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

J. Gavinet, M. Santonja, Virginie Baldy, H. Hashoum, S. Peano, T. Tchong, Stephane Greff, R Gros, Catherine Fernandez, A. Bousquet-Mélou

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AUTHORS

Gavinet J.[†], Santonja M.[†], Baldy V., Hashoum H., Peano S., Tchong T., Gros R., Greff S., Fernandez C.[*], Bousquet-Mélou A.

Aix Marseille Univ, Avignon Université, CNRS, IRD, IMBE, Marseille, France.

† Equal contribution to the work

*Corresponding author

catherine.fernandez@imbe.fr

Tel: +33 (0)4 13 55 12 22

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

ABSTRACT

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

1. INTRODUCTION

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

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

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

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

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

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

2. MATERIAL AND METHODS

2.1. Experimental site and material collection

Field experiment and biological material collection (*Cotinus coggygria* leaves, oak acorns and soils) were performed at the Oak Observatory at the OHP (O_3HP) experimental site located in the research center "Observatoire de Haute Provence", 60 km north of Marseille (43°56'115" N, 05°42'642" E; 680 m a.s.l.). The climate is Mediterranean with a mean annual temperature of 11.9 °C and a mean annual precipitation 830 mm (1967–2000). The site is covered by an old-growth oak forest belonging to the site Natura 2000 "FR9302008 Vachères", which was managed for centuries by coppicing. Downy oak (*Quercus pubescens*: 75% coverage) and Montpellier maple (*Acer monspessulanum*: 25% coverage) are the two dominant tree species, with understory vegetation dominated by smoke tree (*Cotinus coggygria*: 30% coverage). The soil is a pierric calcosol (with S horizon between limestone rocks) or calcarisol when limestone appears less than 25 cm deep.

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

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

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

2.2. Chemical analysis

2.2.1. Total phenolics

Extraction of phenolics was carried out based on the method described by Singleton and Rossi (1965) and adapted to smaller amounts of plant material. Briefly, a dry mass (DM) of 250 mg of crushed leaf was extracted with 20 mL of deionized water. The mixture was left for 1 h under constant shaking at ambient temperature shielded from light. The extract was then filtered on Whatman GF/C paper filter. A volume of 25 µL of the extract were added to 1650 µL of ultrapure water, 200 µL of saturated Na_2CO_3 aqueous solution and 100 µL of Folin-Ciocalteu's reagent. After 30 min, the phenolic index was measured at 765 nm on a spectrophotometer (Spectronic Biomate 3 Thermo Electron Scientific Instrument Corporation ®) and expressed as equivalent of mg of gallic acid per g of plant material (DM).

2.2.2. Flavonoids

Among phenolic compounds, we focused on flavonoids which were quantified in terms of proanthocyanidins and flavonoid index (total flavonoid). Extraction and quantification were based on a previous work (Kaundun et al. 1998) adapted by our laboratory to smaller amounts of plant material. A mass of 0.5 g DM of crushed leaf was suspended in 15 ml of HCl 2N solution and heated to 90 °C in a water bath with reflux for 50 min with every 10 min of air influxes. The acidic treatment generated anthocyanidins from homologous proanthocyanidins and flavonol aglycones from corresponding flavonol glycosides. The solution was left to cool approximately 30 min and filtered (filter porosity 3). Anthocyanidins were quantified spectrophotometrically at 435 nm (Spectronic Biomate 3 Thermo Electron Scientific Instrument Corporation ®) and expressed as mg per g of plant material (DM). Flavonol aglycones were extracted three times with 9 mL of diethyl ether. The extracts were recombined and evaporated to dryness. The residue was then dissolved and mixed with 1.5 mL of methanol. An aliquot of 100 µL was added to 5 mL of 1 % $AlCl_3$ / MeOH solution and let 20 min to react. The flavonoid index was measured at 530 nm and expressed as equivalent of mg of quercetin per g of plant material (DM).

2.3. Bioassays

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

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166 *2.3.1. Experiment 1: Response of understory plant species & microorganisms to C. coggygria*
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 (± 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. coggygria* 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 × leaf type × concentration × soil type). Bioassays were
178 conducted under natural photoperiod and controlled temperature (20.5 °C ± 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): $Cv = 100\ (\Sigma N_i / \Sigma N_i T_i)$, 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. coggygria* 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

placed in 117 mL glass jars and amended with powdered glucose (1000 µg $C.g^{-1}$ soil). After incubation (1 h, 22 °C), a volume of 1 mL of air was sampled in the headspace with a syringe and injected onto the gas chromatograph (ChrompackCHROM 3-CP 9001) to analyze CO_2 production. SIR rates were converted into MB using equations given by Beare et al. (1990).

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

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

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

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

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

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

2.3.3. Experiment 3: response of soil saprophytic fungi and mesofauna to C. coggygria aqueous

extracts

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

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

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

2.4. Data analysis

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

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

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

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

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

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

3. RESULTS

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***

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295 *3.2.1. Soil microorganisms and herbaceous plant responses to C. coggygria 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 × 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 ± 3.0 *vs.* 55.3 ± 3.9 for *S.*
311 *nutans*; 13.7 ± 2.7 *vs.* 34 ± 3.5 for *V. pulverulentum*). Higher germination rates were however
312 observed for *L. sativa* (85.3 ± 1.6 *vs.* 79.3 ± 1.6) and *Satureja montana* (82.0 ± 2.8 *vs.* 74.0 ±
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 ± 2.0 *vs.* 75.3 ± 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 ± 0.7 *vs.* 55.3 ± 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 coggygria* 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. coggygria* 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 coggygria* extracts effects on germination rate and velocity ranged from -38% to

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

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

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

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

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

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

4. DISCUSSION

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

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

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

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

4.3. Soil organisms modulate plant-plant chemical interactions

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

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

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

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

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

ACKNOWLEDGEMENTS

This study was funded by ECCOREV Research Federation (FR3098) and the French National Research Agency (ANR) through the SecPriMe^2 project (ANR-12-BSV7-0016-01). We also gratefully acknowledge the program MISTRALS (Mediterranean Integrated STudies at Regional And Local Scales), particularly the axe BioDivMeX, and the LabEx OT-Med (no

ANR-11-LABX-0061) funded by the «Investissements d'Avenir» program of the French National Research Agency through the A*MIDEX project (no ANR-11-IDEX-0001-02). The field experiment and material sampling was carried out at the Oak Observatory at "Observatoire de Haute-Provence" (O_3HP) platform with the help of Jean-Philippe Orts. The authors also thank Sylvie Dupouyet for her help with the bioassays.

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685

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 ± 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

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

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691

692 **Table 2**: Results of ANOVAs testing the effects of *Cotinus coggygria* 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

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

698
699

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

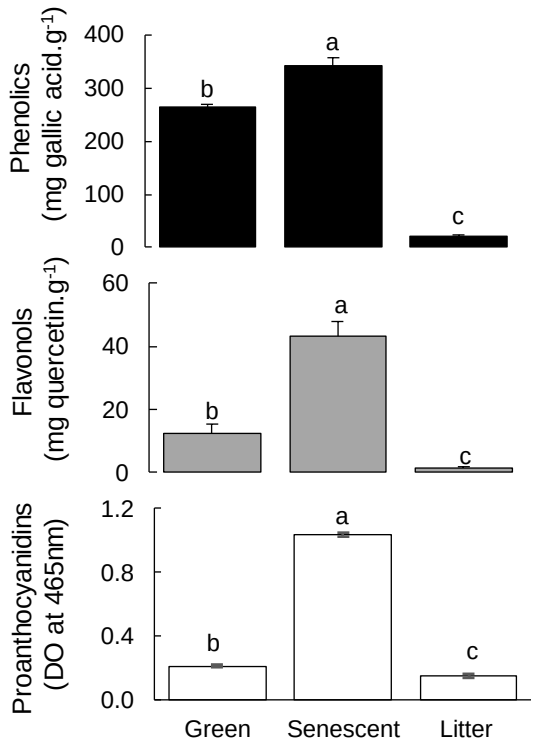
704

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

705

706

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



712 **Figure 2:** Influence of *Cotinus coggygria* 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 ± standard errors; n= 4.
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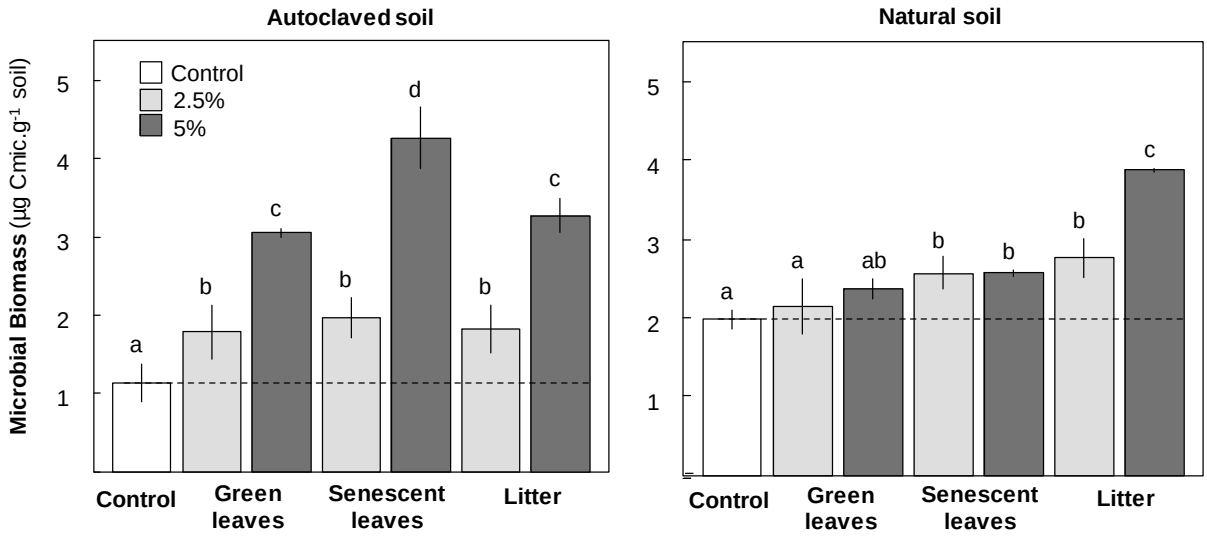

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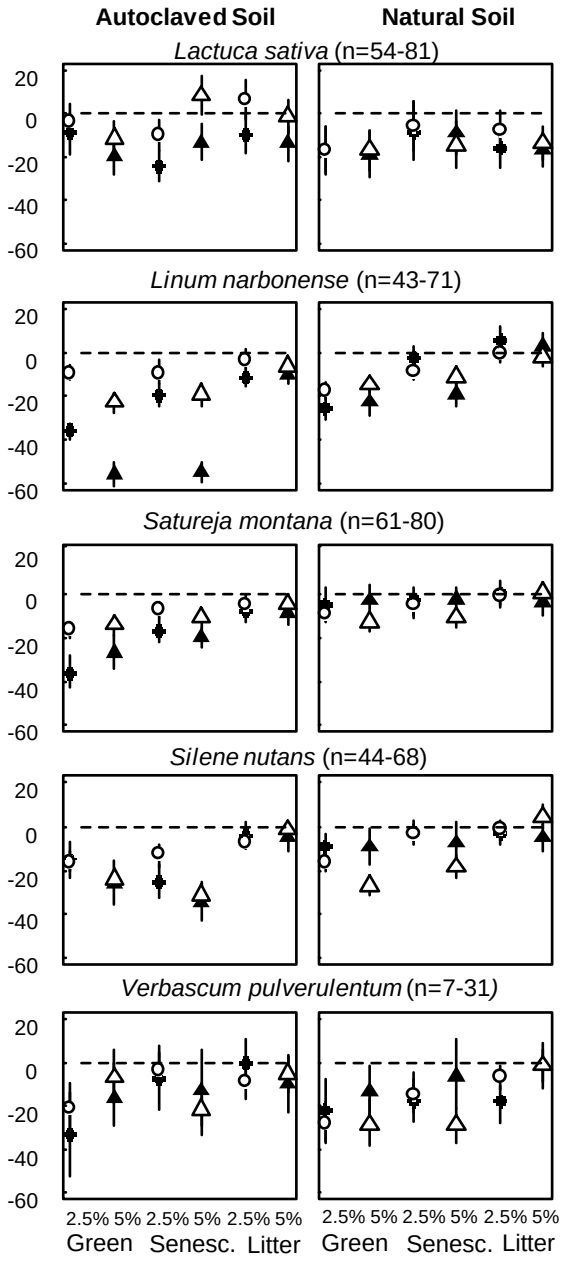
Figure 2: Relative Allelopathic Effects of *Cotinus coggygria* aqueous extracts on herbaceous species root (filled symbols) and shoot (open symbols) lengths. Extracts were prepared with different leaf types (green, senescent and litter) at two concentrations (2.5% and 5% dry matter of plant material,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



728 **Figure 4:** Influence of *Cotinus coggygria* 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 coggygria* on seedling emergence, diameter and lenght. Different letters indicate
732 differences between treatments for each biomass compartment. Values are means ± standard
733 errors; n= 10 for greenhouse experiments, n= 30 – 50 for field experiment.
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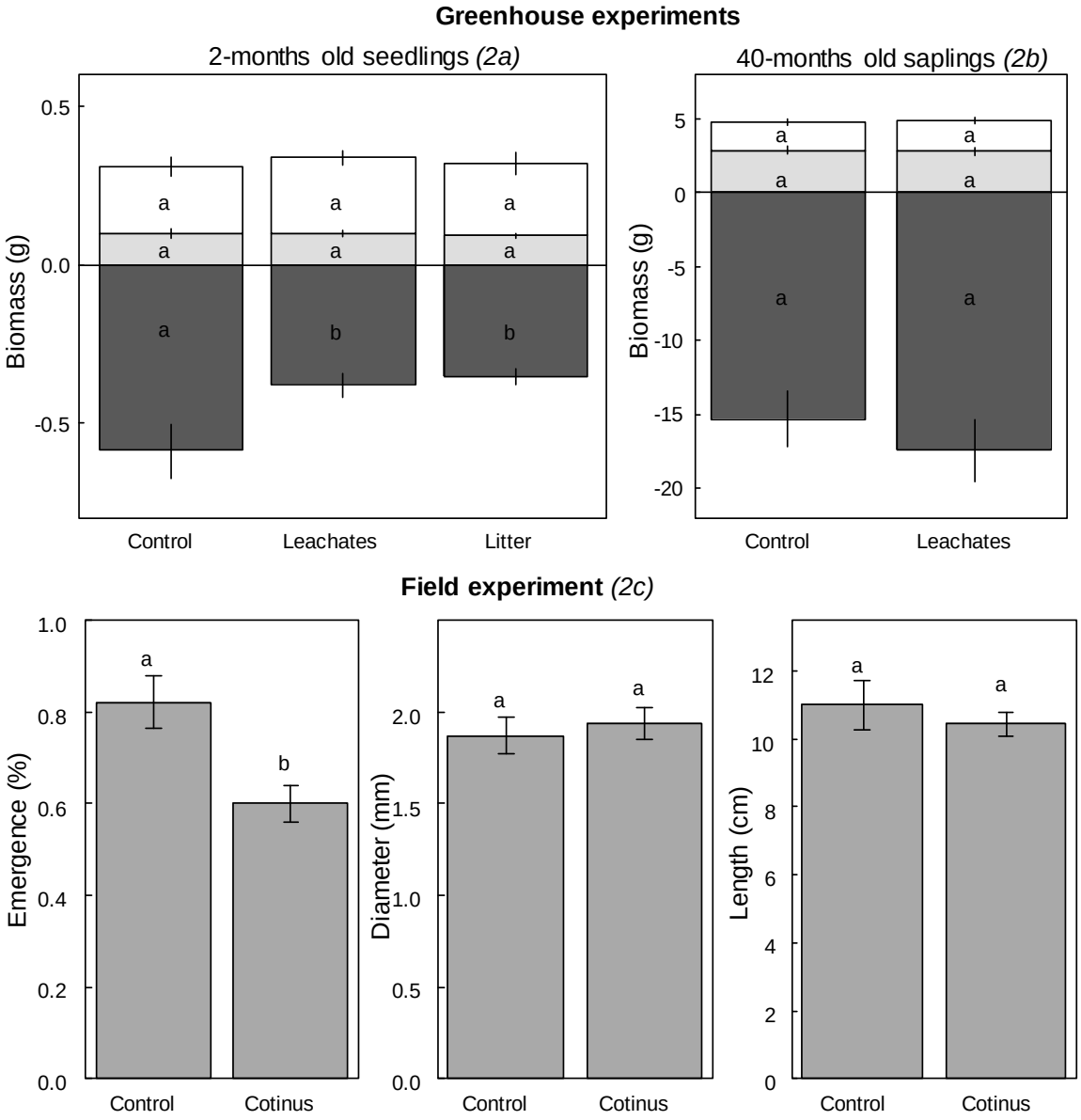

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