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## Phenolics of the understory shrub *Cotinus coggygria* influence Mediterranean oak forests diversity and dynamics

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1 **TITLE:** Phenolics of the understory shrub *Cotinus coggygria* influence Mediterranean oak  
2 forests diversity and dynamics

3

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15 **KEYWORDS:** Biotic interactions; oak seedlings; soil microorganisms; soil mesofauna;  
16 regeneration; allelopathy

17

18 **ABSTRACT**

19 Chemical interactions in forested ecosystems play a role in driving biodiversity and ecosystem  
20 dynamics. Plant phenolics released by leaching can influence surrounding plants and soil  
21 organisms such as bacteria, fungi or arthropods. However, our knowledge about such  
22 chemically-mediated biotic interactions in Mediterranean oak forests is still limited, in  
23 particular whether they play a role in the limited forest regeneration. In this study, we analyzed  
24 how phenolics of *Cotinus coggygia*, a dominant shrub of Mediterranean downy oak (*Quercus*  
25 *pubescens*) forests, influence understory herbaceous plant species, downy oak regeneration and  
26 soil organisms in order to obtain a more integrative view of possible direct and indirect  
27 interactions triggered by this shrub species. We performed a series of experiments testing the  
28 effect of aqueous extracts of *C. coggygia*, mimicking natural leachates, on these organisms.  
29 *Cotinus coggygia* contained a high quantity of phenolics in green and senescent leaves but  
30 much less in leaf litter. Extracts from *C. coggygia* leaves stimulated bacterial communities,  
31 exhibited few effects on both saprophytic and symbiotic fungi, and negatively affected  
32 Collembola. Herbaceous species growth was particularly impaired by extracts from green and  
33 senescent leaves, although these effects were alleviated in the presence of soil microorganisms.  
34 In both greenhouse and field experiments, *C. coggygia* affected early oak seedling  
35 establishment in particular through a reduced root growth, but exhibited no effect on later  
36 seedling and sapling growth. We discussed the implication of these results for the balance  
37 between competition and facilitation in oak forests and concluded that *C. coggygia* has the  
38 potential to strongly alter biotic interactions, understory plant diversity and oak forest dynamics.

39

## 40 1. INTRODUCTION

41 Forests are multi-layered and heterogeneous ecosystems in which canopy trees, understory  
42 plants and soil organisms interact in complex networks (Wardle et al. 2004). Phenolics released  
43 by woody plant species can play a key role in these interactions by influencing the structure and  
44 diversity of plant and soil communities (Chou 1999; Souto et al. 2000a; Wardle et al. 1998; Das  
45 and Joy 2009), with important feedback on forest community composition, richness or  
46 dynamics (Mallik 2008). For instance, autotoxicity of canopy trees on their own seedlings  
47 probably plays a role in forest species turnover along succession in Mediterranean forests  
48 (Fernandez et al. 2008, 2016). However, understory shrub species can also profoundly affect  
49 forest ecosystem functioning and dynamics. For example, phenolics released by the  
50 understory dwarf shrub *Empetrum hermaphroditum* was reported to impair the regeneration of  
51 the dominant tree *Pinus sylvestris* in boreal forests (Nilsson 1994; Nilsson et al. 2000).

52 Soil microorganisms can be directly affected by plant phenolics (Chomel et al. 2014;  
53 Santonja et al. 2018) but they may also use these plant chemicals as carbon source and thus  
54 modify the plant-plant chemical interactions (Fernandez et al. 2013; Souto et al. 2000a). Litter  
55 phenolics can inhibit tree fungal symbionts (Rose et al. 1983; Souto et al. 2000b) which may  
56 have important consequences for tree seedlings development. However, these effects on fungi  
57 are species-specific and only few studies tested whether plant phenolics can decrease tree  
58 mycorrhization in natural soil (Souto et al. 2000b). Phenolics released by plants may also affect  
59 soil arthropods (Poinsot-balaguer et al. 1993; Das and Joy 2009; Asplund et al. 2015), which  
60 play a key role on soil microbial community structure (Berg et al. 2004; Chahartaghi et al. 2005)  
61 and litter decomposition process (Seastedt 1984; Filser 2002; Santonja et al. 2017). However,  
62 to our knowledge, such plant-soil arthropod chemical interactions were poorly studied.

63 The Mediterranean basin has an exceptionally high plant diversity and endemism (Myers  
64 et al. 2000) shaped by a high diversity of human and ecological factors such as geology,

65 topography or perturbation regimes (Blondel 2006). Many Mediterranean plants synthesize a  
66 wide variety of specialized metabolites, which help them withstanding the summer drought and  
67 high radiative stress typical of Mediterranean-type ecosystems (Chaves and Escudero 1999),  
68 and which can also influence ecosystem structure and functioning (Scognamiglio et al. 2013;  
69 Vilà and Sardans 1999). The downy oak (*Quercus pubescens* Mill.) is a long-lived  
70 submediterranean tree that occurs mainly in Southern Europe, from northern Spain to the  
71 Caucasus (Quézel and Médail 2003). Downy oak forests cover about 400 000 ha in  
72 Mediterranean France (IFN, 2014) and were traditionally managed as coppices, but the  
73 abandonment of this practice during the second half of the 20<sup>th</sup> century resulted in ageing stands  
74 with frequent signs of dieback. With the abandonment of vegetative reproduction through  
75 coppices, the future of these stands should depend upon sexual regeneration, but local  
76 observations underline a lack of seedlings and saplings (Prévosto et al. 2013). The role of plant-  
77 plant chemical interactions during the germination or establishment phases have still been  
78 poorly investigated. The understory of downy oak forests is frequently dominated by the shrub  
79 *Cotinus coggygria* Scop. (Anacardiaceae), which has a wide distribution from southern Europe,  
80 the Mediterranean, Moldova and the Caucasus to central China and the Himalayas (Matić et al.  
81 2011). This species has been traditionally used as a dyestuff since antiquity (Valianou et al.  
82 2009) but also widely used in ornamental horticulture. This shrub produces high diversities and  
83 amounts of phenolics and terpenes (Novaković et al. 2007; Hashoum et al. 2017). This species  
84 has consequently been studied for a source of bioactive substances such as those from extracts  
85 or essential oils that present antibacterial, antifungal and antioxidant properties (Marčetić et al.  
86 2013; Novaković et al. 2007; Matić et al. 2011). Considering these characteristics, this species  
87 could play a major role in biotic interactions occurring in Mediterranean downy oak forests.

88         The aim of the present study was to analyze how phenolics of *C. coggygria* influence  
89 understory herbaceous plant species, downy oak regeneration and soil organisms, including

90 both microorganisms and arthropods. More precisely, our objectives were to (i) quantify  
91 phenolics present in *C. coggygia* leaf leachates; (ii) determine whether phenolics of *C.*  
92 *coggygia* affect the germination and seedling growth of understory plant species and downy  
93 oak; (iii) evaluate the impact of these phenolics on soil microorganisms and arthropods; and  
94 finally (iv) assess the role of soil microorganisms for plant-plant chemical interactions.

95

## 96 **2. MATERIAL AND METHODS**

97

### 98 ***2.1. Experimental site and material collection***

99 Field experiment and biological material collection (*Cotinus coggygia* leaves, oak acorns  
100 and soils) were performed at the Oak Observatory at the OHP (O<sub>3</sub>HP) experimental site located  
101 in the research center “Observatoire de Haute Provence”, 60 km north of Marseille (43°56'115”  
102 N, 05°42'642” E; 680 m a.s.l.). The climate is Mediterranean with a mean annual temperature  
103 of 11.9 °C and a mean annual precipitation 830 mm (1967–2000). The site is covered by an  
104 old-growth oak forest belonging to the site Natura 2000 “FR9302008 Vachères”, which was  
105 managed for centuries by coppicing. Downy oak (*Quercus pubescens*: 75% coverage) and  
106 Montpellier maple (*Acer monspessulanum*: 25% coverage) are the two dominant tree species,  
107 with understory vegetation dominated by smoke tree (*Cotinus coggygia*: 30% coverage). The  
108 soil is a pierric calcosol (with S horizon between limestone rocks) or calcarisol when limestone  
109 appears less than 25 cm deep.

110 Three types of *C. coggygia* leaves (according to leaf maturity) were collected to perform  
111 chemical analyses and to prepare the aqueous extracts (mimicking *C. coggygia* leachates) for  
112 bioassays: green and senescent leaves were collected directly on the shrub and leaf litter on the  
113 forest floor. Oak acorns were collected on the ground in autumn, floated and visually screened  
114 to eliminate non-viable acorns. Soil samples used as bioassay substrate were collected in zones

115 without *C. coggygia* (i.e. at least 10 m from shrubs), sieved at 2 mm, and then stored at room  
116 temperature until the start of the experiments.

117 We selected four herbaceous target species naturally present in downy oak forests to  
118 perform the bioassays: *Linum narbonense* L. (Linaceae), *Satureja montana* L. (Lamiaceae),  
119 *Silene nutans* L. (Caryophyllaceae) and *Verbascum pulverulentum* Vill. (Scrophulariaceae).  
120 Seeds were collected from wild populations on the study site, and then stored in a cold chamber  
121 at 5 °C until the start of the experiment. We also selected *Lactuca sativa* L. (Asteraceae) as  
122 target plant species because this species is known for its sensitivity to specialized metabolites  
123 and frequently used for bioassays as a reference (e.g. Chou et al. 1998; Fernandez et al. 2006).  
124 Seeds of *L. sativa* were purchased from a commercial company (Vilmorin ®).

125

## 126 **2.2. Chemical analysis**

127

### 128 **2.2.1. Total phenolics**

129 Extraction of phenolics was carried out based on the method described by Singleton and  
130 Rossi (1965) and adapted to smaller amounts of plant material. Briefly, a dry mass (DM) of 250  
131 mg of crushed leaf was extracted with 20 mL of deionized water. The mixture was left for 1 h  
132 under constant shaking at ambient temperature shielded from light. The extract was then filtered  
133 on Whatman GF/C paper filter. A volume of 25 µL of the extract were added to 1650 µL of  
134 ultrapure water, 200 µL of saturated Na<sub>2</sub>CO<sub>3</sub> aqueous solution and 100 µL of Folin-Ciocalteu's  
135 reagent. After 30 min, the phenolic index was measured at 765 nm on a spectrophotometer  
136 (Spectronic Biomate 3 Thermo Electron Scientific Instrument Corporation ®) and expressed as  
137 equivalent of mg of gallic acid per g of plant material (DM).

138

### 139 **2.2.2. Flavonoids**

140 Among phenolic compounds, we focused on flavonoids which were quantified in terms of  
141 proanthocyanidins and flavonoid index (total flavonoid). Extraction and quantification were  
142 based on a previous work (Kaundun et al. 1998) adapted by our laboratory to smaller amounts  
143 of plant material. A mass of 0.5 g DM of crushed leaf was suspended in 15 ml of HCl 2N  
144 solution and heated to 90 °C in a water bath with reflux for 50 min with every 10 min of air  
145 influxes. The acidic treatment generated anthocyanidins from homologous proanthocyanidins  
146 and flavonol aglycones from corresponding flavonol glycosides. The solution was left to cool  
147 approximately 30 min and filtered (filter porosity 3). Anthocyanidins were quantified  
148 spectrophotometrically at 435 nm (Spectronic Biomate 3 Thermo Electron Scientific  
149 Instrument Corporation ®) and expressed as mg per g of plant material (DM). Flavonol  
150 aglycones were extracted three times with 9 mL of diethyl ether. The extracts were recombined  
151 and evaporated to dryness. The residue was then dissolved and mixed with 1.5 mL of methanol.  
152 An aliquot of 100 µL was added to 5 mL of 1 % AlCl<sub>3</sub> / MeOH solution and let 20 min to react.  
153 The flavonoid index was measured at 530 nm and expressed as equivalent of mg of quercetin  
154 per g of plant material (DM).

155

### 156 **2.3. Bioassays**

157 We chose to test the effects of natural leachates using leaf aqueous extracts because water-  
158 soluble compounds have been shown to be most involved in allelopathy (Fernandez et al. 2013,  
159 2016). These extracts were prepared by soaking entire leaves in deionized water for 24 h at  
160 room temperature (20 ± 1°C) in darkness (Fernandez et al. 2013; Hashoum et al. 2017). After  
161 24 h, extracts were filtered through #42 Whatman® paper filter and stored at 4 °C until use.  
162 New extracts were prepared to prevent compound degradation once a week (Experiment 1,  
163 stock solution at 5% dry weight further diluted at 2.5%) or once a month (Experiment 2, solution  
164 at 5% dry weight) with fresh material.



165

166 2.3.1. Experiment 1: Response of understory plant species & microorganisms to *C. coggygia*  
167 aqueous extracts

168 We compared natural and autoclaved soil bioassays to evaluate the impact of soil microbial  
169 communities in shaping plant-plant chemical interactions (Kaur et al. 2009; Fernandez et al.  
170 2013). Sterilization consisted in autoclaving soil for two cycles of 1 h (24 h apart) at 121 °C to  
171 eliminate a fraction of the microbial community (Alef and Nannipieri 1995; Trevors 1996).

172 Bioassays were conducted in Petri dishes with 50.0 g ( $\pm$  0.1 g) of soil, either natural or  
173 autoclaved, corresponding to a thickness of 0.5 to 0.6 mm (Fernandez et al. 2013). Each Petri  
174 dish was sown with 20 seeds of each target species that were watered every 2 days with 5 mL  
175 of deionized water (control) or *C. coggygia* extracts (2.5% and 5%) from one of the three leaf  
176 types (green leaf, senescent leaf and leaf litter). Five replicates were performed for each  
177 treatment (target plant species  $\times$  leaf type  $\times$  concentration  $\times$  soil type). Bioassays were  
178 conducted under natural photoperiod and controlled temperature (20.5 °C  $\pm$  1 °C) for 40 days.

179 Seed germination was monitored every day and used to compute total germination rate and  
180 germination speed using the velocity coefficient (Mazliak 1982):  $C_v = 100 (\sum N_i / \sum N_i T_i)$ , where  
181  $N_i$  is the number of seeds germinated at time  $i$ , and  $T_i$  is the number of days since the start of  
182 the experiment. The higher the velocity coefficient, the faster the germination. A seed was  
183 considered as germinated when the protruding radicle achieved the length of 1 mm beyond the  
184 seed coat. Lengths of root and shoot were measured for each individual at the same age, i.e. 10  
185 days after germination (accuracy: 1 mm).

186 A further set of Petri dishes containing either natural or autoclaved soils was used to test  
187 the effects of *C. coggygia* extracts on biomass and basal respiration of soil microbial  
188 communities. Microbial biomass (MB) was estimated using substrate-induced respiration (SIR)  
189 rates (Anderson and Domsch 1978). Ten grams (dry weight equivalent) of subsamples were

190 placed in 117 mL glass jars and amended with powdered glucose (1000  $\mu\text{g C.g}^{-1}$  soil). After  
191 incubation (1 h, 22 °C), a volume of 1 mL of air was sampled in the headspace with a syringe  
192 and injected onto the gas chromatograph (ChrompackCHROM 3-CP 9001) to analyze CO<sub>2</sub>  
193 production. SIR rates were converted into MB using equations given by Beare et al. (1990).

194

195 *2.3.2. Experiment 2: Response of oak seedling (2a) and sapling (2b) to C. coggygria aqueous*  
196 *extracts in greenhouse and (2c) to C. coggygria presence in the field*

197 This experiment was conducted in greenhouse to determine if *C. coggygria* aqueous  
198 extracts could alter (i) oak acorns germination and early development and (ii) oak saplings  
199 development and associated mycorrhiza. Finally, a seeding experiment was performed *in situ*  
200 to compare patterns obtained in greenhouse (only chemical interactions) and in the field (all  
201 types of interaction).

202 In the experiment 2a, thirty oak acorns were sown in individual pots filled with natural  
203 soil and vermiculite (2:1, for a total of 150 g of substrate per pot). Three treatments were applied  
204 on each set of 10 pot replicates: i) control, ii) leachates, watered with *C. coggygria* aqueous  
205 extracts at 2.5% DM of plant material, and iii) litter, where soil substrate was mixed with 10 g  
206 of *C. coggygria* leaf litter. Pots were watered every 3 days with deionized water or aqueous  
207 extracts and kept under a 12h-photoperiod for 2 months. At the end of this period, germinated  
208 acorns in each treatment were counted. The seedlings were separated into leaves, stem and  
209 roots, and weighed after drying them at 60 °C for 48 h.

210 The experiment 2b was conducted to assess *C. coggygria* impact on older saplings and their  
211 mycorrhizae. A total of 70 forty-month old individuals of *Q. pubescens* certified mycorrhized  
212 by *Tuber melanosporum* Vittad. were grown in 10 L plastic pots containing a substrate  
213 consisting of mold (pH 6), perlite and vermiculite (1/3 of each). Three grams of magnesium  
214 carbonate per L of substrate were added to obtain an alkaline pH of 7.6 that favors *T.*

215 *melanosporum* development. Half of the saplings were watered monthly with 200 mL of  
216 aqueous extracts at 5% DM of plant material whereas control saplings received 200 mL of  
217 deionized water. In order to mimic as close as possible the natural conditions, *C. coggygia*  
218 extracts were prepared according to the shrub phenology: with senescent leaves sampled from  
219 October to January, litter sampled from February to April or green leaves sampled from May  
220 to September. After 4, 12 and 16 months, 10 saplings per treatment were harvested and divided  
221 into leaf, stem and root after careful removing of soil. A subsample of 10 secondary root  
222 segments (3 cm) per sapling were randomly selected for the analysis of the mycorrhizal  
223 colonization (Garbaye 2013). Root segments were kept in 60 % ethanol to stop the mycelial  
224 development until analysis. Segments were placed in Petri dishes and all root tips were observed  
225 using a binocular scope and classified as mycorrhized or not. We then computed the  
226 mycorrhization rate according to the formula number of mycorrhizal root tip / total root tips  $\times$   
227 100.

228 For the field experiment (2c), 50 sowing points were installed on the *Q. pubescens* forest  
229 understory, either in the presence or absence of *C. coggygia* shrub (100 sowing points in total).  
230 Each sowing point consisted of manually dug holes of about 2 cm in which 2 downy oak acorns  
231 were laid flat, covered with soil and a wire mesh (10 cm x 10 cm, 0.6 cm mesh size) to prevent  
232 predation by rodents. Acorns collection and sowing took place in November 2013. Acorns were  
233 collected on several trees to encompass intraspecific variation and non-viable acorns were  
234 eliminated by floating and visual screening. Sowing points were distributed in 5 blocks in each  
235 treatment (with or without *C. coggygia*). Plots were fenced to limit predation by wild boar.  
236 Emerged seedlings were counted in June 2014, and seedling number and dimensions (diameter  
237 at 2 cm and length) were then recorded yearly in winter until 2017 (4-year-old seedlings).

238

239 2.3.3. Experiment 3: response of soil saprophytic fungi and mesofauna to *C. coggygia* aqueous

240 *extracts*

241 For this experiment, intact soil from the upper 10 cm were sampled, transported to the  
242 laboratory and placed in aluminum mesocosm (20 × 15 cm). The mesocosms were placed in a  
243 culture room with a natural photoperiod, a temperature of about 23 °C and an air humidity of  
244 about 40%. Mesocosms were sprayed each 3 days using 50 mL of deionized water (control) or  
245 aqueous extracts of *C. coggyria* senescent leaves at 2.5% and 5% DM of plant material.

246 After 15 days, soil arthropods were extracted using the Tullgren funnel method, and  
247 stored in 95% ethanol to be counted and separated into Collembola and different suborders for  
248 Acari (Oribatida, Mesostigmata and Prostigmata) (Hopkins 1997; Santonja et al. 2017) using a  
249 binocular scope. Collembola and Acari Oribatida were regarded as microbi-detritivore  
250 mesofauna, whereas Acari Mesostigmata and Prostigmata as predatory mesofauna (Coleman et  
251 al. 2004; Santonja et al. 2017).

252 Fungal biomass was determined by quantifying ergosterol, which is a specific fungal  
253 membrane constituent and thus a good indicator of living fungal biomass (Gessner and Chauvet  
254 1993). Ergosterol extraction and quantification were performed following the method described  
255 in Santonja et al. (2017).

256

#### 257 **2.4. Data analysis**

258 Differences in concentration of phenolics, flavonols and proanthocyanidins according to  
259 *C. coggyria* leaf type (green leaf, senescent leaf and leaf litter) were tested using Kruskal-  
260 Wallis tests followed by post hoc Student-Newman-Keuls tests.

261 For the experiment n°1, differences in plant performance (germination rate and velocity,  
262 shoot and root growths) in the control treatments between species and soil types (autoclaved  
263 and natural) were first analyzed using a binomial GLM for germination rate and two-way  
264 ANOVAs for other response variables, followed by post-hoc Tukey tests. Then, we computed

265 a Relative Allelopathic Effect (RAE) as the relative difference between plant performance in  
266 the control (Pc) and leachates (Pt) treatments:  $RAE (\%) = (Pc - Pt) / Pt$  and calculated a mean  
267 and bootstrapped confidence interval at 95% (n=1000) for each combination of species, leaf  
268 type (green, senescent and litter), concentration (2.5 or 5%) and soil type (autoclaved or  
269 natural). RAE was considered significantly different from zero (i.e. treatment different from the  
270 control) when zero was not included in the bootstrapped confidence interval. Differences in  
271 microbial biomass according to the *C. coggygia* leaf type, concentration and soil type were  
272 tested using a three-way ANOVA followed by post hoc Tukey tests.

273 Concerning the experiment n°2, differences in oak seedling root, stem and leaf biomasses  
274 according to the treatments (control, leachates or litter) were tested using one-way ANOVAs  
275 followed by post hoc Tukey tests. Differences in oak sapling biomass (root, stem and leaf) and  
276 mycorrhization rate according to the treatment, sampling date and their interactions were tested  
277 using two-way ANOVAs followed by post-hoc Tukey tests. For the field experiment, the effect  
278 of *C. coggygia* presence on seedling emergence and survival was tested using binomial GLMs  
279 and using a one-way ANOVA for seedling growth. For all ANOVAs, normality and  
280 homoscedasticity of the residuals were assessed by Shapiro-Wilk and Bartlett tests,  
281 respectively, and data were log or root-squared-transformed when necessary.

282 Finally, concerning the experiment n°3, differences in mesofauna abundance and fungal  
283 biomass according to *C. coggygia* leachates were tested by Kruskal-Wallis tests, followed by  
284 post-hoc Student-Newman-Keuls tests, due to heteroscedasticity.

285 All statistical analyses were performed with R software (R Development Core Team 2017).

286

### 287 **3. RESULTS**

288

289 **3.1. Chemical analysis**

290 Concentrations of total phenolics, flavonols and proanthocyanidins increased from green  
291 to senescent leaves but strongly decreased in leaf litter (Fig. 1).

292

293 **3.2. Bioassays**

294

295 **3.2.1. Soil microorganisms and herbaceous plant responses to *C. coggygia* aqueous extracts**

296 The sterilization process reduced by two-fold microbial biomass on natural soil (*t*-test,  
297  $P < 0.01$ ; Fig. 2). On natural soil, this biomass increased regularly from green leaf to leaf litter  
298 extracts watering with a weak effect of dose (2.5 vs. 5% DM), except for leaf litter extract that  
299 showed a higher dose effect. On autoclaved soil, stimulatory effects of all aqueous extracts were  
300 observed on microbial biomass, especially with extracts at 5% DM with 2 to 3-fold increases  
301 (Fig. 2).

302 Target plant species exhibited highly different germination rate and velocity, and growth  
303 length values in the control treatments (Table 1). *Lactuca sativa* had the highest germination  
304 rate (79-85 %) and velocity (43-44). *Verbascum pulverulentum* presented the lowest  
305 germination rate (14-34 %) concomitant to the lowest growth (1.0-1.4 and 1.0-1.2 cm for root  
306 and shoot, respectively). *Linum narbonense* demonstrated the highest growth (6.0-6.1 and 5.4-  
307 5.6 cm for root and shoot, respectively) with the slowest germination velocity (8.2-8.3). The  
308 effect of soil type on germination rate depended on species (Species  $\times$  Soil interaction,  
309 likelihood ratio  $\chi_2 = 221.8$ ,  $P < 0.001$ ). *Silene nutans* and *V. pulverulentum* presented lower  
310 germination rates on autoclaved soil compared to natural soil ( $47.7 \pm 3.0$  vs.  $55.3 \pm 3.9$  for *S.*  
311 *nutans*;  $13.7 \pm 2.7$  vs.  $34 \pm 3.5$  for *V. pulverulentum*). Higher germination rates were however  
312 observed for *L. sativa* ( $85.3 \pm 1.6$  vs.  $79.3 \pm 1.6$ ) and *Satureja montana* ( $82.0 \pm 2.8$  vs.  $74.0 \pm$   
313 3.2) on autoclaved soil compared to natural soil. *Linum narbonense* was the only species for

314 which germination rate was not affected by the soil treatment ( $74.3 \pm 2.0$  vs.  $75.3 \pm 7.2$  for  
315 autoclaved and natural soils, respectively). As a consequence, the presence/absence of  
316 microorganisms in soils influenced species ranking for germination rate. In addition to lower  
317 germination rate on autoclaved soil, *S. nutans* was the only species presenting a lower  
318 germination velocity on autoclaved soil compared to natural soil ( $F_{4,140} = 8.4$ ,  $P < 0.001$  ;  $15.8$   
319  $\pm 0.7$  vs.  $55.3 \pm 3.9$ , respectively), whereas this parameter seemed to be not affected by soil  
320 treatment for the four other species. Shoot and root growth of all species were not affected by  
321 soil treatment ( $P > 0.05$ , Table 1).

322 *Cotinus coggygia* aqueous extracts generally affected plant growth more than plant  
323 germination, but extract effects depended on target plant species, leaf type, extract  
324 concentration and soil type.

325 The effects of *C. coggygia* extracts on growth ranged from - 57% to no effect, with no  
326 positive effect detected (Fig. 3). Generally, we observed higher inhibitory effects on root growth  
327 (up to -57%) than shoot growth (up to -29%). Inhibitory effects were higher with extracts  
328 prepared with green leaf, followed by those based on senescent leaf. Inhibitory effects were  
329 strongly reduced or totally disappeared with litter extracts and were generally stronger on  
330 autoclaved soil than on natural soil. *Linum narbonense* was the most sensitive species,  
331 especially on autoclaved soil where growth was reduced by more than 50% for several leaf  
332 types and extract concentrations (Fig. 3). *Satureja montana* and *V. pulverulentum* were also very  
333 sensitive to green leaf extracts on autoclaved soil, with almost 40% of root length reduction,  
334 whereas *S. nutans* reached similar reductions with senescent leaf extracts on autoclaved soil.  
335 On natural soil, plant growths were also reduced by about 20% for *L. narbonense*, *S. nutans*  
336 and *V. pulverulentum* in presence of leaf extracts while *S. montana* maintained a similar plant  
337 growth than in control treatment.

338 *Cotinus coggygia* extracts effects on germination rate and velocity ranged from -38% to

339 +26% and from -24% to +17%, respectively (Supplementary Fig. S1). *Lactuca sativa* had more  
340 stable germination rate and velocity across all treatments than the other species. Extracts  
341 reduced the germination rate and velocity of *L. narbonense* to about 38%, but this reduction  
342 was overall lower on natural soil where 2.5% extracts slightly stimulated germination.  
343 Germination rate of *S. montana* was reduced on autoclaved soil, while on natural soil its  
344 germination velocity was stimulated by senescent leaves. Germination rate of *V. pulverulentum*  
345 was inhibited by green leaf extracts on natural soil. No clear hierarchy of extract effects was  
346 evidenced regarding leaf type (Supplementary Fig. S1).

347

### 348 3.2.2. Oak seedling and sapling responses to *C. coggygria* aqueous extracts

349 In the greenhouse, leaf extracts did not influence acorn germination rate as all sowed acorns  
350 germinated (Exp. 2a). Both *C. coggygria* extracts and litter presence caused a 39% reduction  
351 of seedling root biomass ( $F_{2,27} = 6.4$ ,  $P < 0.01$ ) but did not affect stem ( $F_{2,27} = 0.2$ ,  $P = 0.8$ ) or  
352 leaf biomass ( $F_{2,27} = 0.3$ ,  $P = 0.8$ ) of the 2 month-old oak seedlings (Exp. 2a, Fig. 4). However,  
353 aqueous extracts did not affect the 40-months-old oak sapling leaf, stem and root biomasses,  
354 although leaf biomass slightly increased for saplings watered with *C. coggygria* extracts at the  
355 end of the experiment (Exp. 2b, Table 2, Fig. 4). Mycorrhization rates, rather low at 4 months  
356 ( $18.8 \pm 2.6\%$ ), increased after 12 and 16 months of experiment (68-82%) but were not  
357 influenced by *C. coggygria* extracts whatever the sampling date (Table 2).

358 In the field, oak seedling emergence was lower under *C. coggygria* shrubs (LR  $\chi^2 = 8.7$ ,  $P$   
359  $= 0.003$ ), but *C. coggygria* presence did not affect seedling survival (LR  $\chi^2 = 4.1$ ,  $P = 0.5$ , data  
360 not shown), diameter ( $F_{1,70} = 0.1$ ,  $P = 0.7$ ) or length ( $F_{1,70} = 0.4$ ,  $P = 0.5$ ) growth over the  
361 following 3 years (Exp. 2c, Fig. 4).

362

### 363 3.2.3. Saprophytic fungi and mesofauna responses to *C. coggygria* aqueous extracts



364 Saprophytic fungal biomass as well as Oribatida and predatory Acari abundances were  
365 not affected by *C. coggygria* extracts (KW = 1.3, P = 0.5; KW = 1.5, P = 0.5; KW = 0.4, P =  
366 0.8, for saprophytic fungi, Oribatida and predatory Acari, respectively; Table 3). Collembola  
367 abundance was 3.5 times lower in mesocosms watered with *C. coggygria* extracts at 5% than  
368 in mesocosms watered with deionized water (KW = 7.0, P = 0.03; Table 3).

369

#### 370 4. DISCUSSION

371 *C. coggygria* contained a particularly high quantity of phenolics in green and senescent  
372 leaves. For instance, total phenolic and flavonoid contents were at least 10 times higher than  
373 values reported for *Pinus halepensis* (Fernandez et al. 2009; Santonja et al. 2015). Phenolic  
374 quantities were however strongly reduced in litter. Phenolics are water-soluble compounds that  
375 are rapidly leached during the initial phases of decomposition (Chomel et al. 2014; Santonja et  
376 al. 2015). For example, Santonja et al. (2015) reported that *C. coggygria* lost 73% of initial  
377 phenolic content after 100 days of litter decomposition.

378

##### 379 4.1. Impacts of *C. coggygria* on herbaceous species and oak regeneration

380 Bioassays conducted on several target plant species generally highlight species-specific  
381 response to allelochemicals. For instance, Fernandez et al. (2013) found that 40% of 15 target  
382 plant species tested were inhibited by aqueous extracts of *P. halepensis* green needles, while  
383 20% were insensitive and 40% were even stimulated. In the present study, although sensitivity  
384 to *C. coggygria* aqueous extracts varied depending on target species, effects were generally  
385 negative and only a slight positive effect on germination was detected for one species,  
386 highlighting a strong phytotoxic potential of *C. coggygria*. Contrary to our expectations, the  
387 commonly used *L. sativa* was not the most sensitive target species. The high sensitivity of *L.*  
388 *narbonense* is concordant with previous studies that also outlined a high sensitivity of *Linum*

389 *strictum*, a species from the same genus (Bousquet-Mélou et al. 2005; Fernandez et al. 2006,  
390 2013). Interestingly, this finding is contradictory with the general view that specialized  
391 metabolites are less efficient against co-occurring species (Callaway and Ridenour 2004).  
392 Litter, which had by far the lowest phenolic content, also presented the lowest effects. However,  
393 green leaves showed generally a higher effect than senescent leaves despite a lower phenolic  
394 content. Different chemical compositions between these two phenological stages may probably  
395 explain the particularly high effect of green leaves (Hashoum et al. 2017).

396 The negative influence of *C. coggygia* on early oak seedling root development may  
397 explain the lower emergence below shrubs recorded on the field. Later sapling development in  
398 contrast was poorly affected by extracts, both in the field and greenhouse experiments. Older  
399 saplings may be less sensitive to this type of interaction because of a lower quantity of absorbed  
400 phenolics relative to seedling biomass (dilution effect). Alternatively, older seedlings may be  
401 better protected against phytotoxic compounds thanks to their mycorrhizal associations (Mallik  
402 and Zhu 1995; Zeng and Mallik 2006).

403

#### 404 **4.2. Phenolics influence on soil organisms**

405 In the present study, *C. coggygia* extracts generally increased microbial biomass. Hortal  
406 et al. (2015) also found that specialized metabolites of the shrub *Thymus hyemalis* promotes  
407 microbial activity and biomass. Microbial stimulation may be due to the use of these  
408 compounds as energy source by microorganisms (Inderjit 1996; Blum and Shafer 1998), which  
409 could explain the lower inhibition of herbaceous species in natural soils containing  
410 microorganisms. An alternative explanation is that aqueous extracts also contain nutrients and  
411 sugars that stimulate microorganisms, or that microorganisms may have been present in extracts  
412 according to leaf types, adding more microbes to the soil. Increase in microbial biomass was  
413 stronger at higher concentration of extracts, probably because of a higher quantity of

414 compounds or microorganisms. This was particularly the case with high increase of  
415 microorganisms on autoclaved soils, containing less initial microbial biomass, which may be  
416 linked to a higher colonizing capacity of remaining microorganisms and/or extract  
417 microorganisms favored by a lower microbial competition.

418 Saprophytic and symbiotic fungi were not affected by *C. coggygia* extracts. *Ex situ* studies  
419 found that phenolics may inhibit, have neutral effect or even stimulate fungal development and  
420 respiration depending on the source species, on extracts concentration and on fungal target  
421 species (Rose et al. 1983; Souto et al. 2000a,b). Rose et al. (1983) also showed that root  
422 colonization of Douglas fir (*Pseudotsuga menziesii* Mirb.) seedlings by *Rhizopogon sp.* was  
423 inhibited by the mere application of litter on soil surface. In our study, no effect on total  
424 mycorrhization rate was detected but we did not investigate such potential species-specific  
425 effects by examining mycorrhizal species present on oak roots. Even if qualitative changes in  
426 mycorrhizal communities occurred, this did not seem to affect oak seedling development during  
427 the 16 months of our experimentation. In addition, it is worth noting that our experiment was  
428 designed to test chemical effects on already mycorrhized saplings, but it would be interesting  
429 to further study whether initial root colonization by mycorrhizae could be affected.

430 Among soil arthropods, Collembola abundance was negatively affected while Acari  
431 appeared as insensitive to extracts of *C. coggygia* senescent leaves. Previous experiments also  
432 reported that phenolic compounds, including phenolic acids, flavonoids and tannins, can limit  
433 litter colonization, growth and activity of soil Collembola (Poinsot-balaguer et al. 1993; Das  
434 and Joy 2009; Chomel et al. 2014; Asplund et al. 2015). For example, Asplund et al. (2015)  
435 reported a negative effect of lichen phenolics on Collembola abundance and species richness in  
436 boreal *P. sylvestris* forest, while Das and Joy (2009) showed a decrease in litter colonization by  
437 *Cyphoderus javanus* Börner (Collembola: Hexapoda) according to the increase in phenolic  
438 concentration in tropical forest. In the present study, by negatively affecting Collembola,

439 phenolics present in *C. coggygria* senescent leaves could favor microbial and Acari  
440 colonization of leaf litter during the initial stages of litter decomposition. However, in later  
441 decomposition stages positive effects of *C. coggygria* litter on Collembola have been observed  
442 (Santonja et al. 2017), suggesting that the leaching of phenolics remove the inhibitory effect  
443 found here with senescent leaves. In support to this hypothesis, Chomel et al. (2014) also  
444 reported than phenolics present in pine needles delayed the litter colonization by Collembola in  
445 Mediterranean *P. halepensis* forest.

446

#### 447 ***4.3. Soil organisms modulate plant-plant chemical interactions***

448 Aqueous extracts of *C. coggygria* leaves exhibited more negative effects on plant target  
449 species on autoclaved than on natural soils. Microbial activities can influence the persistence,  
450 availability and biological activity of phenolics in soil (Inderjit 2005; Kaur et al. 2009; Meiners  
451 et al. 2012). Here, microbial community present in natural soil released the toxicity of *C.*  
452 *coggygria* extracts, despite a similar microbial biomass than in autoclaved soil. We can  
453 hypothesize that this alleviation of negative effects is due to a qualitative difference in soil  
454 microbial community, i.e. sterilization removed a part of soil microbial community able to  
455 degrade phytotoxic compounds. This highlights the possible importance of microbial  
456 community composition in determining the intensity or direction of plant-plant chemical  
457 interactions.

458

#### 459 ***4.4. Synthesis: potential impacts of C. coggygria on forest regeneration***

460 Our study shows that *C. coggygria* leachates can inhibit both herbaceous species and  
461 early oak seedling development. Herbaceous species are strong competitor for oak seedling  
462 establishment (Rey Benayas et al. 2005; Gavinet et al. 2016) because they form a dense  
463 superficial network of roots that strongly compete with tree seedlings for water in the upper

464 soil layers (Balandier et al. 2006). The inhibitory effect of *C. coggygria* on herbaceous  
465 species could mediate indirect interactions favoring oak establishment. Although we did  
466 not observe such indirect positive interactions in the field, they may take place in systems  
467 with a higher herbaceous cover. In the USA, Petranka and McPherson (1979) found that  
468 *Rhus copallina*, a shrub closely related to *C. coggygria* with high phenolics content, plays  
469 a key role in the prairie – forest transitions as this shrub allows for tree seedling  
470 establishment by inhibiting herbaceous species development. In addition, by stimulating  
471 microbial biomass and reducing the abundance of microbivorous mesofauna, *C. coggygria*  
472 may favor microbial community development and associated functions such as nutrient  
473 mineralization. However, it is difficult to predict feedback effects on plant species without  
474 more knowledge of the type of microbes being stimulated (neutral, mutualistic or  
475 pathogens, Hortal et al. 2015) and our in-situ experiment suggests that modifications of  
476 soil microbes may not play an important role for oak seedling establishment, as also shown  
477 in a pot experiment (Gavinet et al., 2018).

478 Changes in seedling response to neighbor presence with ontogeny have been highlighted  
479 in several studies (e.g. Le Roux et al. 2013) and generally attributed to a change in resource  
480 requirement or availability (Soliveres et al. 2010). The results of our study suggest that  
481 ontogenetic changes in plant-plant interaction outcomes may also result from a change in plant-  
482 plant chemical interactions.

483

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494

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686 **Table 1:** Germination and growth parameters of the 5 target species in the control treatment (watered with deionized water) depending on soil  
687 type. Data are means  $\pm$  standard errors (n = 15 boxes for germination parameters, n = 35-150 seedlings for growth parameters). Different letters  
688 indicate significant differences between species with a>b>c>d ( $P<0.05$ ).

689

	<b>Autoclaved soil</b>				<b>Natural soil</b>			
	Germination rate (%)	Germination velocity	Root growth (cm)	Shoot growth (cm)	Germination rate (%)	Germination velocity	Root growth (cm)	Shoot growth (cm)
<i>Lactuca sativa</i>	85.3 $\pm$ 1.6a	43.1 $\pm$ 0.6a	2.42 $\pm$ 0.08b	3.04 $\pm$ 0.10b	79.3 $\pm$ 1.6a	44.1 $\pm$ 0.0a	2.04 $\pm$ 0.07b	3.44 $\pm$ 0.11b
<i>Linum narbonense</i>	74.3 $\pm$ 2.0b	8.2 $\pm$ 0.2d	6.01 $\pm$ 0.13a	5.40 $\pm$ 0.08a	75.3 $\pm$ 7.2a	8.3 $\pm$ 0.3c	6.13 $\pm$ 0.11a	5.62 $\pm$ 0.09a
<i>Satureja montana</i>	82.0 $\pm$ 2.8a	22.9 $\pm$ 0.9b	2.27 $\pm$ 0.04b	2.16 $\pm$ 0.03c	74.0 $\pm$ 3.2a	23.1 $\pm$ 0.9b	2.31 $\pm$ 0.05b	2.57 $\pm$ 0.04c
<i>Silene nutans</i>	47.7 $\pm$ 3.0c	15.8 $\pm$ 0.7bc	2.16 $\pm$ 0.09b	2.49 $\pm$ 0.05c	55.3 $\pm$ 3.9b	24.3 $\pm$ 1.2b	2.61 $\pm$ 0.07b	2.65 $\pm$ 0.06c
<i>Verbascum pulverulentum</i>	13.7 $\pm$ 2.7d	16.2 $\pm$ 1.5c	1.02 $\pm$ 0.04c	0.99 $\pm$ 0.03d	34.0 $\pm$ 3.5c	19.7 $\pm$ 0.9b	1.41 $\pm$ 0.03c	1.18 $\pm$ 0.03d

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691

692 **Table 2:** Results of ANOVAs testing the effects of *Cotinus coggygia* extracts, sampling date,  
693 and their interactions on oak sapling root, stem and leaf biomasses and mycorrhization rate. *F*-  
694 values and associated *P*-values (\*\*\*) ( $P < 0.001$ ) are indicated. Significant results are highlighted  
695 in bold.

696

697

	<i>Cotinus</i> d.f.=1	Date d.f.=2	<i>Cotinus</i> x Date d.f.=2
Root biomass	0.5	<b>33.3***</b>	0.4
Stem biomass	0.1	<b>11.3***</b>	0.8
Leaf biomass	0.1	2.9	1.1
Mycorrhization rate	0.2	<b>78.2***</b>	1.3

698

699

700 **Table 3.** Saprophytic fungal biomass (expressed as  $\mu\text{g}\cdot\text{g}^{-1}$  soil DM) and abundances of the  
 701 different mesofauna groups (expressed as nb ind. $\cdot\text{g}^{-1}$  soil DM) according to *Cotinus coggygria*  
 702 extract concentrations. Values are means  $\pm$  standard errors; n= 5. Different letters indicate  
 703 significant differences between leachate treatments with a>b (Kruskal-Wallis test,  $P<0.05$ ).

704

Leachate concentration	Saprophytic fungal biomass	Collembola abundance	Oribatida abundance	Predatory Acari abundance
0	116.89 $\pm$ 8.26 a	0.07 $\pm$ 0.01 a	0.22 $\pm$ 0.03 a	0.07 $\pm$ 0.01 a
2.5	129.49 $\pm$ 7.37 a	0.08 $\pm$ 0.04 ab	0.31 $\pm$ 0.06 a	0.08 $\pm$ 0.01 a
5	133.46 $\pm$ 10.29 a	0.02 $\pm$ 0.00 b	0.21 $\pm$ 0.04 a	0.08 $\pm$ 0.01 a

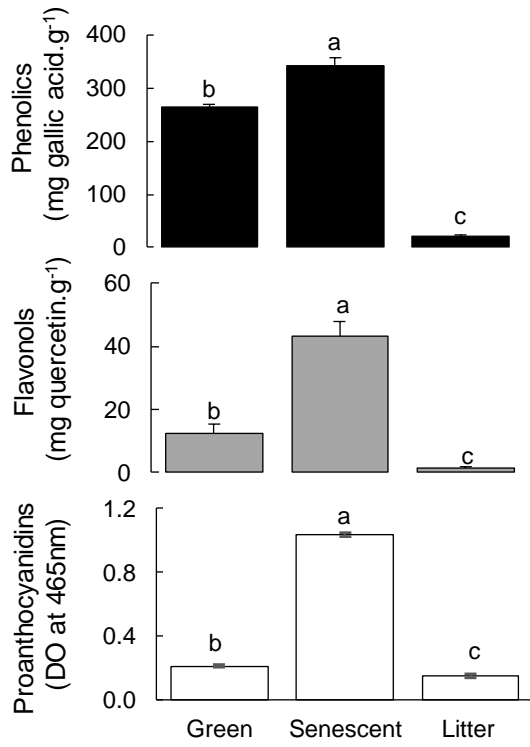
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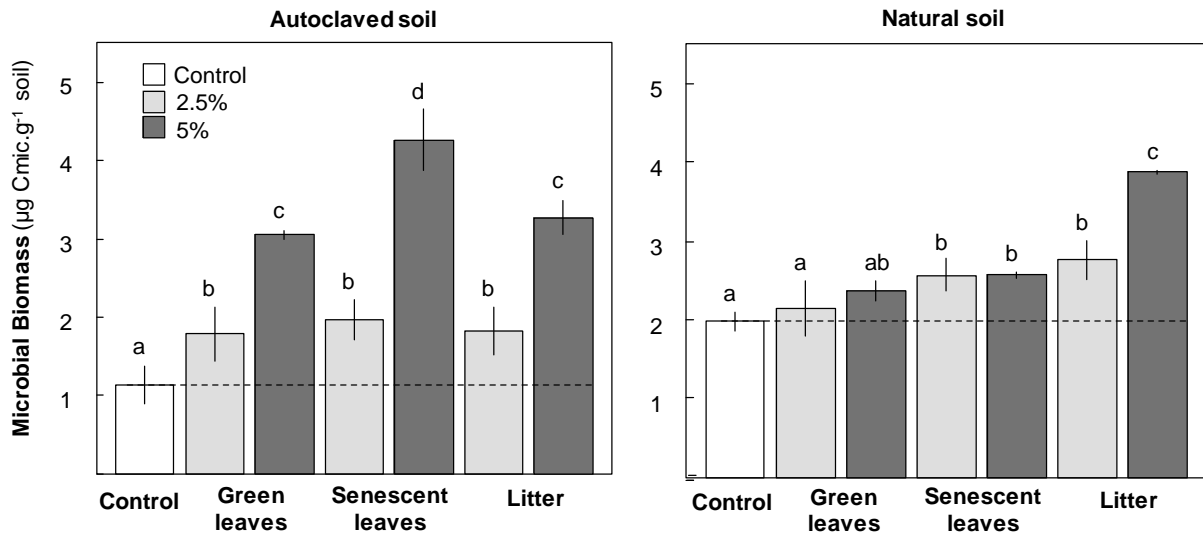
707 **Figure 1:** Chemical composition of *Cotinus coggygia* aqueous extracts of green leaves,  
708 senescent leaves and leaf litter. Values are means  $\pm$  standard errors; n= 6. Different letters  
709 indicate significant differences between leaf types with a>b>c (Kruskal-Wallis test,  $P<0.05$ ).

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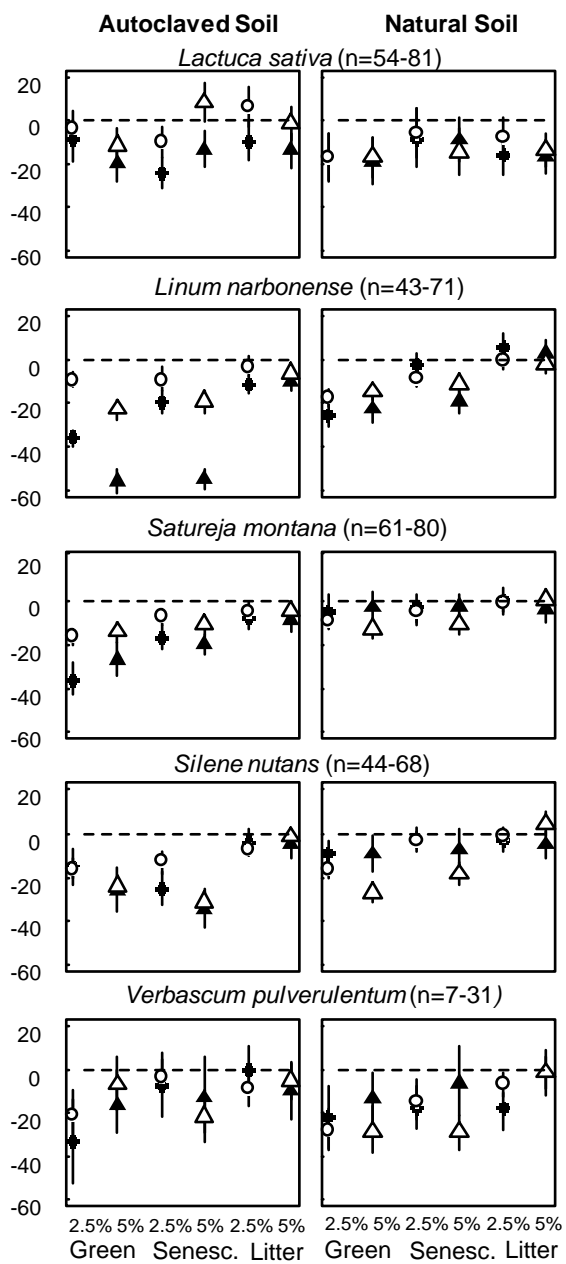
712 **Figure 2:** Influence of *Cotinus coggygia* extracts on microbial biomass of autoclaved (left) or  
713 natural soils (right). Dashed lines represent the mean value of control samples. Different letters  
714 denote differences between treatments with a<b<c (post-hoc Tukey tests,  $P<0.05$ ). Values are  
715 means  $\pm$  standard errors; n= 4.  
716



717  
718

719 **Figure 2:** Relative Allelopathic Effects of *Cotinus coggygia* aqueous extracts on herbaceous  
 720 species root (filled symbols) and shoot (open symbols) lengths. Extracts were prepared with  
 721 different leaf types (green, senescent and litter) at two concentrations (2.5% and 5% dry matter  
 722 of plant material, figured by circles and triangles, respectively) and the target species were  
 723 grown either on autoclaved (left) or natural soils (right). Error bars are bootstrapped confidence  
 724 intervals, number of seedlings (n) are indicated for each species.

725

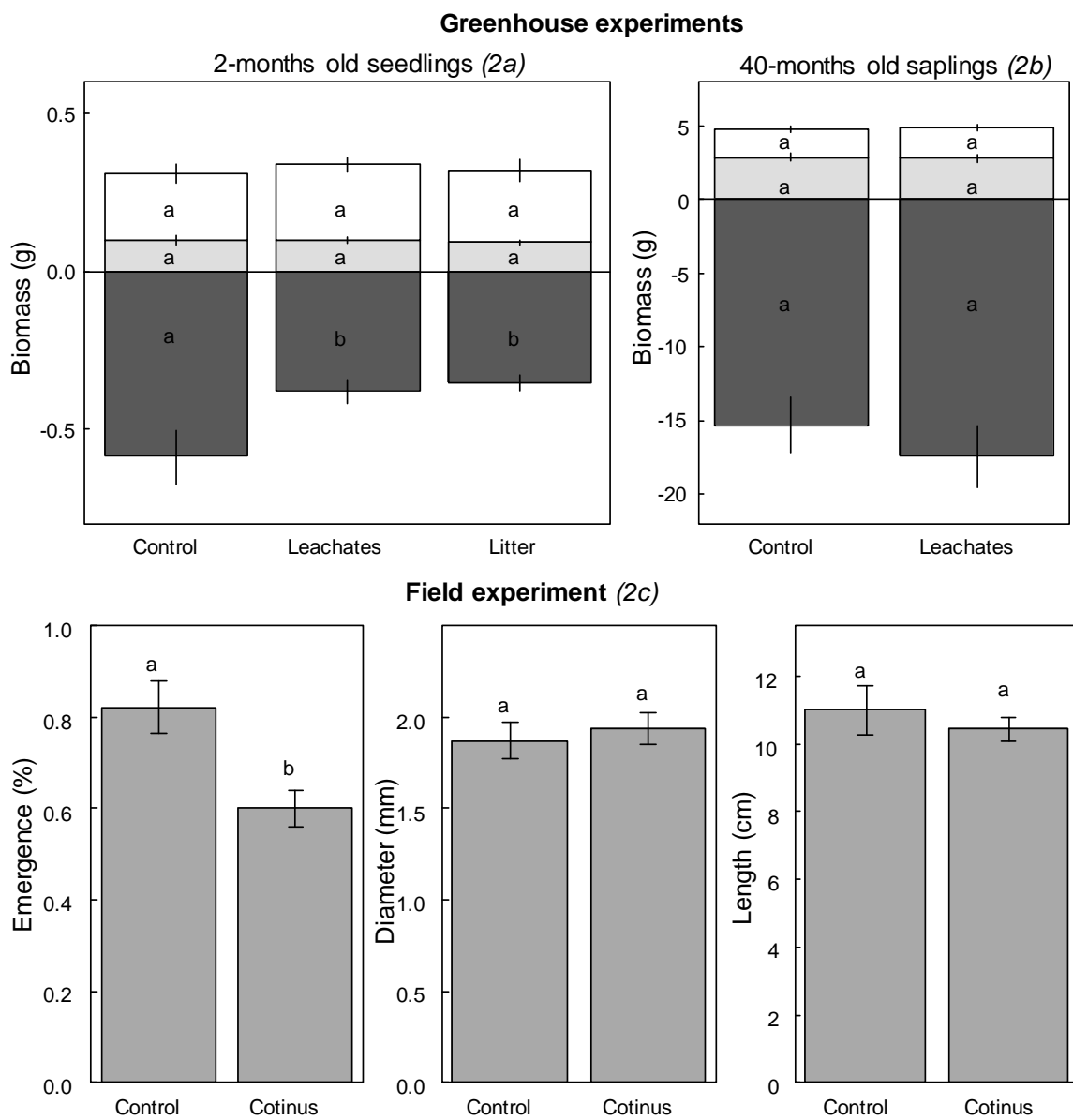


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728 **Figure 4:** Influence of *Cotinus coggygia* on downy oak seedlings development. Upper panel:  
 729 greenhouse experiments showing the influence of allelochemicals on oak leaf (white), stem  
 730 (grey) and root (dark grey) biomasses. Lower panel: field experiment showing the influence of  
 731 *Cotinus coggygia* on seedling emergence, diameter and length. Different letters indicate  
 732 differences between treatments for each biomass compartment. Values are means  $\pm$  standard  
 733 errors; n= 10 for greenhouse experiments, n= 30 – 50 for field experiment.

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