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Do litter-mediated plant-soil feedbacks influence Mediterranean oak regeneration? A two-year pot experiment

Authors:

Jordane GAVINET^{1,2,*}, Bernard PRÉVOSTO¹, Anne BOUSQUET-MELOU², Raphaël GROS², Elodie QUER², Virginie BALDY², Catherine FERNANDEZ²

1 – Institut national de recherche en sciences et technologies pour l’environnement et l’agriculture (Irstea), UR RECOVER, 3275 Route de Cézanne, 13100 Aix en Provence, France

2 - Institut Méditerranéen de Biodiversité et d’Écologie marine et continentale (IMBE), Aix Marseille Université, CNRS, IRD, Avignon Université, UMR 7263, 3 place Victor-Hugo, 13331 Marseille cedex 3, France

* Corresponding author: jordane.gavinet@imbe.fr

Abstract

Background & Aims: Oak seedling establishment is difficult and may be partly explained by litter-mediated interactions with neighbors. Litter effects can be physical or chemical and result in positive or negative feedback effects for seedlings. Mediterranean species leaves contain high levels of secondary metabolites which suggest that negative litter effects could be important.

Methods: Seedlings of *Quercus ilex* and *Quercus pubescens* were grown for two years in pots with natural soil and litter inputs from 6 Mediterranean woody species, artificial litter (only physical effect) or bare soil.

Results: Litter types had highly different mass loss (41-80%), which correlated with soil organic C, total N and microbial activity. Litter of *Q. pubescens* increased soil humidity and oak seedlings aerial biomass. Litters of *Cotinus coggygria* and *Rosmarinus officinalis*, containing high quantities of phenolics and terpenes respectively, decomposed fast and led to specific soil microbial catabolic profiles but did not influence oak seedling growth, chemistry or mycorrhization rates

Conclusions: Physical litter effects through improved soil humidity seem to be predominant for oak seedling development. Despite high litter phenolics content, we detected no chemical effects on oak seedlings. Litter traits conferring a higher ability to retain soil moisture in dry periods deserve further attention as they may be critical to explain plant-soil feedbacks in Mediterranean ecosystems.

Keywords: litter effects, allelopathy, soil microorganisms, secondary metabolites, litter traits

Introduction

The composition and dynamics of plant communities is largely influenced by plant-plant interactions occurring at the local scale (Connell and Slatyer 1977; Lortie et al. 2004). Interaction mechanisms include changes in resource levels, microclimate or indirect interactions with other organisms, resulting in a complex balance between positive and negative effects of neighbor presence (Holmgren et al. 1997; Brooker and Callaghan 1998; Montgomery et al. 2010). Among these mechanisms, leaf litter influence on plant species establishment and growth through plant-soil feedback effects has been shown to have important consequences for vegetation community composition and dynamics (Molofsky and Augspurger 1992; van der Putten et al. 2013) and to modulate the effect of neighbor presence (Ganade and Brown 2002; Violle et al. 2006). These litter-mediated effects can occur through both physical and chemical mechanisms (Facelli and Pickett 1991a). Physical mechanisms include mechanical interference for seed germination and modification of abiotic parameters such as soil humidity, light level or temperature (Facelli and Pickett 1991b). Chemical mechanisms include the release of nutrients (Perez-Moreno and Read 2000; Brearley et al. 2003; Quested et al. 2003) but also secondary metabolites, which can have a direct phytotoxic effect on target plant (Bonanomi et al. 2011) or indirectly reduce plant growth by altering nutrient cycling and soil properties (Kuiters 1990; Hättenschwiler and Vitousek 2000; Inderjit and Weiner 2001). Recent studies indicate an uttermost importance of these plant chemical traits for plant-soil feedbacks, with positive effects of litters with high N content and decomposability and negative effects of litters with high contents of phenolics (Dorrepaal et al. 2007; Bonanomi et al. 2011; Lopez-Iglesias et al. 2014). However, Xiong and Nilsson (1999) showed that deciduous tree litter with higher decomposability had higher negative effects on seedlings, that they interpreted by a major release of allelochemicals through decomposition, which underline that the relation between litter decomposability, chemistry and effects on target plants is not clear yet.

Mediterranean plants are particularly rich in secondary metabolites, both terpenoids and phenolics, which help them cope with Mediterranean climate stress such as summer drought, high radiations or temperatures but also lead to high allelopathic potential (Scognamiglio et al. 2013). Negative chemical interactions are thought to play an important role in Mediterranean ecosystems (Vilà and Sardans 1999)

and forest succession (Alías et al. 2006; Fernandez et al. 2008, 2013, 2016). In southern France, the two main oak species forming late-successional forests, the evergreen *Quercus ilex* L. (Holm oak) and the deciduous *Quercus pubescens* Willd. (Downy oak), experience a difficult regeneration. This is due to many factors including variability in seed production, nature and abundance of the seed dispersers, importance of the pre- and post-dispersal predation (e.g. Gómez 2003; Espelta et al. 2008; Pérez-Ramos and Marañón 2008). A major constraint is also related to the high heterogeneity of establishment success at the micro-site level, which is influenced by the presence and identity of neighboring plants (e.g. Gómez-Aparicio et al. 2004; Prévosto et al. 2016). Chemical interactions with neighbors may participate in explaining their regeneration pattern (Gómez-Aparicio et al. 2004) but have been poorly explored, in particular as regards litter effects. Laboratory bioassays have shown that leaf litter from a wide range of Mediterranean species can negatively influence herbaceous seedling germination or growth (Bonanomi et al. 2006; Alrababah et al. 2009; Lopez-Iglesias et al. 2014) which was attributed to the release of phytotoxic compounds (Bonanomi et al. 2011; Lopez-Iglesias et al. 2014). Litter effects on late-successional oak species remain to be tested as litter effects strongly depend on both litter type and target seedlings identity (Xiong and Nilsson 1999). These species-specific effects can be of high importance for explaining plant succession (van der Putten et al. 2013) and may participate in defining the two oak species regeneration niches. For instance, Li and Romane (1997) showed that soil beneath *Q. ilex* stands had a negative effect on *Q. ilex* but not on *Q. pubescens* germination, which could be due to a modification of soil properties by *Q. ilex* litter decomposition.

In this study, we examined the 2-year litter effect of 6 Mediterranean woody species dominant in the main successional stages – shrublands, pioneer pine forests and downy oak forest - on soil chemical and microbial properties and seedling growth, biomass allocation and chemistry of the two oaks *Q. ilex* and *Q. pubescens*. Our aim was to characterize medium-term litter effects on oak seedlings and to gain insight into underlying mechanisms by exploring links between litter effects, litter chemical traits and soil properties, because litter effects are often mediated by a change in soil characteristics (Facelli and Pickett 1991a; Inderjit and Weiner 2001). Specifically, we asked:

- i) do two co-occurring oak species respond in the same way to litter additions?

- ii) are litter effects related to species successional stage or litter chemistry, in particular N and secondary metabolites content?
- iii) are litter effects mediated by a change in soil humidity, nutrient content or microbial communities?

Material & methods

Plant and soil material

We selected 6 Mediterranean woody species representing the dominant species in the 3 main successional stages of Southern France: shrublands with *Rosmarinus officinalis* L. and *Cistus albidus* L., pioneer pine stands with *Pinus halepensis* Mill. and *Quercus coccifera* L. (an understory shrub which can also be found in shrublands) and late-successional downy oak forests with *Quercus pubescens* Willd. and the understory shrub *Cotinus coggygria* Scop. Senesced leaves were collected either with litter traps or by shaking plants at the periods of leaf abscission of each species, then were air dried and stored at room temperature in the dark until further use. Natural soil was collected with a flat shovel from a calcareous, sandy-loam soil on terraces at Saint-Mitre-les-Remparts (43°46'N; 5°04'W, Bouches-du-Rhône, France) in the mineral soil layer (20-60cm deep) and mixed with coarse sand and perlite (ratio 3:1:1) in order to improve soil drainage.

Oak acorns of *Q. pubescens* and *Q. ilex* were collected in autumn 2012 at Saint-Mitre-les-Remparts on several individuals per species. Non-viable acorns were eliminated by the floating method and visual screening. Acorns were then sown in an organic substrate in moist conditions. Seedlings emerged in April 2013. Eighty emerged seedlings of similar dimensions per species were selected in June 2013 (3 months-old seedlings) and each seedling was transplanted in 10L pots (30cm Ø x 26cm deep) filled with the soil mixture. Root length at the time of transplantation was about 2-3cm.

Experimental design

Eight litter treatments were tested: no litter (control, bare soil), artificial litter in fiber glass which mimics physical effects of leaf litter without chemical effects (Schlatterer and Tisdale 1969) and leaf litter of each of the 6 woody species selected. Each treatment was replicated in 10 pots for each of the 2 oak species, resulting in 160 pots in total. For litter treatment, soils were covered with 10g of leaf litter,

corresponding to 263g/m², and this input was renewed every 6 months during 2 years (i.e. 526 g/m²/an). As a comparison, litter accumulation below the studied species in the field ranged between 400 and 1300g/m². Pots were covered with a net to prevent litter losses or addition, stored in the outside and watered with automatic sprayers about twice a week in summer and once a month the rest of the year. The experiment started in July 2013 and lasted 2 years until June 2015.

Litter chemistry and decomposition

Initial litter quality was determined on 5 samples of freeze-dried and grounded litter per species.

Litter carbon (C) and nitrogen (N) concentrations were analyzed by thermal combustion on a Flash EA 1112 series CHN elemental analyzer (Thermo Scientific, USA).

Litter phosphorus (P) was extracted with 20ml of nitric acid from remaining dry ash after combustion of 0.5g of litter at 500°C for 5h in a muffle furnace. pH was adjusted to 8.5 with a 40% NaOH solution. 1ml of each sample, 0.2ml of mixed reagent (emetic tartar and ammonium molybdate solution), 0.004ml of ascorbic acid and 0.76ml of distilled water were placed in a spectrophotometer microcuvette. After 150min, phosphorus concentration was measured at 780nm with an UV/Vis spectrophotometer (Thermo Scientific, USA).

Terpenoid compounds were extracted using 0.5g of litter and a mixture of dichloromethane and hexane (90:10) as organic solvents. Terpene concentration was calculated in a dry mass (DM) basis. Water-soluble terpenoids compounds were also measured as this fraction may be particularly important for allelopathic effects (Fischer et al. 1994). Two grams of litter were placed in 20 ml water during 24h and filtered. The resulting water extracts were soaked for 30min with 1ml of the organic extraction solution, and the organic layer sampled after phase separation. Organic solution was injected on a gas chromatograph (Hewlett Packard GC6890®) coupled to a mass selective detector (MSD; HP 5973N). Compounds separation was achieved on a HP-5MS capillary column (30 m x 0.25 mm x 0.25 µm, J&W Scientific) with helium as carrier gas. After sample injection, the initial temperature (40°C for 5 min) was increased up to 270°C at a rate of 4°Cmin⁻¹, then maintained for 2.5min. Identification of terpenes was established by comparison of the retention index and the mass spectrum of detected compounds

with those of authentic reference samples (Sigma-Aldrich®) when available and/or database (NIST2008) and literature. Terpenes were quantified based on internal standard (dodecane - 100ng/2µl of injected solution).

Total folin phenolics were measured colorimetrically using gallic acid as a standard. A 0.25g leaf sample was dissolved in 20ml of a 70% aqueous methanol solution, shaken for 1h and then filtered (0.45µm filter); 0.25 ml of filtered extract was mixed with 0.25 Folin-Ciocalteu reagent, 0.5ml of saturated aqueous Na₂CO₃ to stabilize the color reaction, and 4ml of distilled water. After 60min, concentration of phenolics was measured at 765nm on an UV/Vis spectrophotometer (Thermo Scientific, USA).

Litter water holding capacity (WHC) was measured on 5 sample for each species. Intact leaf litter samples were placed in distilled water for 24h, drained and weighed moist. Samples were then dried at 60°C for 3 days and weighed dry. WHC was calculated as the ratio of moist weight to dry weight × 100.

Ash free dry mass of each litter was calculated as the difference between intact litter dry weight and weight of litter ashes after combustion at 500°C for 5h in a muffle furnace. At the end of the experiment, litter was removed from each pot, freeze-dried, manually cleaned of soil particles and weighed. We used a part of each litter sample to calculate ash free dry mass after combustion at 500°C for 5h in a muffle furnace. We calculated litter mass loss as the difference between litter ash free dry mass inputs and litter ash free dry mass at the end of the experiment.

Soil analysis

Soil humidity was measured at each litter input (i.e. at 6, 12 and 18 months of experiment) with a portable TDR probe (Wet2, Delta-T Devices, UK) by averaging 2 readings per pot.

At the end of the experiment, after removing litter fragments, the upper 4cm of soil were collected and sieved at 2mm on 5 randomly selected pots per treatments. A subsample of sieved soil was used to determine water content by weighing fresh weight and oven-dried weight (3 days at 60°C). Another subsample of soil was air-dried for chemical analysis. Air-dried soil was grounded and analyzed for total C and N content as described for litter. Inorganic C concentration was measured using a Bernard

calcimeter. Briefly, the method consists of quantifying the CO₂ released when the sample is treated with hydrochloric acid: in a closed system, under a constant pressure and temperature, the quantity of CaCO₃ is directly proportional to the volumetric increase resulting from the release of the CO₂. Organic C was then calculated from the difference between total and inorganic C.

Soil microbial basal respiration (BR) and substrate induced respiration (SIR) were measured to assess the ecophysiological state of soil microbial communities. Ten grams (dry weight equivalent) of fresh soil were placed in 117ml glass jars which were then closed with hermetic rubber septa and incubated for 4h at 24°C. After incubation, 1 ml of air was sampled in the head space with a syringe and injected into a gas chromatograph (Chrompack CHROM 3 – CP 9001) equipped with a thermal conductivity detector and a packed column (Porapack) to analyse CO₂ production. Ambient CO₂ concentrations were subtracted from sampled CO₂ concentrations, and the values were adjusted to 24°C according to Ideal Gas Laws with Q₁₀=2. To measure SIR, ten grams (dry weight equivalent) of fresh soil were placed in 117 ml glass jars, amended with powdered glucose (1,000µg C g⁻¹ soil). After glucose amendment, samples were incubated during 1.5h, then air flushed and the glass jars were closed and incubated during 1.5h. One ml of air was then sampled with a syringe and injected into the gas chromatograph to analyze CO₂ production as described above.

Catabolic profiles of cultivable microbial populations were assessed with Biolog EcoPlates (Biolog Inc., USA) using a procedure adapted from Garland and Mills (1991). Briefly, 5g (dry mass equivalent) of soil were shaken in 50ml of a sterile 0.1% solution of tetrasodium pyrophosphate for 1h at 500 rpm to suspend microbial communities, then extracts were diluted at 1:100. Each 96-well plate contains 3 replicate blocks of 31 individual carbon sources, which are considered to represent root exudates (Preston-Mafham et al. 2002), with a water blank for each block of replicates. A 125µL aliquot of diluted extract solution of each sample was added to all 31 wells in each EcoPlate. The plates were incubated at 30°C for 7 days, and absorbance was measured at 595nm on a microplate reader (Metertech Σ960, Avantec, France). Colorimetric reaction reveals substrate use intensity by the microbial community.

Seedlings measurements

Non-destructive measurement of seedling dimensions (diameter, height and cumulative length of stems) were performed at each litter input (i.e. at 0, 6, 12 and 18 months after the beginning of the experiment) and used to compute increments between each sampling date (Table S1 Supp. Mat.) At the end of the experiment, all seedlings were harvested and separated into leaves, stems and roots. Leaf area was measured with WinFOLIA software (Regent Instruments, Canada). Roots were gently washed and separated into fine and coarse roots. All plant parts were freeze-dried, and weighed. Data were used to calculate seedling total biomass and Root/Shoot ratio. A randomly selected subsample of 5 individuals per treatment, corresponding to the pots used for soil analysis, was selected for analysis of leaves C, N and phenolic content, fine roots C and N content, following the same methods described for initial litter quality. On the same subsample of seedlings, 5 segments of 3cm of fine roots were analyzed for percentage ectomycorrhizal root length colonization (PRLC) following the gridline intersect method (Giovannetti and Mosse 1980).

Data analysis

Differences in initial litter chemistry and litter mass loss were analyzed with one-way ANOVA, followed by a post-hoc Tukey test. Differences in soil humidity depending on date, litter type and their interaction were tested with a linear mixed model including pot as random factor to account for the repeated measures. Similarly, differences in oak diameter, height and length increment were tested with a linear mixed model. For soil microbial catabolic diversity assessed by Biolog Ecoplates, the average well colour development (AWCD) of each internal replicate block of 31 wells in each plate was calculated at each reading to determine the incubation time corresponding to AWCD = 0.4 absorbance units ($t_{0.4}$). The absorbance value at $t_{0.4}$ in each well was then normalized. A Shannon index was calculated to assess microbial catabolic diversity. The effect of oak species, litter treatment and their interactions on soil N and organic C content, microbial respiration, SIR and catabolic diversity, seedling biomass and allocation, seedling leaves and roots chemistry and percentage mycorrhizal root length were analyzed with two-way ANOVA followed by post-hoc Tukey tests. Normality and homoscedasticity were tested using Shapiro-Wilk and Bartlett tests, respectively, and data were log-transformed when necessary (i.e. for leaf biomass and leaf area). Correlations between litter initial

characteristics, decomposition, and soil parameters averaged by species were also tested with Pearson correlation. A Partial Least Square Discriminant Analysis (PLS-DA) was conducted to reveal differences of substrate utilization profiles between litter treatments and oak species. Difference between groups was tested with a permutational test based on cross validation, with 1000 permutations. All statistical analyses were conducted using R software 3.2.1.

Results

Litter quality and mass loss

Litter species had contrasted nutrient and secondary metabolites content (Table 1). *Q. coccifera* and *R. officinalis* had the highest N content and *C. albidus* and *C. coggygia* the poorest, while P content was less contrasted between species. All species were rich in phenolics with particularly large amounts for *C. coggygia*. Litters of *R. officinalis* and *P. halepensis* were rich in terpenes, more solubles for the former species.

Litter mass loss differed also strongly between species with a high mass loss of 70-80% for *C. coggygia* and *R. officinalis*, 60% for *C. albidus*, and about 40-50% for the other species.

Litter effect on underlying soil

Soil humidity was affected by date ($F=212$, $P<0.001$), litter treatment ($F=6$, $P<0.001$) but not their interaction ($F=1$, $P=0.12$). All litter treatments except *R. officinalis* and *C. albidus* increased average soil humidity compared to the control pots (bare soils), but only the litter of *Q. pubescens* induced higher soil humidity than the artificial litter treatment (Table 2). Litter effects on soil humidity compared to bare soils tended to increase with time, reaching a +65% increase under the litter of *Q. pubescens* at the last sampling date (Fig. S1 Supp.Mat.).

Litter treatment influenced all measured soil parameters (Table 2). Litter mass loss positively correlated with soil N and organic C content, soil microbial basal respiration (Figure 1) and SIR ($r=0.79$, $P=0.02$) but not microbial catabolic diversity ($r=0.35$, $P=0.4$). Litter effect on soil properties was not related to any measured initial litter traits (data not shown). The two oak litter treatments and, to a lesser extent, the litter of *P. halepensis* favored the highest catabolic diversity (Shannon index). The discriminant analysis revealed a difference between the types of substrates consumed by microbes in the soils

amended by litters of *C. coggygia* – with a high consumption of a carbohydrate (i-erythrol), a carboxylic acid (malic acid) and a polymer (Tween 80) – and *R. officinalis* – with a high consumption of easily degradable carbohydrates (Figure 2, $P=0.001$).

Oak species and litter effect on seedlings

Seedling relative growth decreased along the experiment but was not influenced by oak species identity neither litter treatment (Table S1 Supp. Mat.). In two years, the mean seedling diameter increased by 3.8 ± 0.1 mm, seedling height increased by 11.2 ± 0.1 cm and seedling length by 16.3 ± 0.1 cm.

Seedling biomass and biomass allocation strongly differed between the two oak species (Table 3, Figure 3). Leaf biomass was higher for the evergreen *Q. ilex* while stem, root and fine root biomass were higher for *Q. pubescens*, resulting in a higher root/shoot ratio for the latter species. Litter treatment influenced leaf area, leaf and stem biomass and to a lower extent Root/Shoot ratio (Table 3). For both species, leaf biomass was higher in pots with *Q. pubescens* or artificial litter than with *C. coggygia* litter or in control pots and stem biomass was higher with *Q. pubescens* litter than with *C. coggygia* litter or in control pots. Root/Shoot ratio tended to be lower with *Q. pubescens* litter than in control pots ($P=0.09$, Table 3, Figure 3). Aerial (leaf+stem) biomass correlated with mean soil humidity ($r=0.80$, $P=0.02$). Interactions between oak species identity and litter treatment were never significant, however *Q. pubescens* seedlings responded with a higher intensity to litter treatments (Figure 3). For instance, leaf and stem biomass increase with *Q. pubescens* litter compared to the control pots was +43% and +53% respectively for *Q. pubescens* seedlings but only +22% and +18% for *Q. ilex* seedlings.

Seedling leaves N and phenol and fine root N concentrations differed between species but not between litter treatments. *Q. pubescens* had a higher leaf and fine root N but a lower leaf phenol concentration than *Q. ilex* (Figure 4). Percentage ectomycorrhizal root length colonization was slightly higher for *Q. pubescens* ($70.7 \pm 0.2\%$) than for *Q. ilex* ($65.0 \pm 0.3\%$) but was not modified by litter treatment (Table 3).

Discussion

Litter inputs generally increased soil humidity, N and organic matter content and soil microbial respiration. Litters of *Q. pubescens* and to a lesser extent artificial litter had positive effects on aerial seedling biomass, while other litter types had no effects compared to bare soil.

Leaf litter influence on oak seedling establishment and forest succession

Positive effect of litter on soil humidity and its beneficial effects on seedlings have already been outlined, particularly in dry conditions (Fowler 1986; Violle et al. 2006; Donath and Eckstein 2008). Here, seedlings increase in aerial biomass and decrease in Root/Shoot ratio with the litter of *Q. pubescens*, corresponding to the highest soil humidity, suggests that the main litter effects were physical rather than chemical (Navarro-Cano et al. 2010). However, the low seedling aerial biomass with *C. coggygria* litter may result also from a negative effect of phenolics release as soil humidity was not particularly low with this species. Interestingly, the highest positive effects are found for *Q. pubescens* litter on its own seedlings. While conspecific litter effects have been suggested to be generally negative and long-lasting due to the accumulation of self-DNA fragments (Mazzoleni et al. 2015), this mechanism did not seem to be predominant in our experiment.

The two oak species studied differed in several traits, revealing different strategies: *Q. pubescens* higher leaf and root N content and lower leaf phenol content suggest a more acquisitive strategy, while *Q. ilex* is more conservative and invests more in defense. These results are in line with the expectation that *Q. pubescens* is less tolerant to drought stress but able to grow faster in mild conditions (Miglioretti 1987; Quézel and Médail 2003). This difference may explain why *Q. pubescens* seedlings responded with a higher intensity to the improvement of soil humidity by its own litter. Similarly, Demey et al. (2013) found that nitrogen release from leaf litter benefited most to species with an acquisitive strategy. Most studies of Mediterranean litter effects used sensitive herbaceous species as phytometers (e.g. Bonanomi et al. 2006; Lopez-Iglesias et al. 2014), while large-seeded and woody species seem to be much less sensitive to chemical litter effects than annual ones (Loydi et al. 2013; Bonanomi et al. 2017). Oaks are late-successional species with slow growth rates and important seed reserves, which may explain their overall poor reaction to litter addition and chemical composition. In addition, they quickly form a deep tap-root which may allow them to escape from litter influences. The link between species

strategy and allelopathic interactions is just beginning to be investigated (see also Meiners 2014 for an attempt to link functional traits to allelopathic potential), but seems to be of high importance to include allelopathy into a broader ecological context (Blanco 2007; Meiners et al. 2012).

In this study, we found no relation between litter species successional stage and their effects. Comparing grasslands and woodlands, Donath and Eckstein (2008) concluded that litter effects slow-down succession because of both a difference in litter effects and species sensitivity, but among 5 litter types from a successional gradient including grassland to forest sub-arctic species, Quedsted and Eriksson (2006) found no relation between litter effect and successional stages. In addition, we found no negative litter effects in spite of reported allelopathic potential of several of the studied species. For instance, *Cistus* allelopathy may occur through root exudates of some species such as *Cistus ladanifer* (e.g. Alías et al. 2006) but remains to be demonstrated for other species (Leiva et al. 2015). Leachates of *P. halepensis* fresh needles have also been shown to affect seedlings of several species (Fernandez et al. 2008, 2013, 2016), but Aleppo pine litter had only physical effects on 2 woody species (Navarro-Cano et al. 2010) and no effect on holm oak seedling germination or growth (Broncano et al. 1998), maybe because fresh needles are more allelopathic than senescent or decaying needles (Nektarios et al. 2005). Despite having a high allelopathic potential, the studied species does not seem to hamper oak regeneration through litter decomposition, although these results do not preclude that chemical interactions occur through other mechanisms than litter decomposition or with more sensitive species. Increased soil humidity under litter, in turn, could speed up Mediterranean forest succession.

Disentangling litter effects from other neighbor effects in the field is far from easy. In this study, we chose to examine litter effects on 3-month-old seedlings and their subsequent growth in a controlled pot experiment with a common soil material. However, to extrapolate our results to forest dynamics it would be interesting to perform a field experiment by seeding oaks under the same species tested here, including a litter removal treatment and a monitoring of soil humidity. Furthermore, we assumed that litter effects in the first 3 months of germination were negligible as seed reserves are the main determinant of early-seedling performance (Jensen and Gutekunst 2003; Loydi et al. 2013) but we may have missed early litter effects which could be relevant for the taproot early development.

Links between litter traits, decomposition and effects on soils and seedlings

In this study, litter mass loss determined N release and litter effect on soil chemical and microbial properties (Carrera et al., 2005). However, litter mass loss was not related to C:N, N, WHC or total phenolics (Chomel et al. 2016), suggesting that in our set of species the drivers of decomposition were either not measured here or multifactorial. Fast decomposing litters of *C. coggygia* and *R. officinalis* had strong and positive effects on soil N and organic C and on soil microbial respiration and SIR, probably because of a high N release and despite high secondary metabolites content and reported antimicrobial properties of the former species (Marčetić et al. 2013). The toxicity of secondary compounds for microorganisms have often been emphasized (e.g. Langenheim 1994; Ormeño et al. 2006), but phenolics (Bowman et al. 2004; Hättenschwiler and Jørgensen 2010) or terpenes (Vokou et al. 2002; Ehlers 2011) can also act as C-sources and stimulate soil respiration. Litters of *C. coggygia* and *R. officinalis* led to specific microbial catabolic profiles, which may be linked to a selection of microorganisms able to degrade specific phenolic or terpenoid compounds (Chomel et al. 2014). Litter from different species thus strongly influenced soil microbial communities, modifying their activity, catabolic diversity or profile in a species-specific way (Fanin et al. 2014; Chomel et al. 2014).

However, litter effects on soil microbial communities and N content did not correspond to effects on oak seedlings, which was probably more influenced by increased soil humidity as discussed above. To our knowledge, there is no recognized litter trait influencing litter effects on soil humidity, but this would deserve further attention. Interestingly, litters with low decomposition rates could improve soil humidity in dry periods by maintaining a thick layer of litter over time, thereby reducing soil evaporation (Monnier et al. 2012), which may result beneficial for seedlings. Traits such as ratio of thickness per mass may also influence litter ability to retain soil water (Donath and Eckstein 2008). Contrary to previous studies (Dorrepaal et al. 2007; Lopez-Iglesias et al. 2014), we found no link between litter chemistry (N or secondary metabolites content) and effects on oak seedlings development. In this study, litters with the highest amounts of potentially phytotoxic secondary metabolites (i.e. *C. coggygia* and *R. officinalis*) were also the faster decomposing species and released high N amounts. Thus, negative effects of allelopathic compounds may have been outbalanced by positive effects of N enrichment,

resulting in neutral interaction outcome. In addition, these litters led to the development of an active and specialized microbial community, which may have degraded and detoxified allelochemicals (Inderjit 2005; Kaur et al. 2009; Ehlers 2011). Moreover, oaks were highly mycorrhized which could have protected seedlings from phytotoxic compounds (Mallik and Zhu 1995; Zeng and Mallik 2006).

Litter traits explaining feedback effects on seedlings may also change through time (Dorrepaal et al. 2007). Several studies found short-term negative litter effects driven by litter phenolics (e.g. Lopez-Iglesias et al. 2014) which shifted to neutral or positive effects at longer term (Xiong and Nilsson 1999; Bonanomi et al. 2017) probably in link with positive N effects (Dorrepaal et al. 2007). Here, we chose to examine medium-term litter effects with a design mimicking repeated litter amendment in natural systems. By doing such, we may have missed some temporary negative litter effects, however, we show that litter secondary metabolites do not impair seedling development over this critical 2-year period. In addition, oaks are slow growing species that may need more time to respond to soil modifications by litter: Fernandez et al. (2016) found a root inhibition of downy oak seedlings in response to *P. halepensis* fresh needle leachates only after 1.5 year of treatment. In forests, litter removal experiments have induced a reduction in adult tree growth only at long-term (>15 years, Sayer 2005).

Conclusion

Litter mass loss differed widely between 6 important woody Mediterranean species. Litter decomposition, but not chemical traits, correlated with an increase in soil organic C, total N and microbial activity. Higher soil humidity under the slow decomposing *Q. pubescens* litter is probably responsible for its positive effects on aerial seedling biomass, while other litter types had neutral effects. Despite high secondary metabolite contents, we found no evidence for negative litter effect, which may have been outbalanced by positive effects on soil humidity or soil N and microbes. Predicting litter effect on seedlings on the basis of their chemistry appear far from easy due to the multifactorial drivers of decomposition and nutrient release and the importance of microorganisms in determining the fate of allelochemicals. Structural litter traits that could influence their effects on soil humidity and seedlings growth should be integrated in further studies (Donath and Eckstein 2008), as they may prove critical in explaining plant-soil feedbacks in dry ecosystems. Our study suggest that increased soil humidity under

litter could speed up Mediterranean forest succession, but that litter-mediated allelopathy seems unlikely to impair oak regeneration.

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Tables

Table 1: Initial litter chemistry, litter mass loss and N release. Data are means \pm standard error of 5 replicates per species for initial litter traits, 20 replicates for litter mass loss and N release. Different letters indicate differences between species, Tukey post-hoc test at $p < 0.05$.

	Artificial litter	<i>C. albidus</i>	<i>C. coggygia</i>	<i>P. halepensis</i>	<i>Q. coccifera</i>	<i>Q. pubescens</i>	<i>R. officinalis</i>
N (mg/g)	-	3.7 \pm 0.3 d	4.8 \pm 0.2 c,d	5 \pm 0.2 c	9.2 \pm 0.3 a	5.8 \pm 0.4 c	7.8 \pm 0.3 b
C/N	-	103.1 \pm 6.4 a	99.4 \pm 4.2 a	91 \pm 3.6 a	42.8 \pm 0.9 c	65.6 \pm 3 b	62 \pm 1.3 b
P (mg/g)	-	0.47 \pm 0.02 a	0.54 \pm 0.02 a	0.36 \pm 0.03 b,c	0.53 \pm 0.02 a	0.24 \pm 0.02 c	0.45 \pm 0.04 a,b
Terpens (mg/g)	-	0.1 \pm 0.04 d	0.6 \pm 0.3 c	8.8 \pm 1.8 b	-	-	15.7 \pm 0.9 a
Soluble terpenes (μ g/g)	-	0.6 \pm 0.1 d	6.4 \pm 0.4 c	18.1 \pm 1.4 b	-	-	77.5 \pm 3.8 a
Phenolics (mg/g)	-	49 \pm 7.4 b,c	272.7 \pm 31.1 a	31.2 \pm 4.7 c	60.9 \pm 11.3 b	31.8 \pm 3.6 c	58.9 \pm 4.8 b
WHC (%)	70.3 \pm 5.4 e	293.0 \pm 8.9 a	223.4 \pm 11.9 b	83.6 \pm 4.9 e	126.3 \pm 1.7 d	186.8 \pm 3.2 c	171.3 \pm 4.0 c
Mass loss (%)	-	61.7 \pm 0.7 c	79.6 \pm 1.0 d	41.2 \pm 0.9 e	45.4 \pm 0.9 d	48.6 \pm 0.9 d	67.7 \pm 0.6 a

Table 2: Litter effects on underlying soil properties. Data are means \pm standard error of 9-10 replicates per litter treatment. Different letter indicate differences between species in the order a>b>c>d, Tukey post-hoc test at p<0.05.

Litter treatment	Average soil humidity (%)	Total N (mg/g)	Organic C ($\mu\text{g/g}$)	Basal Respiration ($\mu\text{gC/g soil/h}$)	SIR ($\mu\text{gC/g soil/h}$)	Microbial catabolic diversity
Control	10.3 \pm 0.4 c	0.65 \pm 0.03 b,c	107.9 \pm 4.4 c,d	0.61 \pm 0.06 c	3.8 \pm 0.4 c	1.90 \pm 0.14 c
Artificial litter	11.9 \pm 0.3 b	0.54 \pm 0.01 c	89.1 \pm 7.9 d	0.53 \pm 0.02 c	3.6 \pm 0.4 c	2.15 \pm 0.21 b,c
<i>C. albidus</i>	11.5 \pm 0.4 b,c	0.73 \pm 0.03 b,c	147.9 \pm 6.4 a,b	0.88 \pm 0.07 b	4.8 \pm 0.5 b,c	2.32 \pm 0.14 a,b,c
<i>C. coggygria</i>	11.7 \pm 0.4 b	0.80 \pm 0.03 a,b	157.0 \pm 7.2 a,b	0.92 \pm 0.07 b	6.8 \pm 0.5 a,b	2.13 \pm 0.13 b,c
<i>P. halepensis</i>	12.2 \pm 0.3 b	0.67 \pm 0.02 b,c	130.7 \pm 3.1 b,c	0.83 \pm 0.05 b	5.1 \pm 0.3 b,c	2.50 \pm 0.07 a,b
<i>Q. coccifera</i>	12.7 \pm 0.3 a,b	0.61 \pm 0.03 c	126.8 \pm 4.9 b,c	0.70 \pm 0.04 b,c	4.2 \pm 0.3 c	2.81 \pm 0.03 a
<i>Q. pubescens</i>	13.7 \pm 0.3 a	0.71 \pm 0.04 b,c	130.0 \pm 5.9 b,c	0.78 \pm 0.05 b,c	4.8 \pm 0.5 b,c	2.74 \pm 0.04 a
<i>R. officinalis</i>	10.4 \pm 0.3 c	0.89 \pm 0.04 a	165.5 \pm 5.7 a	1.17 \pm 0.05 a	7.8 \pm 0.5 a	2.40 \pm 0.03 a,b,c

* Microbial catabolic diversity is calculated with a Shannon index from the standardized consumption of the 31 substrates of Biolog Ecoplates ©

Table 3: Oak species identity (S), litter treatment (L) and their interactive (S x L) effects on seedlings growth, biomass allocation (leaf, stem, root and fine root fraction relative to total biomass: LMF, SMF, RMF and FRMF, respectively), chemistry and mycorrhization. Results of two-way ANOVAs, F-values are shown and test significance is indicated as follow: +p<0.10, *p<0.05, **p<0.01, ***p<0.001.

	S	L	S x L
<i>Degrees of Freedom</i>	1	7	7
Growth			
Diameter	3.4	1.2	0.7
Height	0.6	1.1	0.6
Length	0.1	1.4	0.8
Biomass			
Leaf	54.7***	4.4**	0.5
Stem	42.8***	3.1**	0.6
Root	84.8***	0.4	0.6
Fine roots	17.0***	1.1	1.1
Root/Shoot	80.4***	2.0 ⁺	0.8
Chemistry			
Leaf N content	216.0***	0.5	0.3
Root N content	24.2***	0.8	0.8
Leaf Phenol content	53.4***	1.3	0.5
Mycorrhization			
Percentage Root Length Colonized	7.1*	0.9	1.1

Figures

Figure 1: Correlations between litter mass loss and soil parameters. C= Control, A=Artificial litter, Ca = *Cistus albidus*, Cc = *Cotinus coggygria*, Ph = *Pinus halepensis*, Qc = *Quercus coccifera*, Qp = *Quercus pubescens*, Ro = *Rosmarinus officinalis*.

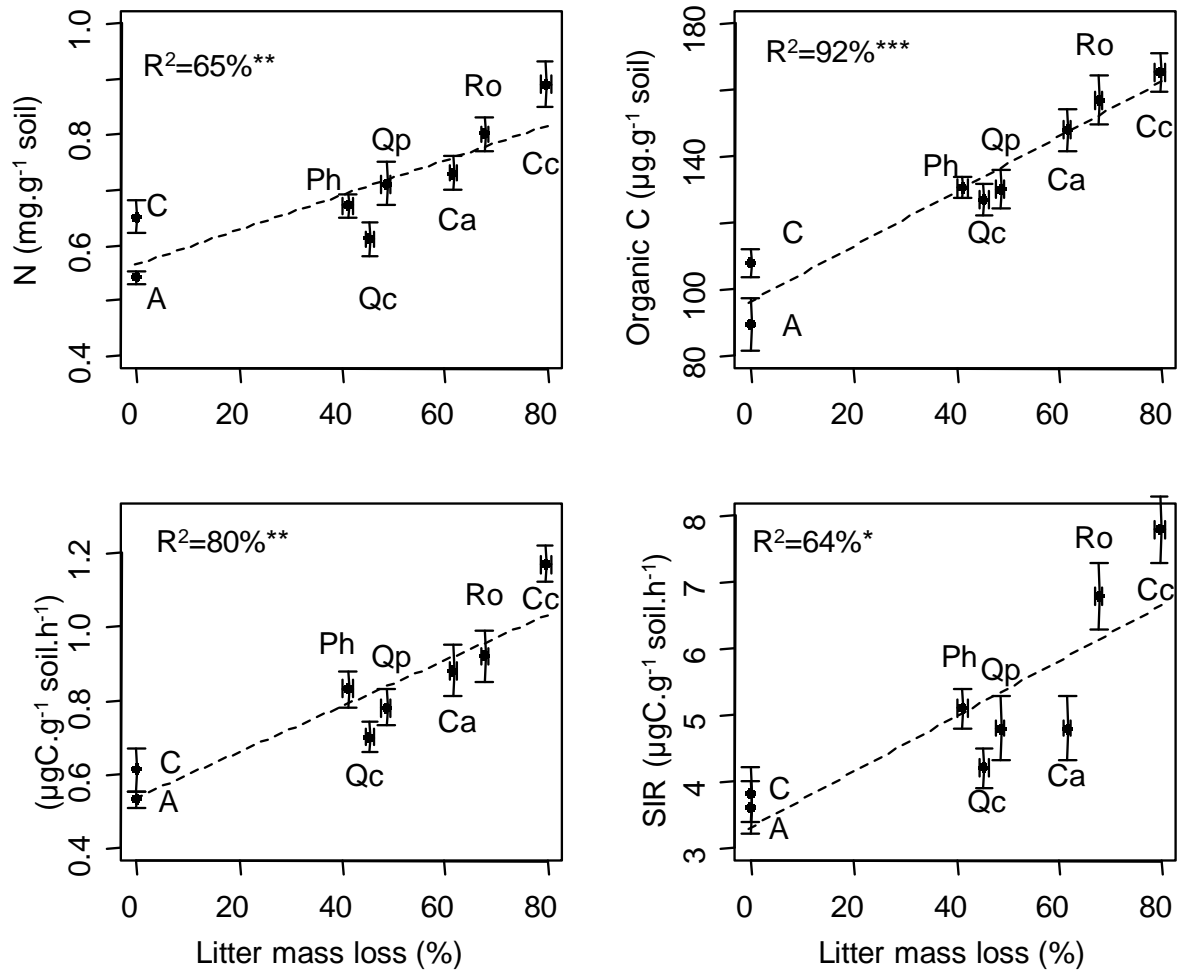


Figure 2: Partial Least Square Discriminant Analysis (PLS-DA) of microbial use of the Biolog Ecoplate substrates according to litter treatments (left) and correlation circle of the substrates (right). Results of a permutation test based on a double cross-validation and number of misclassification (NMC) are shown.

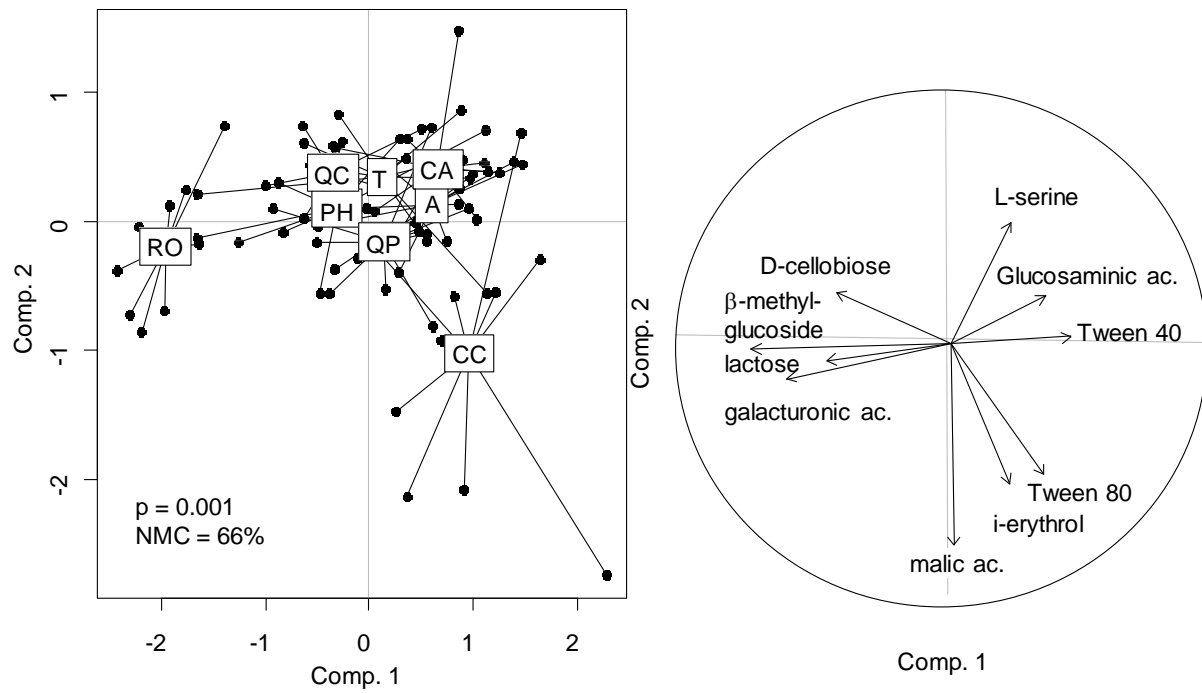


Figure 3: Seedling biomass as a function of litter treatments for both oak species. *Q. pubescens* biomass is higher than *Q. ilex* biomass for all compartments. A star indicate a significant difference of biomass from the Control treatment. Litter species code : C= Control, A=Artificial litter, Ca = *Cistus albidus*, Cc = *Cotinus coggygria*, Ph = *Pinus halepensis*, Qc = *Quercus coccifera*, Qp = *Quercus pubescens*, Ro = *Rosmarinus officinalis*. Data are means \pm standard errors of 10 seedlings per species and litter treatment.

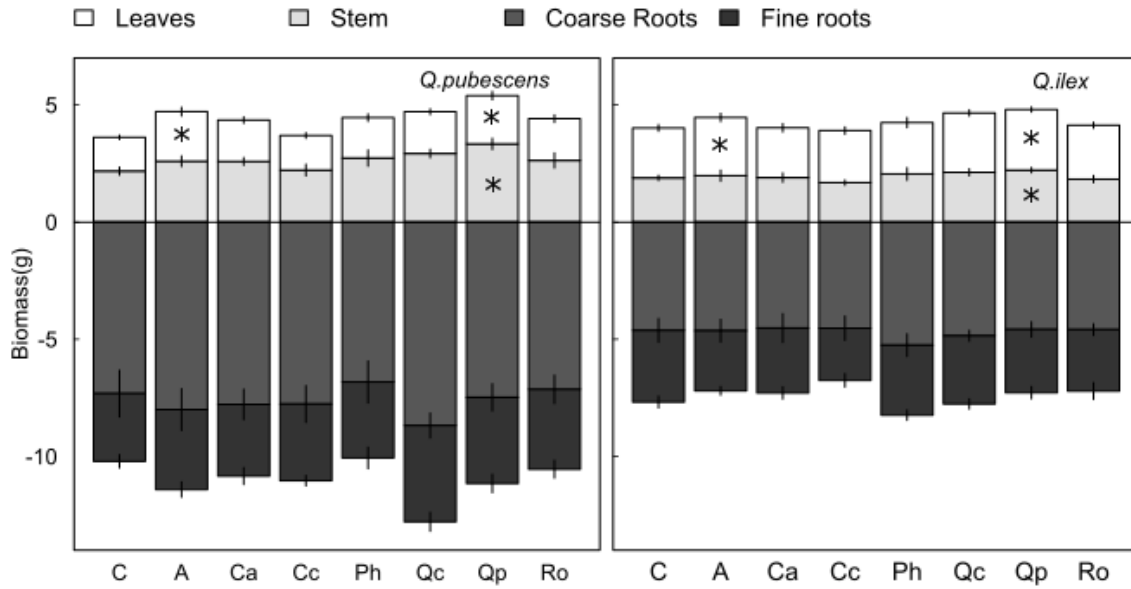
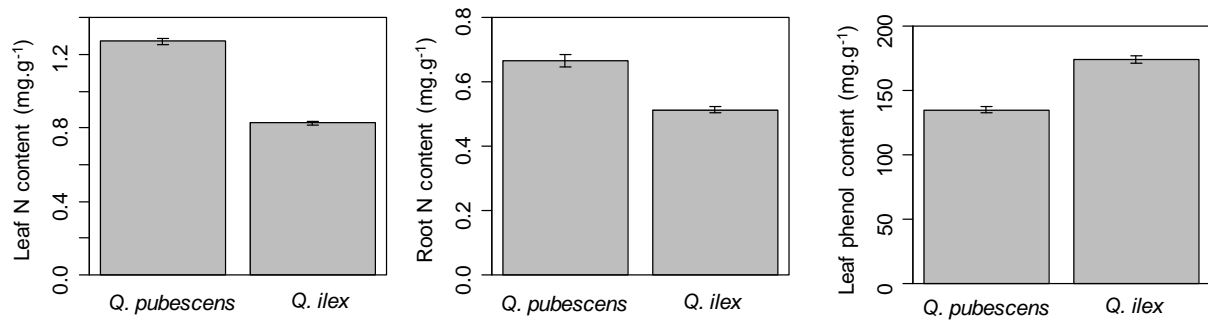


Figure 4: Species differences in leaf and root chemistry. Data are means \pm standard error of 80 individuals per species. All difference are significant at $p < 0.001$ (ANOVA, Table 3).



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Supplementary Material

Figure S1 : Relative humidity of soils under different litter treatments during the experiment. C= Control, A=Artificial litter, Ca = *Cistus albidus*, Cc = *Cotinus coggygria*, Ph = *Pinus halepensis*, Qc = *Quercus coccifera*, Qp = *Quercus pubescens*, Ro = *Rosmarinus officinalis*.

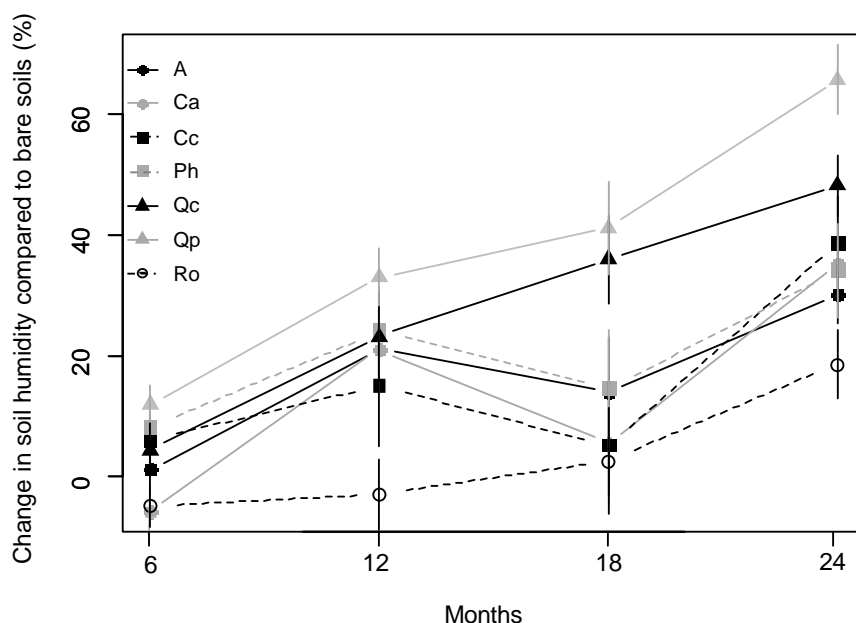


Table S1: Diameter, Height and Length relative increment as a function of sampling date (every 6 months), oak species identity, litter treatment and their interaction. Relative increment of the dimension X is calculated as: $X_{inc} = (X_{n-1} - X_n) / X_{n-1}$, with X_{inc} increment in dimension X, X_n dimension at current date and X_{n-1} dimension at previous date. Results of a linear mixed model accounting for repeated measures (R package nlme, procedure lme with pot as a random factor).

	Diameter			Height		Length	
	DF	F	P	F	P	F	P
Date	1	97.8	<0.001	250.8	<0.001	88.6	<0.001
Sp	1	0.1	0.7	0.1	0.8	2	0.2
Lit	7	0.5	0.8	0.9	0.6	1.4	0.2
Date x Sp	1	0.2	0.6	0.1	0.7	0.5	0.5
Date x Lit	7	1.2	0.3	1.8	0.08	1.6	0.1
Sp x Lit	7	0.6	0.8	0.3	0.9	1.1	0.4
Date x Sp x Lit	7	0.8	0.6	0.3	0.9	0.9	0.5