Biotic interactions in a Mediterranean oak forest: role of allelopathy along phenological development of woody species

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Abstract Plant–plant chemical interactions in forests can have a strong impact on the biodiversity and dynamics of these ecosystems, particularly in Mediterranean forests where plants exhibit a high secondary metabolite diversity. Allelopathic interactions in Mediterranean ecosystems have been mostly studied in the first stages of ecosystem dynamics, shrublands and pine forests, but little is known about these interactions in mature oak forests. In this study, the allelopathic effect of three main woody species of downy oak forests (*Quercus pubescens*, *Acer monspessulanum* and *Cotinus coggygria*) on germination and growth of two herbaceous species (*Festuca ovina* and *Linum perenne*) was tested through aqueous extracts obtained from different leaf phenological stages (green, senescent and litter). The germination velocity of the two target species was inhibited by the aqueous extracts of senescent leaves from all the woody species. The growth of *F. ovina* seedlings was affected by aqueous extracts of green leaves of all the woody species, while the growth of *L. perenne* was only affected by aqueous extracts of green leaves of *A. monspessulanum.* This shows that (i) allelochemicals released by leaf leachates of the dominant woody species could control the dynamic of the herbaceous species, and then their potential competition with trees and (ii) allelopathic effects of woody species are related to their phenological stage and seem consistent with the development stage of target species.

Keywords Allelopathy · Forest ecosystem · Plant–plant interaction · Phenological stage

Introduction

Plant communities within ecosystems are governed by biotic interactions, especially plant–plant interactions. For instance, chemical interactions play a key role on germination and growth of plant species (Callaway and Walker 1997; Fernandez et al. 2013) demonstrating the potential implication of allelopathy in ecosystem functioning (Muller 1969; Wardle et al. 1998; Inderjit et al. 2011). Allelopathy is defined as the beneficial or harmful influence that a plant exerts over other plants through the release of secondary metabolites into the environment (Rice 1984). This process is particularly studied in agrosystems (Narwal 2000; Cheng and Cheng 2015) as a natural alternative to herbicides (Cheng and Cheng 2015). However, much less attention has been given to the role of allelopathy in natural ecosystem functioning (Inderjit et al. 2011; Meiners et al. 2012). Forest ecosystems are particularly complex, multi-layered ecosystems where trees and understory plants can influence each other through resource-mediated interactions such as light interception (Beaudet et al. 2011) but also chemical interactions (Mallik 2008; Fernandez et al. 2013).

Mediterranean forest ecosystems have a highly discontinuous functioning related to alternating dry and wet

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periods (Gauquelin et al. 2016). Mediterranean plants produce numerous plant secondary metabolites (PSMs) which help them to cope with drought stress (Chaves and Escudero 1999) but can also be involved in allelopathic interactions (Scognamiglio et al. 2013). Evidence is accumulating that allelopathy is indeed an important mechanism shaping plant community diversity and dynamics in Mediterranean ecosystems. Several Mediterranean shrubs have a phytotoxic activity (Vokou 1992; Araniti et al. 2012) which probably influence surrounding communities, like *Cistus ladanifer* (Alías et al. 2006; Herranz et al. 2006) or *Thymus vulgaris* (Ehlers et al. 2013). Fernandez et al. (2013) also showed that herbaceous species composition in the understory of Aleppo pine (*Pinus halepensis*) forests is strongly influenced by allelochemicals released by pine needle leachates. The allelopathic activity of Aleppo pine has also been suggested to be involved in pine forest regeneration failure (through autotoxicity, Fernandez et al. 2008) and in pine–oak succession (Fernandez et al. 2016). Thus, chemical interactions probably influence plant composition and dynamics in the first stages of Mediterranean successional dynamics, i.e. shrublands and pioneer pine forests. However, there is a lack of studies on the occurrence of allelopathic interactions in later stages of plant succession, i.e. in mature oak forests.

Herbaceous species in the understory can influence forest regeneration (Gavinet et al. 2016). Regeneration failure has been observed in Mediterranean oak forests of Southwestern Europe, North Africa and California, and this has been linked to overgrazing, lack of management (Dias et al. 2016) or to the lack of safe site for seedling establishment due in particular to competition with grasses (Rey Benayas et al. 2005; Gavinet et al. 2016). Herbaceous understory species indeed strongly compete with tree seedlings for belowground resources, due to a dense root network in the superficial soil layers (Coll et al. 2003; Balandier et al. 2006). Herbaceous competition for water can be particularly harmful to seedlings in water-limited ecosystems such as the Mediterranean (Rey Benayas et al. 2005; Van Der Waal et al. 2009). Research on forest ecosystems has emphasized the role of light competition by overstory tree to limit the development of understory weeds and indirectly facilitate seedling establishment (McCarthy et al. 2011), but little attention has been paid to the role of chemical interactions. A better knowledge of woody species allelopathic effects on herbaceous vegetation would be useful for understanding but also for managing forest vegetation dynamics.

Seed germination and seedling performance are the main life stages usually affected by allelochemicals (Gallet and Pellisier 2002). Frequent negative allelopathic effects are inhibition of seed germination (Vivanco et al. 2004; Fernandez et al. 2013), delay of germination (Fernandez et al. 2013) and inhibition of seedling growth (Linhart et al. 2015). Neighbouring plants can either be affected directly (*e.g.* photosynthesis, nutrient uptake, cell division or elongation; Inderjit and Duke 2003) or through indirect effects at the soil level by the disruption of nitrogen mineralization and inhibition of the ectomycorrhizal fungi (Mallik 1995; Wardle et al. 1998; Walker et al. 1999; Mallik 2003).

The quantity and quality of allelochemicals and therefore their effective influence on other species depends on leaf chemical composition, which is highly variable among species and even among life stages of a given species (Fernandez et al. 2009). During the life and senescence of leaves, different organic and inorganic metabolites are produced (Facelli and Pickett 1991) and released into the environment by volatilization, leaching, root exudation and litter decomposition (Inderjit and Keating 1999). In deciduous forests, these leaf phenological stages occur in separated time period throughout the year. This is of prime interest to take into account when studying allelopathic interactions, because different phenological stages will match temporally with different life stages of understory species. Thus, it is necessary to know at which phenological stage the effects of leaf allelochemicals are the strongest and notably to separate their effects on the germination and growth stages of the target species.

In this study, we examined allelopathic interactions occurring between woody species and understory herbaceous species in the downy oak forest. Our main objectives are:

i. To quantify the allelopathic effect of three dominant woody species of the downy oak forest (*Quercus pubescens*, *Acer monspessulanum* and *Cotinus coggygria*) on germination and seedling growth of two herbaceous species (*Linum perenne* and *Festuca ovina*) occurring in the same community.
ii. To quantify which phenological stage presents the highest allelopathic effect for each of the three species studied.
iii. To characterize the allelochemicals that may be responsible of this effect.

Materials and methods

Material collection

Plants and soil were collected at the Oak Observatory at the "Observatoire de Haute Provence" (O_3HP), an experimental site located 60 km at the North of Marseille, South of France (43°56′115″N, 05°42′642″E). The site is 680 m above sea level and presented a mean annual temperature

of 11.9 °C and a mean annual precipitation of 830 mm (1967-2000). This forest was managed for centuries by coppicing, dominated by *Quercus pubescens* (75% coverage) and *A. monspessulanum* (25% coverage), with woody understory vegetation dominated by *C. coggygria* (30% coverage).

Green, senescent and litter leaves were collected for each species in order to prepare aqueous extracts for bioassays. Green leaves were harvested in summer 2013 and kept in the freezer at −20 °C. Senescent and litter leaves were collected in autumn and winter, respectively, air-dried and stored in darkness at room temperature. The soil used for bioassays was collected from the study site, sieved to a mesh size of 2 mm and kept at room temperature.

Bioassays

We chose to test the effects of natural leachates by using foliar aqueous extracts because water-soluble compounds have been shown to be most involved in allelopathy (Vyvyan 2002). Aqueous extracts of green, senescent and litter leaves of the three sources species (*Q. pubescens*, *A. monspessulanum* and *C. coggygria*) were prepared by soaking 2.5% dry mass of leaves in deionized water at room temperature in darkness for 24 h in order to limit their degradation (Souto et al. 1994). Aqueous extracts were then filtered through a filter paper (Whatman#1®), diluted at 2.5 and 1% from stock solution and then stored at 4 °C. Aqueous extracts were renewed every week during bioassays. We chose to test two low concentrations of leaf aqueous extracts (1 and 2.5%) in order to get closer as possible to realistic concentrations of natural leachates.

The allelopathic effect of the three woody species was tested using seeds of two target herbaceous species: *Festuca ovina* L. (Poaceae) and *L. perenne* Mill. (Linaceae). Seeds of *F. ovina* and *L. perenne* were purchased from Vilmorin SA (La Ménitré, Limagrain, France). These species often used in allelochemical bioassays (Lovett 1986; Rotherham and Read 1988; Bousquet-Mélou et al. 2005; Bulut and Demir 2007) were selected because they naturally grow in Mediterranean oak forests.

Bioassays were conducted in Petri dishes with 50 g (±0.1 g) of soil corresponding to a thickness of ±0.5 cm to allow root development (Fernandez et al. 2013). Bioassays were performed on natural soil in order to take into account the activity of microorganisms (Inderjit 2005). We sowed 25 seeds per Petri dish of each target species and watered every 2 days with 5 mL of aqueous extracts (1 or 2.5%) or 5 mL of deionized water as control. Four replicates were performed for each treatment (source species × phenological stage × extract dose × target species). Bioassays were conducted under natural photoperiod and controlled temperature (19 °C ± 1 °C) during 10 days after germination.

Final germination percentage was calculated as the number of germinated seeds/the number of sown seeds × 100. Speed of germination was calculated by using the Kotowski velocity coefficient (Mazliak 1982), $Cv = 100\ (\Sigma N_i/\Sigma N_i T_i)$, where N_i is number of seeds germinated at time i and T_i the number of days since the start of the experiment. Concerning seedling growth, lengths (mm) of root and shoot were measured for each individual 5 days after germination (FITOMED 6.0 software).

We also defined a relative allelopathic effect (RAE) index to determine the intensity of allelopathic effect on seed germination and seedling growth. RAE is defined as (O − C)/C × 100% where O is the mean measured value when a target species is exposed to allelopathic compounds and C is the mean value measured for control. Negative RAE indicates an inhibitory effect, whereas positive RAE indicates a stimulatory effect.

Chemical analysis

Total leaf phenolics

Leaf phenolic concentrations were measured colorimetrically according to the Folin–Ciocalteu colorimetric method (Folin and Denis 1915) and detailed in Santonja et al. (2015a). Leaf samples were first freeze-dried and ground using a ball mill to a fine powder before chemical analyses. Leaf sample (0.25 g) was suspended in 20 mL of a 70% aqueous methanol, shaken for 1 h and then filtered (0.45 µm PTFE filter, Restek®). Filtered extracts (0.25 mL) were mixed with 4 mL of distilled water, 0.5 mL of saturated aqueous Na_2CO_3 and 0.25 mL of Folin-Ciocalteu reagent. After 60 min, leaf phenolic concentrations were determined at 765 nm on the same UV–Vis spectrophotometer (Biomate 3, Thermo Electron Corporation®). Quantitative results were expressed as mg of gallic acid/g MS.

Metabolomic analyses

Aqueous methanolic extracts of leaves used in bioassays were analysed by liquid chromatography coupled to high-resolution mass spectrometry. Two hundred milligrams of ground leaves was mixed in 4 mL H_2O:MeOH (50:50) and sonicated for 1 min. The solution was then filtered (PTFE syringe filter 0.2 µm, Restek®), and 10 µL was injected in an UHPLC–QqToF instrument equipped with RS Pump, autosampler and thermostated column compartment and UV diode array (Dionex Ultimate 3000, Thermo Scientific®) coupled to a high-resolution mass spectrometer (HRMS) equipped with an ESI source (Impact II, Bruker

Daltonics®). Mass spectra were acquired in negative mode from 50 to 1200 amu. The elution rate was set to **0.5 mL min^{-1} at a constant temperature of 45 °C. Chromatographic solvents were composed of A: H_2O with 10 mM ammonium formate, and B: acetonitrile:H_2O (95:5) with 10 mM ammonium formate. UHPLC separation occurs on an Acclaim RSLC C18 column (2.1 mm × 100 mm, 2.2 µm, Thermo Scientific®). The program consisted of: 2% B during 2 min followed by a linear gradient up to 20% B during 8 min and then 4 min in isocratic mode. The analysis was followed by an elution of 100% B during 3 min until a return to initial conditions for column equilibration during 3 min for a total runtime of 20 min. Spectrometer was calibrated with formate/acetate solution forming clusters on studied mass range. The same solution was automatically injected before each analysis to ensure mass accuracy.

Data were exported in netCDF files using Bruker Compass DataAnalysis 4.3. All analyses were then processed using XCMS software (Smith et al. 2006) under R (R_Core_Team 2013), using the different steps necessary to generate the final data matrix: (1) peak picking for the detection of different features (peakwidth = c(2,20), ppm = 2, prefilter = c(0,0)), (2) retention time correction (method = "obiwarp"), (3) grouping (bw = 10, minfrac = 0.3, minsamp = 1), (4) fillpeaks processing to integrate portions where peaks were initially absent and finally (5) report and data matrix generation transferred to Excel. Home-made scripts allowed to clean the matrix relatively to (1) ion presence in blanks, (2) ion coefficient of variation above 25% in pooled samples and (3) ion redundancy by removing adducts and isotopes. Biomarkers were compared to Metlin online database (https://metlin.scripps.edu/landing_page.php?pgcontent=simple_search). A GNPS network (https://gnps.ucsd.edu/ProteoSAFe/static/gnps-splash.jsp; Wang et al. 2016) was also realized with default parameters as a tentative of biomarker annotation by comparison of DDA fragmentation pattern of the three major precursors per scan with available database. The network was finally managed under Cytoscape 3.5.0 (Shannon et al. 2003).

Statistical analysis

Statistical analyses were performed with the R software (version 3.3.1, The R Foundation for Statistical Computing, Vienna, Austria). Significance was evaluated in all cases at $P < 0.05$. Normality and homoscedasticity of the data were first checked using Ryan–Joiner and Levene tests, respectively.

The effects of source species (*Acer*, *Cotinus* and *Quercus*) and phenological stage (green leaf, senescent leaf and leaf litter) on leaf phenolic concentrations were tested by two-way ANOVAs followed by Tukey test for post hoc pairwise comparisons. Chemical fingerprints of plant extracts according to source species and phenological stage were studied by principal component analysis (centred and scaled data) using the package "ade4" (Dray and Dufour 2007).

Differences of seed germination (final percentage and velocity) and seedling growth (root and shoot) parameters between *Festuca* and *Linum* in the control treatment were assessed using Student's *t* tests. Three-way ANOVAs, followed by Tukey post hoc pairwise comparisons, were used to test the effects of source species (*Acer*, *Cotinus* and *Quercus*), phenological stage (green leaf, senescent leaf and leaf litter) and extract dose (0, 1.0 and 2.5%) on seed germination (final percentage and velocity) and seedling growth (root and shoot) of target species.

One-sample Student's *t* test was used to test whether RAE was significantly different from zero for each combination of source plant × leaf phenological stage × extract dose.

Results

Chemical analysis

Total leaf phenolics

Phenolic concentrations varied according to the phenological stage and to the identity of the plant species (Fig. 1). We generally observed an increase in leaf phenolic concentrations according to phenological stage in the following order leaf litter < green leaf < senescent leaf ($F = 533.9$, $P < 0.001$). We also observed an increase in phenolic concentrations according to the gradient *Quercus* < *Acer* < *Cotinus*, but only for green and senescent leaves (phenological stage × species identity interaction,

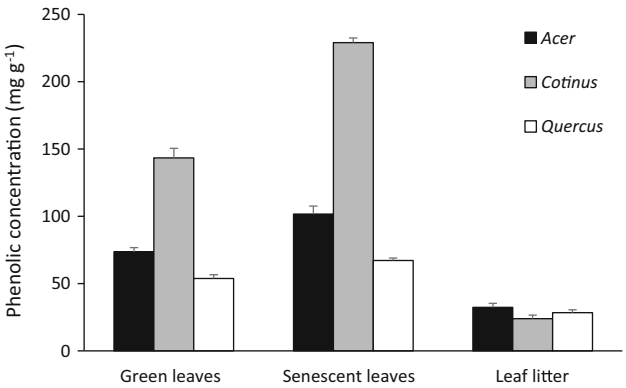

Fig. 1 Leaf phenolic concentrations according to the phenological stage (green leaf, senescent leaf and leaf litter) and to the woody species (*Acer*, *Cotinus* and *Quercus*). Values are mean ± SE

$F = 127.5$, $P < 0.001$; Fig. 1). Phenolic concentrations in leaf litters were not different between the three species (Fig. 1).

Metabolomic analyses

Metabolomic fingerprints were based on 6321 ions detected in negative mode. Fingerprints of the three leaf litters varied less than those of the green and senescent leaves (PCA, total variance 32.5%; Fig. 2). The first axis (20.3%) separated senescent leaves from the three grouped leaf litter. The second axis (12.2%) differentiated green leaves from senescent leaves and leaf litter. Leaf litter chemical fingerprints were characterized by only three biomarkers (see Supplementary Table S1 for details). At senescent stage, fingerprints of *Cotinus* and *Acer* distinguished by the presence of numerous biomarkers compared to the one of *Quercus* closer to leaf litter. Green and senescent leaves of *Acer* shared common ions, while green leaves of *Cotinus* seem to distinguish by the absence of the same ions.

Tentative annotation of PCA biomarkers suggested the presence of linear fatty acids (i.e. traumatin), phenolic derivatives (i.e. ferulic acid methyl ester or anthriscinol) and terpene glycosides (i.e. blumenol glycoside) (Supplementary Table S1). However, these biomarkers could not be detected in the molecular network based on fragmentation data, probably due to their low intensities in chemical fingerprints. The molecular network indicated the presence of flavonoids (Supplementary Fig. S1). Myricetin was found mostly in *Cotinus* extracts and luteolin mostly in *Acer* extracts, and other flavonoids such as quercetin, isorhamnetin glycosides or aglycones as naringenin were found in extracts of all species. Simple glycosylated phenols (i.e. chlorogenic acid) were also detected in all species and phenological stages. Several biomarkers seem to be specific of *Cotinus* and *Acer* green and senescent leaves extracts, while very few biomarkers were specific of *Quercus* fingerprint and litter extracts.

Allelopathy bioassays

Festuca and *Linum* differed in germination and growth parameters (Table 1). *Festuca* showed a higher germination velocity ($t = 4.0$, $P = 0.007$), a higher root length ($t = 2.6$, $P = 0.012$) as well as a higher shoot length ($t = 13.6$, $P < 0.001$) compared to *Linum*.

Effects on seed germination

The germination percentage of *Festuca* was on average 10% higher in the presence of leaf aqueous extracts compared to the control treatment (Table 2), whereas the germination percentage of *Linum* was not affected by the aqueous extracts (Table 2).

Germination velocity of the two target species decreased with increasing extract dose, and senescent leaf extracts had stronger negative effects compared to litter and green leaves extracts (Table 2; Fig. 3). Germination velocity of *Linum* was more sensitive to aqueous extract from *Cotinus* than from *Quercus*, whereas germination velocity of *Festuca* was affected by the three source species in a similar way (Fig. 3). Leaf phenological stage and extract dose interacted in their effects on germination velocity of *Linum* (Table 2). Green leaf extracts had a neutral or positive effect on *Linum* germination velocity at low dose but a negative effect at high dose (Figs. 3d–f).

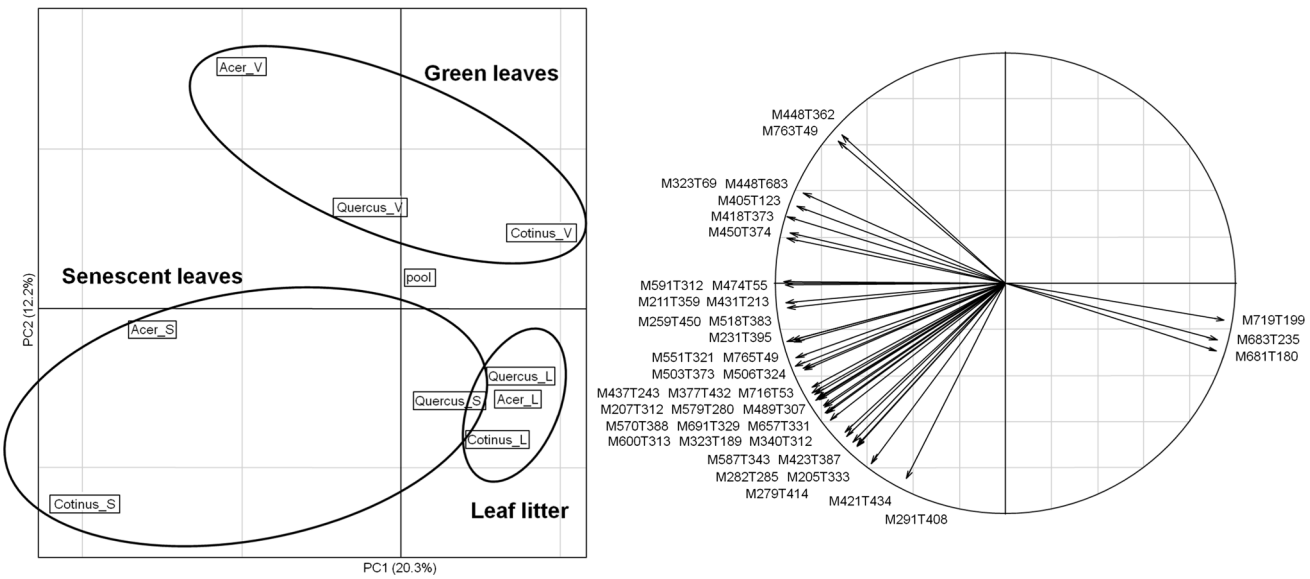

Fig. 2 Principal component analysis of the different aqueous extracts analysed by liquid chromatography/mass spectrometry (negative mode)

Table 1 Seed germination (percentage and velocity) and seedling growth (root and shoot) parameters of *F. ovina* and *L. perenne*

	Festuca ovina	*Linum perenne*
Germination percentage	75.0 ± 4.4	80.0 ± 8.5
Germination velocity	15.7 ± 0.2	12.5 ± 0.8
Root length (nm)	35.3 ± 1.8	28.8 ± 1.7
Shoot length (mm)	40.4 ± 1.2	20.8 ± 0.8

Values are mean ± SE

Effects on seedling growth

Root and shoot growth was negatively affected by the leaf phenological stage, with effects increasing in the order leaf litter < senescent leaf < green leaf (Table 2; Figs. 4, 5). This differed from the order observed for germination velocity.

Allelopathic effects on *Festuca* seedlings growth appeared higher compared to their effects on germination velocity, especially for shoot growth. Root growth of *Festuca* was reduced by both *Acer* and *Quercus* green leaf at high-dose extracts as well as *Quercus* senescent leaf at low dose (Fig. 4a, c; Table 2, leaf phenological stage × -dose interaction). *Festuca* shoot growth was reduced by the green leaf extracts of the three source species, by the senescent leaf extract of *Acer* and *Cotinus* (Fig. 5a–c) and to a lesser extent by litter extracts of *Acer* and *Quercus* at low dose (Fig. 5a, c).

Concerning *Linum,* allelopathic effects are weaker on root and shoot growth than on germination velocity occurred only for some combinations of source species, phenological stage and dose (Table 2; Fig. 3). *Linum* root growth was inhibited by green *Acer* leaves at high dose and *Cotinus* litter at low dose, leading in both cases to a 20% reduction in root growth (Fig. 4d, e). Similarly, we found interactions in the effects of source species and dose as well as leaf phenological stage and dose on *Linum* shoot growth (Table 2). Low-dose extracts did not affect *Linum* shoot growth, while high-dose extracts of Acer green leaves had negative effects (Fig. 5d) and both *Acer* and *Cotinus* litter extract had positive effects (Fig. 5d, e) on this parameter.

Discussion

Leaf leachates of three woody species (*Q. pubescens*, *C. coggygria* and *A. monspessulanum*) showed allelopathic effects on the germination and growth of two herbaceous target species occurring in the same community (*L. perenne* and *F. ovina*). However, the allelopathic effects varied according to the extract dose, the leaf phenological stage and the target species. *F. ovina* was more sensitive to allelochemicals than *L. perenne*. Woody species extracts stimulated germination percentage of *F. ovina* but delayed the germination velocity of both species. In nature, this delay could affect survival or consequent growth of the target species' seedlings. In Mediterranean ecosystems, germination must take place during the period in which temperature and humidity are suitable for plant growth before the summer drought period (Gulias et al. 2004). A delay in germination could thus have negative consequences on seedling fitness by reducing the favourable period for subsequent seedling establishment and growth (Chaves et al. 2001).

Interestingly, the phenological stage with highest allelopathic effect was not the same for germination and growth parameters of the target species. Germination velocity was most affected by senescent leaves extracts,

Table 2 Results of three-way ANOVAs to test the effects of plant source species (*Acer*, *Cotinus* and *Quercus*), leaf phenological stage (green leaf, senescent leaf and leaf litter) and extract dose (0, 1.0 and 2.5%) on seed germination (final percentage and velocity) and seedling growth (root and shoot) of *F. ovina* and *L. perenne*

	Source species (S)	Phenological stage (P)	Extract dose (D)	S × P	S × D	P × D	S × P × D
Festuca ovina							
Germination percentage	0.17	2.12	13.47***	0.72	0.08	0.61	0.85
Germination velocity	2.48	3.33*	23.42***	0.80	0.68	1.41	0.74
Root growth	1.47	1.81	6.23**	0.32	1.01	3.28*	0.96
Shoot growth	0.92	8.98***	18.47***	1.99	0.65	2.48*	1.03
Linum perenne							
Germination percentage	0.22	1.92	0.10	0.18	0.40	0.70	0.33
Germination velocity	3.20*	38.24***	41.98***	1.07	1.64	10.67***	0.87
Root growth	0.12	0.27	3.05*	0.84	1.78	1.14	1.65
Shoot growth	0.31	6.81**	4.70**	0.74	3.91**	2.65*	0.53

F values and associated *P* values (with the respective symbols * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$) are indicated

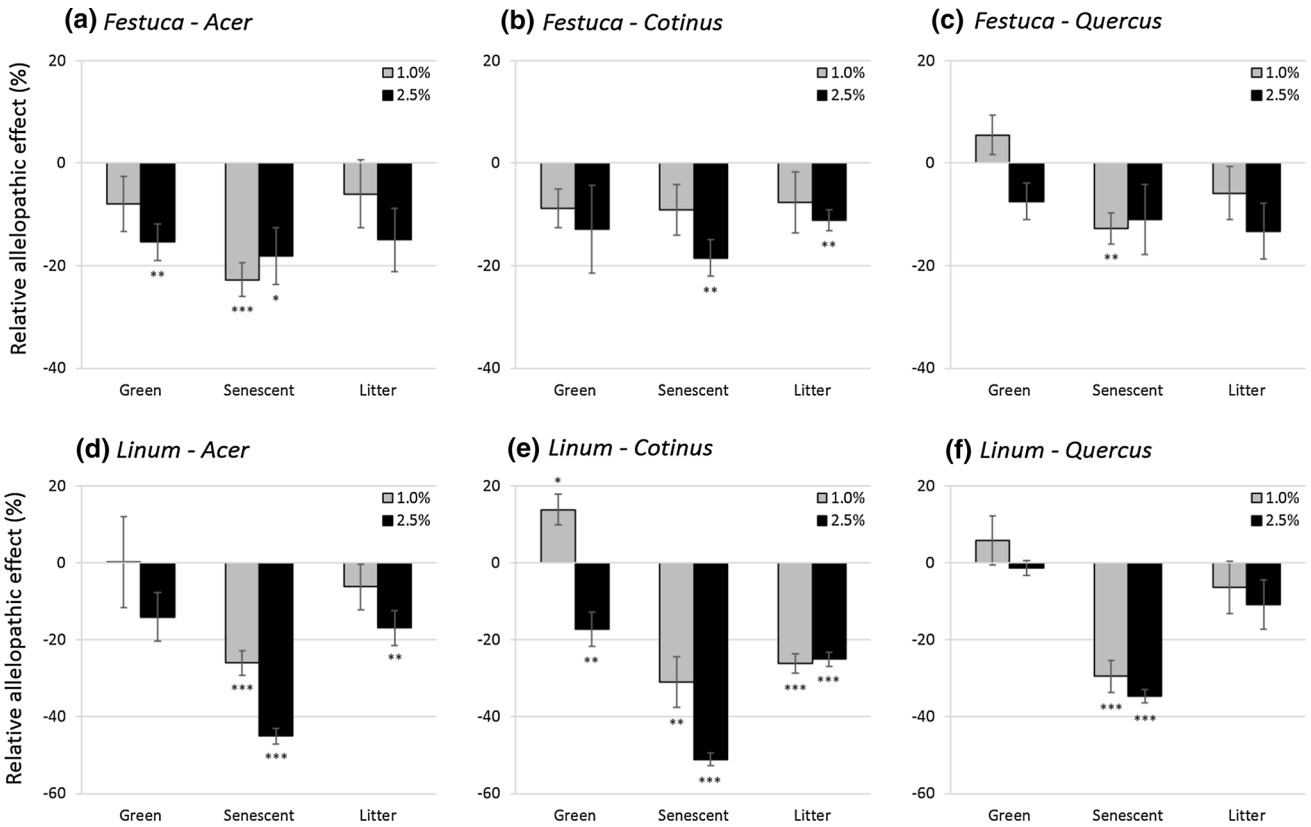

Fig. 3 Relative allelopathic effect (RAE) on germination velocity of *Festuca* (**a**, **b**, **c**) and *Linum* (**d**, **e**, **f**) according to the source species (*Acer* (**a**, **d**), *Cotinus* (**b**, **e**) and *Quercus* (**c**, **f**)), the leaf phenological stage (green leaf, senescent leaf and leaf litter) and the extract dose (low and high). Values are mean ± SE. RAE significantly different from 0 is indicated with the respective symbols * for $P < 0.05$, ** for $P < 0.01$ and *** for $P < 0.001$. *Negative values* of RAE indicate an inhibitory effect, whereas *positive values* indicate a stimulatory effect

while growth was mainly affected by green leaves extracts. The differences in allelopathic effects according to phenological stages may be associated with the amount of phytotoxic compounds released in the leachates and their chemical composition (Silva et al. 2014). Phytotoxic compounds released in leaf leachates belong usually to phenolics (Macias 1995). Here, litter extracts had a much lower phenolic concentration than green or senescent leaves which may explain the poorer allelopathic effect of litter extracts. Santonja et al. (2015b) showed, in the same Mediterranean oak forest as the present study, that 63, 89 and 53% of phenolic compounds disappeared after 6 months of decomposition for *A. monspessulnanum*, *C. coggygria* and *Q. pubescens*, respectively. Similarly, Chomel et al. (2014) demonstrated, in Mediterranean pine forests, that 80% of phenolic compounds disappeared after 6 month of decomposition and Kainulainen and Holopainen (2002) showed that the concentration of total phenolics was 27% lower in *Pinus sylvestris* needle litter than in green needles. This can be explained by the water solubility of phenolic compounds that can be readily leached by rainwater notably at the beginning of the litter decomposition process (Kuiters 1990; Chomel et al. 2016). Nektarios et al. (2005) also found that green needle extracts of *Pinus halepensis* had a more pronounced negative effect on the growth of grass species than decayed pine needles extracts. In consequence, the higher negative effect of green and senescent leaves could be related to a higher amount of phenolics.

However, quantitative differences in phenolic concentration cannot fully explain the differences found in allelopathic effects. Indeed, green leaves had a lower phenolic concentration than senescent leaves but a higher impact on seedling growth than senescent leaves. Differences in allelopathic effect may arise not only from a quantitative difference of allelopathic compounds but also from a qualitative difference. The qualitative analysis of extracts showed that flavonoids such as quercetin, isorhamnetin and naringenin were present in all species and phenological stages, while others were more specific of a given species such as myricetin (*Cotinus*) or luteolin (*Acer*). All these flavonoids are known as allelopathic compounds. For example, quercetin and myricetin isolated from *Abutilon theophrasti* inhibited the germination and root growth of *Glycine max*, *Lepidium sativum* and *Raphanus sativus* (Paszkowski and Kremer 1988).

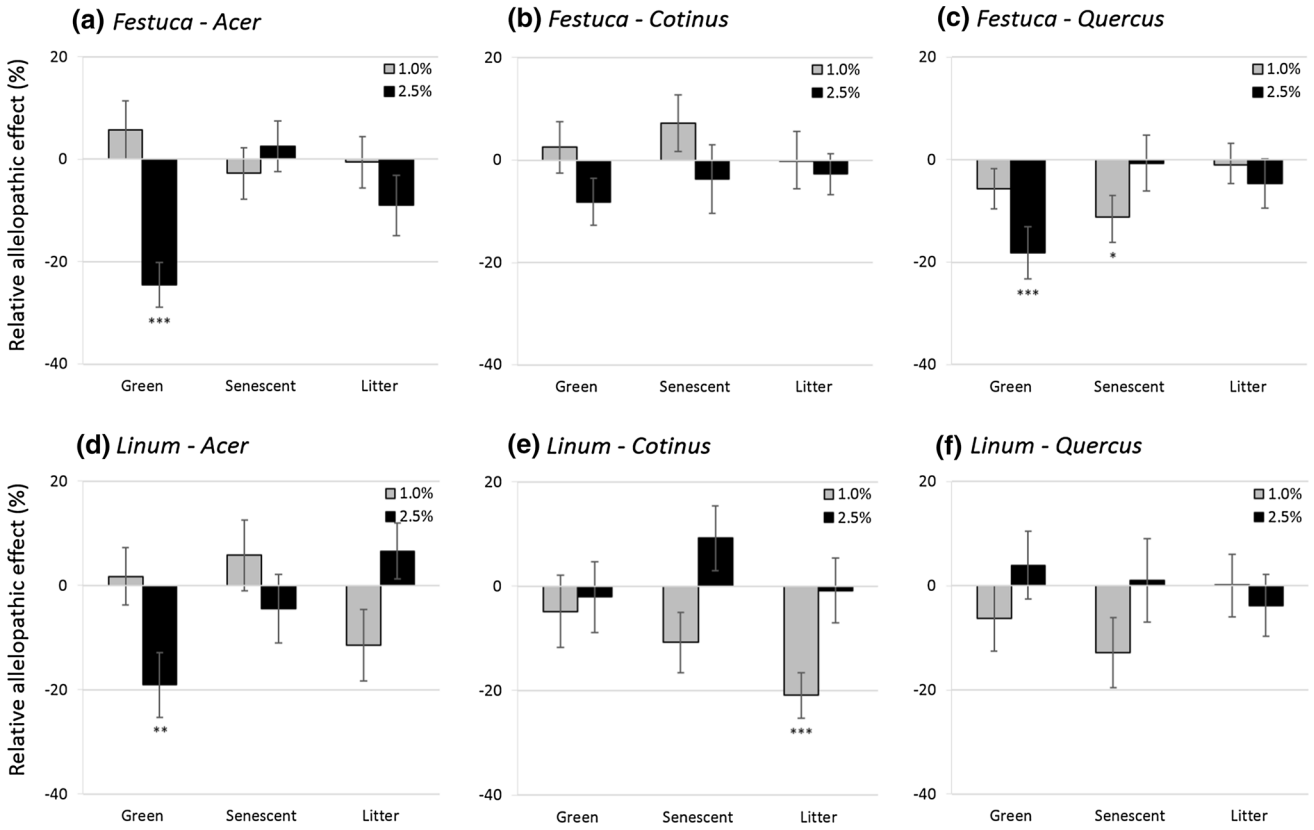

Fig. 4 Relative allelopathic effect (RAE) on root growth of *Festuca* (**a**, **b**, **c**) and *Linum* (**d**, **e**, **f**) according to the source species (*Acer* (**a**, **d**), *Cotinus* (**b**, **e**) and *Quercus* (**c**, **f**)), the leaf phenological stage (green leaf, senescent leaf and leaf litter) and the extract dose (low and high). Values are mean ± SE. RAE significantly different from 0 is indicated with the respective symbols * for $P < 0.05$, ** for $P < 0.01$ and *** for $P < 0.001$. *Negative values* of RAE indicate an inhibitory effect, whereas *positive values* indicate a stimulatory effect

Isorhamnetin-3-O-glucoside isolated from *Melilotus neapolitana* inhibited the germination and the root and shoot growth of *Petrorhagia velutina* (Esposito et al. 2008). Deng et al. (2004) showed that naringenin inhibited the growth of *Oryza sativa*, *Zea mays* and *Echinochloa oryzicola* seedlings. Luteolin 7-O-β-glucuronide isolated from *Chrysanthemum morifolium* reduced the frond number and chlorophyll content of *Lemna gibba* plants (Beninger and Hall 2005). In addition, the chemical fingerprints of plant extracts showed qualitative differences according to the leaf phenological stage, discriminating in particular green and senescent leaves. Kandil et al. (2004) also showed a qualitative difference in phenolic compounds in *Rhizophora mangle* leaves during maturation and senescence; for example, the aglycone quercetin, known as an allelochemical (Tian et al. 2016), was found only in senescent leaves. This qualitative difference between senescent and green leaves may explain their different allelopathic impacts on germination or growth, respectively. Finally, litter extracts and *Quercus* senescent leaves had a similar fingerprint characterized by few biomarkers, suggesting that their low allelopathic potential may be linked to a low diversity of allelochemicals.

Interestingly, these differential allelopathic effects of the source species according to the phenologic stage of their leaves are consistent with the phenology of target plants in nature. When *F. ovina* and *L. perenne* germinate in early spring, they are confronted with the accumulation of allelopathic compounds released by the litter and senescent leaves which exhibit the most pronounced allelopathic effects. Later, herbaceous seedlings develop when woody species have begun their leaf expansion. Thus, during growth, herbaceous species are in contact with the green leaf leachates which exhibit the most pronounced allelopathic effects on growth. Carballeira and Reigosa (1999) also found that *Acacia dealbata* presents a peak of toxic compound production during flowering in early spring. This increase in toxicity during this period seems to happen together with the germination period of understory species. This timing could enhance allelopathic effects during all the early development stages of the herbaceous species and then limit this layer in the forest.

Herbaceous plants can have negative effects on the establishment of tree seedlings, by decreasing oak seedling survival or growth (Gavinet et al. 2016; Prévosto et al. 2016). More precisely, herbaceous plants can exert

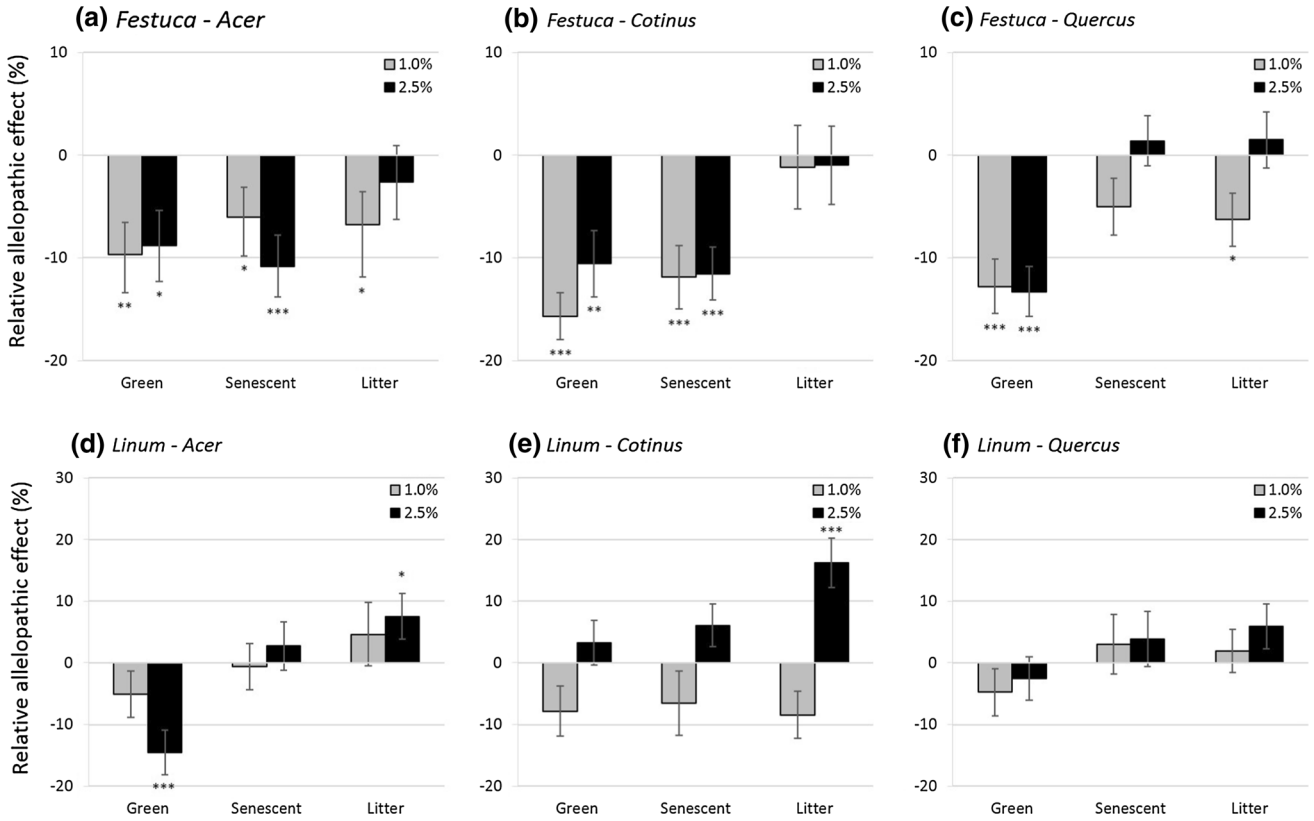

Fig. 5 Relative allelopathic effect (RAE) on shoot growth of *Festuca* (**a**, **b**, **c**) and *Linum* (**d**, **e**, **f**) according to the source species (*Acer* (**a**, **d**), *Cotinus* (**b**, **e**) and *Quercus* (**c**, **f**)), the leaf phenological stage (green leaf, senescent leaf and leaf litter) and the extract dose (low and high). Values are mean ± SE. RAE significantly different from 0 is indicated with the respective symbols * for $P < 0.05$, ** for $P < 0.01$ and *** for $P < 0.001$. *Negative values* of RAE indicate an inhibitory effect, whereas *positive values* indicate a stimulatory effect

negative effects on seedlings through the physical barrier created by their root system but also by their uptake of water and mineral resources (Balandier et al. 2006). *F. ovina*, belonging to the grasses (Poaceae), appeared to be more affected by allelochemicals than *L. perenne*. Perennial grasses are considered to be particularly competitive for seedlings (Balandier et al. 2006). Coll et al. (2003) compared the development of root systems of different herbaceous communities dominated by either grasses or forbs. The grass root system was more efficient for water uptake and had stronger growth under low soil moisture conditions. The herbaceous community dominated by grasses was thus more competitive for seedlings of the tree species *Fagus sylvatica*. In our study, the three main woody species of the downy oak forest inhibited germination velocity and growth of *F. ovina*. Indirect facilitation occurs when a given species increase the establishment of a target species by inhibiting a third more competitive species. This phenomenon has been widely documented in the Mediterranean where woody species facilitate seedling establishment by limiting grass development (e.g. Cuesta et al. 2010). Our results suggest that such indirect interactions can be due not only to resource competition but also to allelochemicals released by woody species (Mallik 2008). However, such chemical indirect interaction would require that allelopathic effects of the studied species on tree seedlings are lower than on grass seedlings, which remains to be assessed.

Conclusion

Besides competition for resources, other types of interactions such as allelopathy may affect the establishment and persistence of species (Gioria and Osborne 2014). This process is well known for alien species in invaded communities (Bais et al. 2003; Hierro and Callaway 2003) or in agrosystems (Chou 1999; Trezzi et al. 2016) but less documented in natural forest ecosystem functioning (Mallik 2008).

Here we showed that the dominant tree *Q. pubescens* and its companion species *A. monspessulanum* and *C. coggygria* may limit the growth of understory herbaceous plants. The leaf phenological stage of the woody species was a main determinant of the allelopathic effects. Our results suggest that temporal changes in quantity and

quality of leaf secondary compounds may enhance the allelopathic effects of woody species by affecting the current development stage of herbaceous species.

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