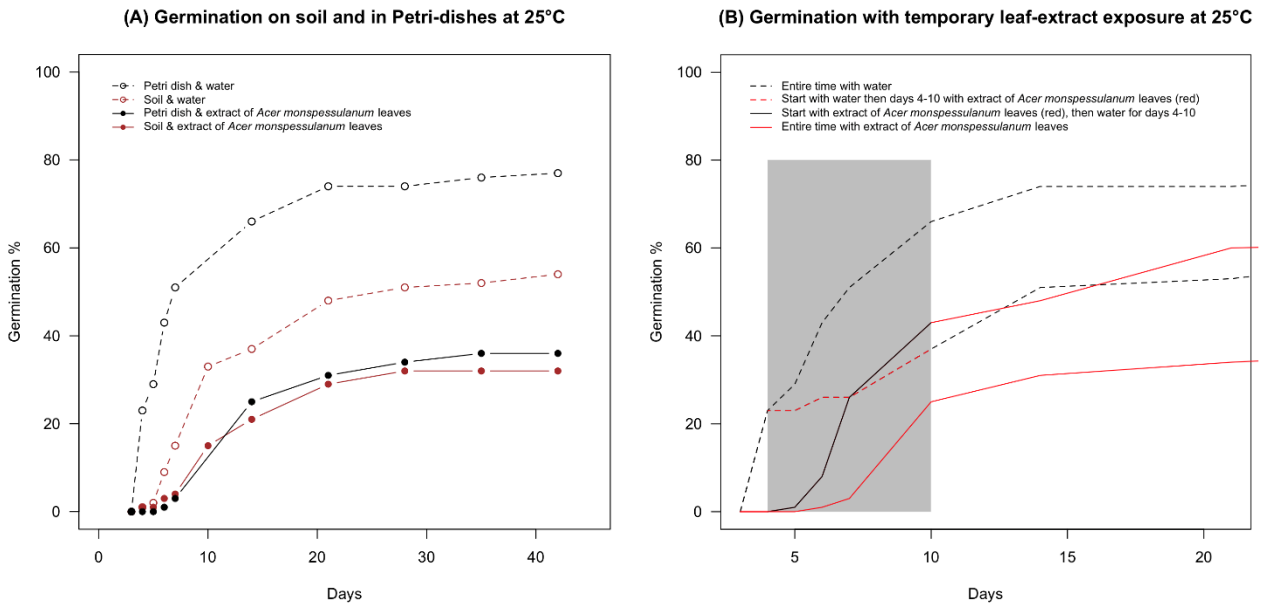


Fig. 4 (A) Change of base water potential with temperature for *L. perenne* seed germination without plant specialized metabolites; **(B)** Germination and hydrotime model for *L. perenne* seed germination in control and *A. monspessulanum* treatment for different water potentials fixed by polyethylene glycol.



375

376 **Fig. 5 (A)** Interaction of soil and plant aqueous extracts on germination of *L. perenne* seeds (mean of
377 four replicates); **(B)** Temporary exposure to aqueous *A. monspessulanum* leaf-extracts and
378 germination for *L. perenne* seeds, the grey zone corresponds to temporary condition inversion at days
379 4 to 10 (mean of four replicates).
380

381 Our germination tests using natural soil showed that *L. perenne* seeds germinated faster and to higher
382 percentages on filter paper compared to natural soil. Aqueous extracts of *A. monspessulanum* leaves
383 had similar effects on germination on soil and filter paper, with slower germination and lower final
384 percentages (Fig. 5A).

385 We also studied how a temporary exposition to *A. monspessulanum* leaf-extracts acted on
386 germination of *L. perenne* seeds, which indicated that beginning germination is halted (Fig. 5B) and
387 even after re-transfer to pure water did not recover initial percentages of germination. The inversed
388 conditions indicate that germination during phases of pure water after exposure to leaf-extracts is fast
389 and recovers partly compared to continuous exposure to leaf extracts (Fig. 5B). Interestingly,
390 germination speed is only altered in the inversed conditions compared to the continuous conditions,
391 and final percentages were intermediate and comparable for both temporary exposure treatments.

392 ***Interaction GA3 and PSM at high temperature***

393 The two-way ANOVA of germination percentages, GA3 and leave extracts of *A. monspessulanum*
394 showed a significant interaction: when GA3 was added together with leave extracts, germination
395 percentages were twice as high as with leave extracts alone and they approached the GA3 and
396 control treatments (Fig. 6).
397

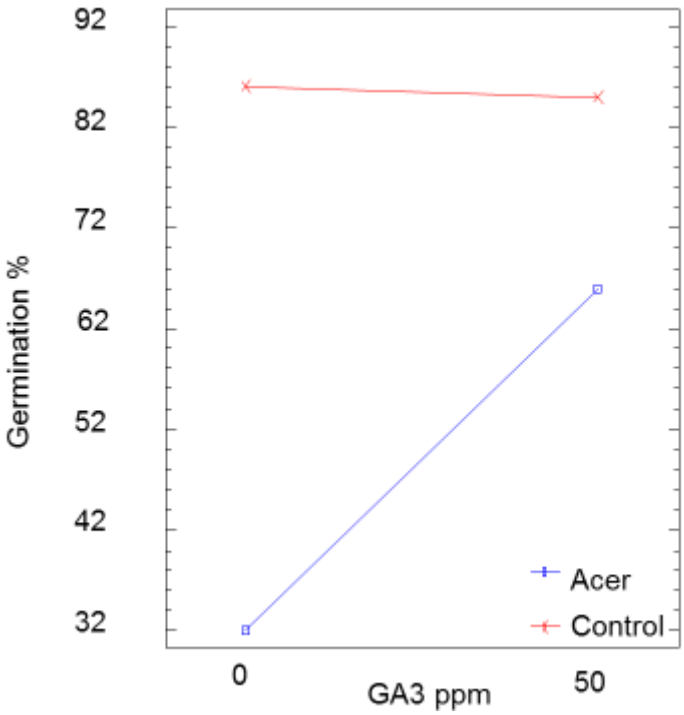


Fig. 6: Significant interaction of AG3 (50ppm) with aqueous *A. monspessulanum* leaf-extracts on germination of *L. perenne* seeds at 25°C (df = 1.12, F = 8.3, p = 0.0138).

398
399
400

401 **Discussion**

402 We showed that plant specialized metabolites (PSM) from leaf extracts of *Q. pubescens, A.*
403 *monspessulanum, C. cogyggria* and *P. halepensis* modified germination with increasing effects at
404 high temperature and low water potentials. When PSM extracts were added, *L. perenne* seeds thus

405 germinated under narrower conditions of water potential and upper range temperatures compared to
406 control experiments. Differences exist between PSM; for example aqueous extracts from *P.*
407 *halepensis* needles only affect little thermal and hydric conditions for germination compared to the
408 more limiting effect of *C. coggygria*, *A. monspessulanum* and *Q. pubescens*. The thermal time model
409 for temperatures below the optimum showed an increase in thermal time needed for germination
410 when PSM are added. Whereas the effect of PSM on germination timing has never been quantified,
411 these data join previous observations from Einhellig and Eckrich (1984) who found that the amount
412 of ferulic acid needed to inhibit germination in grain sorghum and soybeans was lower under higher
413 temperatures. Similarly, Ruprecht *et al.* (2008) found that the allelopathic effect of grasses on
414 germination of forbs was stronger at higher temperature. Together with our data, this suggest that
415 allelopathic effects tend to be stronger at higher temperatures for some ecosystems, a finding for
416 which we now provide a quantified model showing the gradual increase of PSM with increasing
417 temperatures.
418 Besides, our data highlight that PSM affect the use of water during germination of *L. perenne* seeds.
419 Aqueous extracts from *C. coggygria, A. monspessulanum* and *Q. pubescens* leaves increased the base
420 water potentials for germination of *L. perenne*. To date, no study has provided a quantitative model
421 on how PSM interact with lowered water potentials on germination. The only other work by
422 Einhellig (1989) showed that a lowered water availability interacted with PSM by increasing impacts
423 on germination. Our models now quantify, for several source-species, how water potentials and PSM
424 interact and highlight that germination percentages decrease still linearly with water potentials when
425 PSM are added (Fig. 3A).
426 Our analysis also suggests that high temperatures and low water potentials interact with PSM in their
427 effect on germination: *L. perenne* seeds germinate over much narrower conditions in terms of water
428 potentials and upper range temperatures in presence of PSM. Our data show how high temperatures

429 increase the water potential needed to trigger germination. In this setting, PSM have stronger effects
430 or even inhibit completely germination in combined temperature and drought stresses. No previous
431 work has considered the combined effects of PSM, temperature and drought on germination.
432 However the addition of both processes discussed above and their consistency with previous works
433 (Einhellig and Eckrich 1984; Ruprecht et al. 2008) suggest that increased effects of PSM at
434 simultaneously high temperatures and low water potentials are probably a general phenomenon.
435 Negative effects of PSM of a range of source species on seed germination have been documented for
436 various target plants (Bogatek et al. 2006; Herranz et al. 2006; Jefferson and Pennacchio 2003;
437 Ridenour and Callaway 2001; Ruprecht et al. 2008; Souto et al. 2001). Our data precise this picture
438 by showing that effects are smaller at low temperature and concern germination speed while at high
439 temperature and low moisture, PSM effects are strongest and impede a fraction of seeds to
440 germinate. Since there are many studies that document negative effects of PSM on germination, it
441 seems reasonable to assume that effects of PSM of four Mediterranean woody plants on seed
442 germination of *L. perenne* might also be observed in other systems of source and target species.
443 In our system, natural soil did not decrease the effect of aqueous leaf extracts, although several
444 works indicated that PSM might be adsorbed to soil particles or transformed to less active
445 compounds (Kobayashi 2004; Shaw et al. 2006). Aqueous extracts of *A. monspessulanum* leaves
446 have very similar effects on germination under soil and filter paper. However, *L. perenne* seeds
447 germinate faster and to higher percentages on filter paper compared to natural soil. This finding
448 corroborates the previous work by Herranz and coworkers (2006) who showed that soil from below
449 *Cistus* shrubs does not impede the effect of PSM and shows even stronger effect on germination
450 compared to simple aqueous extracts. These findings underline that the gradual increasing effect of
451 temperature and drought stresses together with PSM play a role in the field, and that PSM might
452 decrease germination in concert with climatic conditions for herbaceous plants in many ecosystems.

453 Finally, temporary exposure to leaf extracts can also impact germination, since leaf-extracts
454 decreased germination speed and final percentages and even after re-transfer into environments
455 without PSM, germination recovers only partly, a finding that is very interesting in the context of
456 how PSM act in the field. A part of the seed population is permanently inhibited to germinate even
457 after short exposure and subsequent absence of PSM. This ensures that PSM decrease germination of
458 plants in the field, even if high rainfall events might dilute PSM. Moreover, it also suggests that PSM
459 intervene in the physiological control of germination by changing the germination inducing
460 pathways. It would be interesting to study further on if the non-germinated seeds die or if dormancy-
461 release mechanisms such as chilling or drought might release these seeds from the inhibitory effects
462 of PSM.
463 One may argue that PSM might only interact with water usage by plants and not directly with how
464 temperature affects plants, since temperature decreases base water potentials for germination in most
465 species. This decrease in base water potential would then be sufficient to explain the effects of PSM
466 on the high and dry limits of germination. A single signaling pathway targeted on sensing of water
467 potential could achieve this. Previous work shows that PSM can increase anti-gibberellin effects and
468 ABA-synthesis (Courtois and Olofsdotter 1998; Kruse et al. 2000) that are known to limit water
469 uptake during germination (Schopfer and Plachy 1984). This is also in congruence with our finding
470 that GA3 reduces the effects of PSM suggesting PSM to block GA3 synthesis in an early stage of
471 germination and to prevent germination as sustained by other works (Terzi and Kocaçalişkan 2010;
472 Zhang et al. 2012). This mechanism may be based on reduced water uptake impairing the GA3
473 synthesis. Previous studies showed that allelochemicals (e.g. 3,4-dihydroxy-acetophenone, DHAP)
474 can have an impact on hormonal balance in plants (Kaya et al. 2015; Turker et al. 2008) increasing
475 ABA and decreasing GA3 levels (Kaya et al. 2015; Yang et al. 2017). However, Ruan *et al.* (2016)
476 also showed that DHAP has more important effects at high temperature, maybe because at high

477 temperature, allelochemicals can be transformed into more active compounds that interfere with
478 hormonal balance (Pedrol et al. 2006). In our data, we also observe stronger effects of PSM at higher
479 temperatures (Fig. 2), resulting in steeper slopes for the relation between germination percentages
480 that germinate at the high temperature limits, when PSM are present. An exception were aqueous
481 extracts from *P. halepensis* which increased germination germination speed (Table 3) but did not had
482 a significant effect on final percentages (Fig. 2).
483 We cannot exclude that the source species used here have been in contact with *L. perenne* in its
484 recent evolutionary history and that PSM were implicated in biological interactions encompassing
485 signalization and defense between these plants and *Linum*. This might have induced some adaptation
486 of *Linum* to these source plants as has been elucidated by Ehlers & Thompson (2004) for *Bromus*
487 *erectus* and *Thymus vulgaris* chemotypes in more open Mediterranean habitats. It is also possible
488 that our –commercial- seed source is more sensible to PSM than wild populations that evolved in
489 direct contact with the woody plants studied here, and we think that this is a very interesting question
490 for future studies. Since low germination speed enhances the building of a permanent soil seed bank
491 (Saatkamp et al. 2011), our data might alternatively suggest that detection of PSM at the germination
492 stage is a way for subordinate species to avoid negative effects of dominant woody plants on the later
493 seedling and adult plant stages. These results highlight that chemical interactions of dominant and
494 subordinate species below ground need to receive greater attention in view of improving our
495 understanding of seed trait function (Saatkamp et al. 2019). Together with earlier work (Fernandez et
496 al. 2013) data presented here suggest that Mediterranean woody species can permanently limit
497 germination of subordinate plants in their habitat by decreasing germination in limiting conditions
498 such as during drought and high temperatures.
499
500 **Acknowledgments**

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Author Contributions statement

- HH, AS, TG, CF and ABM conceived and designed the research;
- HH and AS collected the data;
- HH and AS analyzed and interpreted the data;
- HH, AS, led the writing and HH, AS, TG, CF, JR, ABM revised the manuscript.

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