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**Chemical interaction between *Quercus pubescens* and its companion species is not emphasized under drought stress**

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**Abstract**

How plant-plant interactions will interact with global change drivers such as increased drought during the regeneration phase is a key question to forecast future vegetation dynamics. Chemical interaction and especially allelopathy and drought have been suggested to affect plant performance synergistically, i.e. that plant under drought stress would be more sensitive to allelochemicals and that exposure to allelopathic interactions could increase drought sensitivity through an inhibition of root development and mycorrhization. In this paper, we tested these hypotheses by using a controlled experiment with *Quercus pubescens* Mill. as a target species and three co-occurring species plus itself as source species. Allelopathic treatments consisted of annual provision of litter and monthly watering with green leaf aqueous extracts during 2 vegetation seasons starting from oak acorns. During the second vegetation season, a drought stress treatment was added on half of the seedlings. Allelopathy of co-occurring species reduced seedlings dimensions while *Q. pubescens* treatment increased it. During the second vegetation season, seedling growth rate and physiology were reduced by drought but poorly affected by allelopathic treatment. At the end of the experiment, drought stress and allelopathy from *Cotinus coggygia* and *Pinus halepensis* both reduced seedling biomass but had opposite effects on the root/shoot ratio. Drought and allelopathy did not interact significantly and, contrary to our hypothesis, there was a tendency of lower allelopathic effects under drought. Our results suggest that drought and allelopathy could additively alter seedling development, but the opposite effects of allelopathy and drought on the root/shoot ratio call for further experiments testing the interaction between these two factors.

**Keywords**

Mediterranean oak; chemical interactions; regeneration; water stress; plant-plant interactions

### **Declarations**

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Availability of data and material: the data will be available upon request to the corresponding author

## 1. Introduction

Plant regeneration plays a central role in the dynamics of plant populations and communities, particularly early phases including germination, growth and establishment of seedlings (Nathan and Muller-Landau 2000; Nathan and Ne'eman 2004; Grubb 1977). These demographic stages are influenced by both biotic and abiotic factors (Lortie et al. 2004). In Mediterranean forests, summer drought is among the most constraining factors for seedling establishment, which often represent an important bottleneck for species regeneration (e.g. Marañón et al. 2004; Pérez-Ramos et al. 2012). Furthermore, summer drought stress is expected to increase with climate change consequently to globally rising temperatures and changing precipitation patterns (IPCC 2014, Giorgi and Lionello 2008) which may reinforce the drought limitation for seedling establishment (Pérez-Ramos et al. 2013).

Biotic factors such as plant-plant interactions are also particularly important for plant regeneration. The surrounding vegetation can influence seedling establishment processes by a change of resource availability and microclimate (Gómez-Aparicio et al. 2005; Prévosto et al. 2016; Gavinet et al. 2016a), but also through chemical interactions (Fernandez et al. 2009; Gavinet et al. 2019; Weir et al. 2004). Plant-plant chemical interactions, i.e. allelopathy (Rice 1984), can be positive - typically through nutrient release - or negative due to the release of phytotoxic compounds. Negative chemical interactions play an important role in Mediterranean ecosystems (Vilà and Sardans 1999) and in particular in forest succession dynamics (Alías et al. 2006; Fernandez et al. 2008, 2013, 2016). For example, specialized compounds produced by plants (terpenoids and/or phenolic compounds) can delay seed germination, inhibit seedling growth (Mallik 2003) or affect root symbionts (Einhellig 1999; Mallik and Zhu 1995) which play a key role during summer drought by improving seedling water and nutrient uptake (Sardans and Peñuelas 2013). Specialized compounds can also indirectly affect seedling development through modified nutrient cycles during the litter decomposition process (Chomel et al. 2014; Kuiters 1990; Kainulainen et al. 2003; Gavinet et al. 2019). Phenolic compounds are mostly soluble and can be released from plant leaves during rainy events or during the litter decomposition process. Terpenoids in contrast are highly volatile and may interact with surrounding plants in their gaseous phase after volatilization, either from living leaves or from litter (Santonja et al. 2019). Allelopathic potential has long been examined through isolation of compounds and laboratory tests on model species such as *Lactuca sativa*, with the aim of identifying potential natural herbicides. However, the investigation of allelopathic interactions in natural ecosystems, especially forests, require more realistic experiments, testing the effects of natural extracts of plant materials on co-occurring species, taking into account the phenological stages of the interacting species (Inderjit & Weston 2000).

The interaction between drought and plant-plant interactions has been intensively debated (Maestre et al. 2005, 2009; Michalet 2007) and this debate becomes particularly important when trying to forecast global change impact

on plant distribution (Michalet and Pugnaire 2016). The interaction between drought and shading has been intensively studied (e.g. Sánchez-Gómez et al. 2006; Holmgren et al. 2012; Laanisto and Niinemets 2015) but drought and chemical interactions have rarely been studied jointly. Drought could impact plant-plant chemical interactions by increasing the production of allelochemicals by source plants (Einhellig 1999; Pedrol et al. 2006; Gatti et al. 2014) or fostering the production of compounds with greater phytotoxic activities (Oueslati et al. 2005; Gatti et al. 2014). Plants may be more sensitive to allelochemicals under drought stress (Pedrol et al. 2006) and allelopathy may aggravate drought stress by antagonistic effects on root development: allelopathy is often inhibiting root development (e.g. Gavinet et al. 2019), while increased biomass allocation to roots is a well-known morphological response to drought aiming at increasing seedling ability to capture belowground water (Dickson and Tomlinson 1996; Lloret et al. 1999; Poorter et al. 2012).

*Quercus pubescens* Willd. (downy oak) forests represent more than one million hectares in France. Downy oak often occurs in the transition of several climatic influences and is sensitive to drought at the seedling stage (Morin et al. 2010), which translates into frequent regeneration failure in the Mediterranean (Prévosto et al. 2013; Gavinet et al. 2016b). The natural regeneration from seeds of Mediterranean oaks is uncertain, as many obstacles can hinder the process: poor seed supply, lack of an effective dispersal vector, high predation on seeds or difficulties in setting up seedlings (Puerta-Piñero 2010; Pausas et al. 2009). Emergence, survival and growth of oak seedlings are highly variable depending on the microsite (Prévosto et al. 2011; Pérez-Ramos et al. 2013). This variability is often related to competition for resources with existing vegetation, but allelopathic interactions may also contribute to the definition of oaks regeneration niche (Li and Romane 1997; Gavinet et al. 2019). Downy oak acorns are likely to be dispersed in places surrounded by species with important contents of phenolics or terpenoids and known to exhibit allelopathic potential such as *Pinus halepensis* (Fernandez et al. 2008), *Acer monspessulanum* L. or *Cotinus coggygria* Scop. and *Q. pubescens* itself. Indeed, in Mediterranean forest, *A. monspessulanum* is a companion species of downy oak in the tree stratum while the shrub *C. coggygria* can dominate the understorey in supramediterranean conditions (Hashoum et al. 2017). *P. halepensis* also coexists with Downy oak in warmer and drier environments (Santonja et al. 2015a). The chemical content of the four source species has been analyzed in several previous studies. Aqueous extracts from needles, roots and litter of *P. halepensis* contain large amounts of compounds belonging to different functional phytochemical groups likely to induce allelopathic effects (monoterpenes, sesquiterpenes, phenolics; Fernandez et al. 2009; Gavinet et al. 2018). Allelochemicals from *P. halepensis* are known to slow down the germination of herbaceous species (Fernandez et al. 2006; Fernandez et al. 2013; Santonja et al. 2019) or the growth of tree species such as *Q. pubescens* especially in early stage of its development (Fernandez et al. 2016) but also the growth of *P. halepensis* via autotoxicity (Fernandez et al. 2008).

Aqueous extracts from green leaves, senescent leaves and litter of *A. monspessulanum*, *C. coggygria* and *Q. pubescens* contain flavonoids such as quercetin, isorhamnetin and naringenin, compounds with reported allelopathic activities (Paszkowski and Kremer 1988; Macias 1995). Notably, allelopathic effects from those aqueous extracts have been reported on the germination and growth of two herbaceous target species occurring in the same community (*Linum perenne* and *Festuca ovina*) with senescent leaves extract that inhibited seeds' germination velocity while green leaves extract impacted seedlings growth (Hashoum et al. 2017). Moreover, total phenolics, terpenes and nutrients contents of the litter are also described in Gavinet et al. 2018 and Santonja et al. 2015a.

In this study, we performed a greenhouse experiment to test how *Q. pubescens* seedlings establishment is affected by plant-plant chemical interactions with itself and three co-occurring species (*A. monspessulanum*, *C. coggygria*, and *P. halepensis*), and how these chemical interactions interact with drought. We hypothesized that i) chemical interactions alter oak seedlings development by decreasing root biomass and mycorrhization, ii) exposure to chemical interactions consequently increase seedlings sensitivity to drought and iii) oak seedlings under drought stress are more sensitive to allelochemicals.

## **2. Material and Methods**

### *2.1 Plant and soil material*

Plants and soils used in this experiment were collected at the Oak Observatory (O<sub>3</sub>HP) at the “Observatoire de Haute Provence” (Hashoum et al. 2017), in a supramediterranean forest site. Acorns of *Q. pubescens* from different trees were collected in autumn 2014 and visually sorted to eliminate those damaged, infested or aborted and to select acorns of roughly similar size. The selected acorns were layered in a moist substrate consisting of sand and organic substrate (50:50) at 4°C until the radicle appeared. Litter from the four source species used (*Q. pubescens*, *A. monspessulanum*, *C. coggygria* and *P. halepensis*) was collected in autumn 2014 directly after the fall of the senescent leaves using tarpaulins placed under the trees, separated according to the different species in order to obtain pure litters from each species, and stored in the laboratory in the dark at ambient temperature. Green leaves of the four source species were harvested each month of the growing season (April to October) during all the experiment duration.

The soil used for the experiment was collected in January 2015 and used directly to prepare the substrate for plantations.

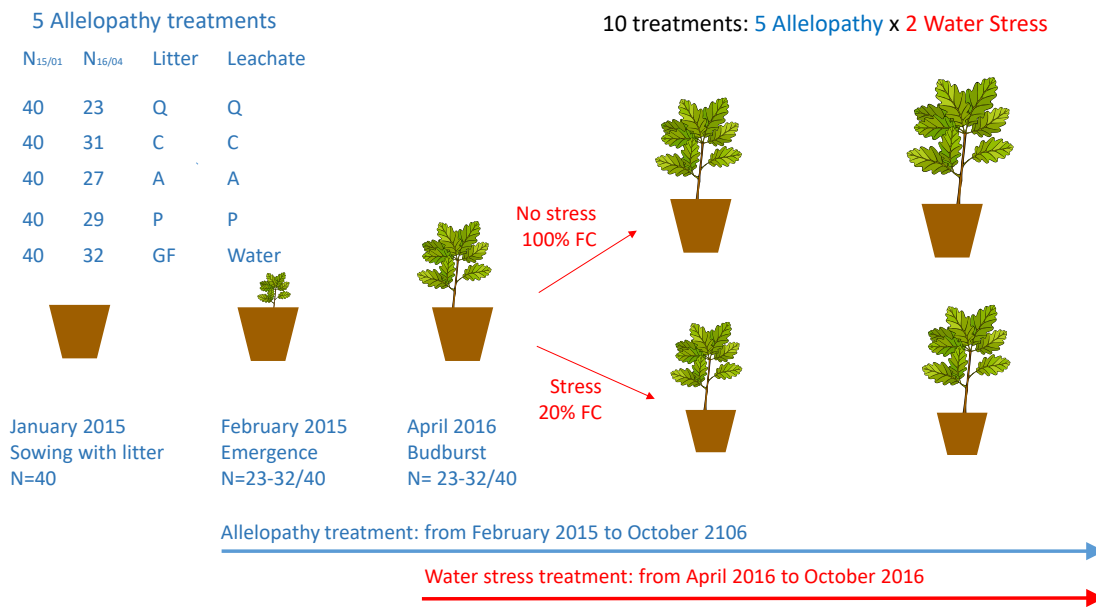
## 2.2 Experimental design

The experiment took place at the botanical garden of Mediterranean Institute of Biodiversity and Ecology, Aix-Marseille University, Marseille, South of France. The climate is typically Mediterranean with a mean annual temperature of 14.9°C and mean annual precipitations of 536 mm (1921 - 2019), mainly distributed in spring and autumn with a dry summer season. Two hundred acorns were sowed in February 2015 in 6L pots (1 acorn per pot, 18.3 cm wide, 25.5 cm high) filled with a substrate composed of 50% natural soil, 25% peat moss substrate, and 25% perlite. The use of natural soil ensures that soil microorganisms naturally present in *Q. pubescens* forests of the O<sub>3</sub>HP site were in the pot.

In order to relate as closely as possible to natural conditions, we considered two of the main release modes of allelochemicals into the environment (Rice 1984): litter decomposition and leachates, i.e. leaching of water-soluble compounds from leaves during rain events. Allelopathic treatments are thus the addition of litter of source species (litter decomposition mode) and aqueous leaf extracts of source species (leachates). The use of aqueous extracts is justified because it is widely recognized that water-soluble compounds are those most often involved in allelopathy (Vyvyan 2002). Allelopathic treatments were applied from February 2015 to April 2016 on 40 pots per source species + a control (5 treatments applied on 40 pots each). For each source species, the pot was supplied with 25g of litter in February 2015 and 15g in October 2015 in order to simulate the annual leaf fall, and watered with leaf extracts every month. Depending on the phenological stage of the plant on the O<sub>3</sub>HP, leaves used for extract preparation were green leaves (vegetation season), senescent leaves (autumn) or litter (winter). The two processes of chemical interactions through leachates and litter have thus been investigated jointly here to mimick natural conditions. The control was supplied with fiberglass cloth used to imitate the physical litter effect but without releasing allelochemicals (Sydes and Grime 1981; Gavinet et al. 2018) and watered with water. To determine the quantity of leaf litter supply, we measured the natural leaf litter amount of each species on the forest site in ten 400 cm<sup>2</sup> blocks, then a common average value of 25g per pot was used for all source species. A net was placed on each pot to prevent litter loss or addition. Aqueous extracts were prepared with fresh foliar material macerated in water for 24 hours at ambient temperature. To determine aqueous extracts concentrations on a leaf dry mass basis, a sample of fresh green leaves were dried and weighed to determine the leaf water content of each species.

During the first year (from February 2015 to April 2016), 500 ml of aqueous extracts at 2.5% (weight/volume) were added to each pot of the allelopathic treatment every month. The second year (from April 2016 to October 2016), half of the pots of each treatment were submitted to a water stress treatment by reducing water supply to maintain the pots at 20% of field capacity, while the other half were maintained at field capacity (not stressed). Soil moisture was maintained through an automatic drip irrigation system. Water-stressed pots submitted to allelopathic treatments were supplied monthly with 125 ml of aqueous extracts at 10%, aiming at keeping the same

amount of compounds but less diluted than the allelopathic pots under no-stress treatment. Our design thus mimick a first year with no drought stress followed by a second year with drought stress, keeping the allelopathic treatment constant (Figure 1). Treatments of chemical interactions are referred hereafter by the Genus name of the source species, i.e. *Acer*, *Cotinus*, *Quercus* and *Pinus*.



**Fig1:** Schematic drawing of the experimental design used to test biotic interactions between companion species and *Q. pubescens* under drought. The biotic interactions applied through the decomposition of litter and the supply of aqueous extract are designated by the initial of the genus name (Q: *Q. pubescens*; C: *C. coggygia*; A: *A. monspessulanum*; P: *P. halepensis*; GF: glass fiber; FC: field capacity; N<sub>15/01</sub>, N<sub>16/04</sub>: number of replicates followed by the corresponding date). In April 2016, each treatment was then divided in two subsamples, one submitted to water stress and the other kept at field capacity.

### 2.3 Plant growth response

Seedling size (height and diameter at ground level) was measured at the end of the first growing season, in November 2015, and at the end of the second growing season, in October 2016. At the end of the second growing season, seedlings were harvested, and root systems were gently washed above a sieve to remove soil particles without losing roots. Seedlings were separated between leaves, stems, coarse and fine roots (diameter < 2mm), oven-dried at 60°C for 4 days and weighed. The total number of leaves was also recorded for each seedling.



#### *2.4 Plant physiological response, mycorrhization rate and litter decomposition*

CO<sub>2</sub> and H<sub>2</sub>O exchanges were measured four months after the application of water stress, in August 2016, on a subsample of 10 seedlings per treatment and on one leaf per seedling. Each leaf was clipped in a PLC3 Universal Cuvette (diameter 18 mm, 2.5 cm<sup>2</sup> surface) related to an Infrared Analyzer (Synergy, CIRAS 3 PPSsystem). Gas-exchange measurements allowed direct calculations of net photosynthesis ( $A$ ,  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ), stomatal conductance to water vapor ( $g_s$ ,  $\text{mmol.m}^{-2}.\text{s}^{-1}$ ) and transpiration ( $E$ ,  $\mu\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ ). Measurements were performed under 30°C temperature, constant CO<sub>2</sub> concentration (400 ppm) and saturating PAR (1000  $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ ). Five measurements were recorded (one measurement every ten seconds), and the average of the five recordings was used for statistical analysis.

In this experiment, we also evaluated the percentage of ectomycorrhizal colonization. This type of symbiotic association is largely represented in Mediterranean forest species where fungi develop mainly around the root, forming a mycelian mantle from which hyphae develop (Duponnois et al., 2013). More, they can modify the sensitivity of plants to allelopathic substances. (Gallet and Pellissier, 2002). We took root samples from each modality directly after the seedlings were collected, at the end of the experiment. Then these samples were stored in 60% alcohol until the time of analysis. We randomly selected 5 samples per modality for analysis, for a total of 50 samples. To quantify the percentage of ectomycorrhizal colonization, we used the line intersection method (Giovannetti and Mosse, 1980). This semi-quantitative method is based on the control of mycorrhization at all points of intersection between the roots present and a grid (1x1cm) placed under a Petri dish. 5 fragments of 3 cm of roots are therefore analyzed in a Petri dish with a few milliliters of distilled water to prevent drying the roots. The counting is done under a binocular magnifying glass at a 4x40 magnification with high illumination to ensure good visualization. The mycorrhization rate is obtained as the ration of the number of mycorrhized root intersection points to the total number of root intersection points.

The litter from each pot was collected at the end of the experiment, freeze-dried and weighed to calculate the decomposition rate as follows: (total mass supplied - final mass) / total mass supplied.

#### *2.5 Statistical analyses*

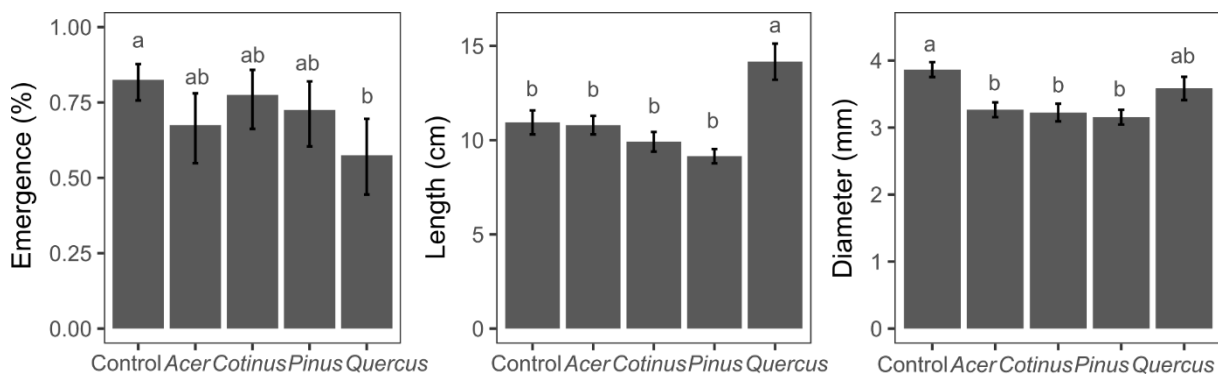
Allelopathic effects on oak seedling emergence were tested using a generalized linear model with a binomial distribution. Allelopathic effects on early oak growth (length and diameter after 10 months of allelopathic treatments, before drought treatment application) were tested through 1-way ANOVA followed by Tukey post-hoc tests. Seedling length and diameter relative growth rate during the second year was computed as  $\text{RGR} = (\log(X_2) - \log(X_1)) / (t_2 - t_1)$ , with  $X_2$  is seedling dimension (height or diameter) at the last measurement date  $t_2$

(i.e. November 2015), and  $X_1$  the same dimension at the first measurement date  $t_1$  (i.e. in October 2016). Given that growth during the winter season is negligible, this value essentially represents the growth rate during the second season, when water stress was applied on half of the seedlings. The effects of allelopathy, drought treatment and their interactions on oak RGR, physiology, biomass, biomass allocation and mycorrhization rates were tested through two-ways ANOVAs. Litter mass loss was calculated as the difference of final litter mass and all litter inputs and expressed in percentage. Conditions of normality and homoscedasticity were checked using the Kolmogorov-Smirnov and Levene tests, respectively, and the data was log-transformed if necessary (seedling length, diameter and RGR).

### 3. Results

#### 3.1 Allelopathic effects on oak seedlings emergence and early growth

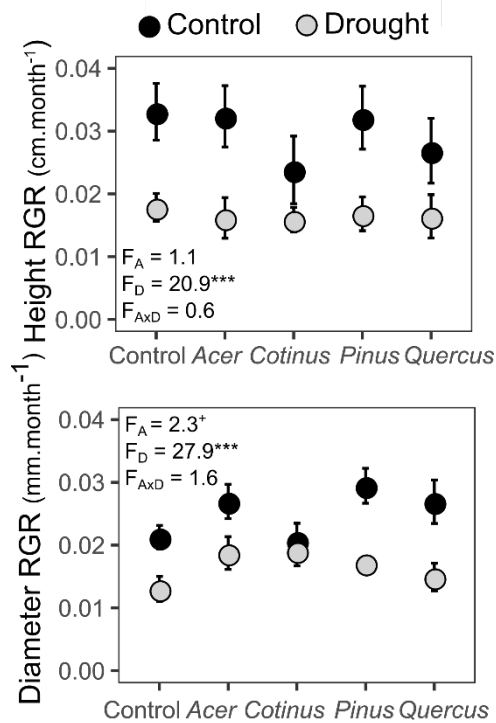
Oak seedling emergence was only marginally influenced by allelopathic treatment ( $\chi^2 = 7.3$ ,  $P = 0.06$ ), with a tendency of higher emergence in control conditions than in the *Quercus* treatment (Figure 2). Allelopathic treatment influenced seedling length ( $F=18.9$ ,  $P < 0.001$ ) and diameter ( $F=3.6$ ,  $P = 0.009$ ). Seedlings had a smaller diameter in the *Acer*, *Cotinus* and *Pinus* treatments and a higher length in the *Quercus* treatment (Figure 2).



**Fig2:** Allelopathic treatment effects on *Q. pubescens* acorn emergence and early seedling growth after the first vegetation season. Different letters represent significant differences ( $P < 0.05$ , Tukey post-hoc tests). Data are model predictions of emergence probability and means of growth variables  $\pm$  standard errors ( $n=40$  acorns per treatment for emergence,  $n= 27 - 32$  acorns per treatment for growth).

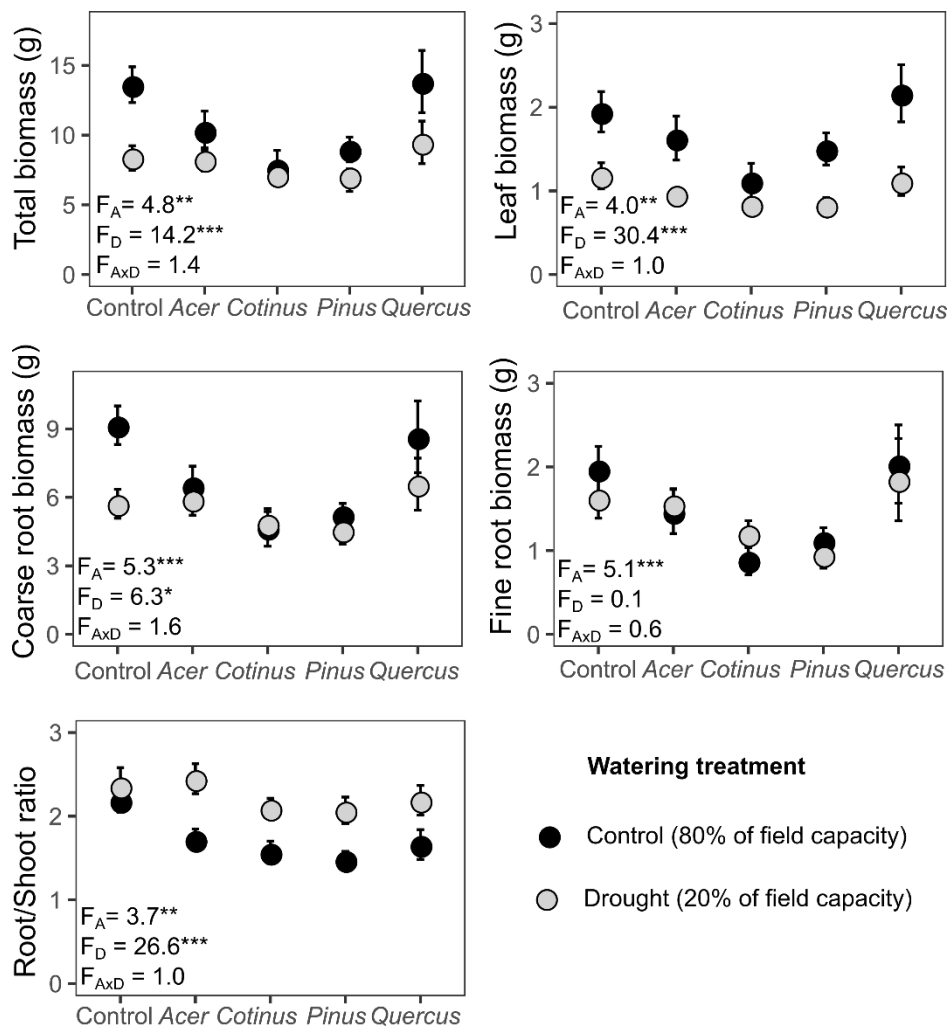
#### 3.2 Allelopathy and drought effects on *Q. pubescens* growth and biomass allocation

Seedling RGR during the second growing season was affected by drought for both height and diameter growth ( $P < 0.001$ ) but not by allelopathy nor by the interaction between these factors (Figure 3), although allelopathy marginally influenced diameter RGR ( $P = 0.07$ ; Figure 3) with a tendency of better growth under *Pinus* treatment.



**Fig 3:** Allelopathy and drought effects on seedling relative growth rate (RGR) in length and diameter during the second growing season. Data are means  $\pm$  standard errors ( $n=11-17$  seedlings per treatment). Results of two-ways ANOVA testing the effect of allelopathy ( $F_A$ ,  $DF = 4$ ), drought ( $F_D$ ,  $DF = 1$ ) and their interaction ( $F_A \times F_D$ ,  $DF = 4$ ) are indicated (with the symbols + for  $P < 0.1$ , \* for  $P < 0.05$ , \*\* for  $P < 0.01$  and \*\*\* for  $P < 0.001$ ).

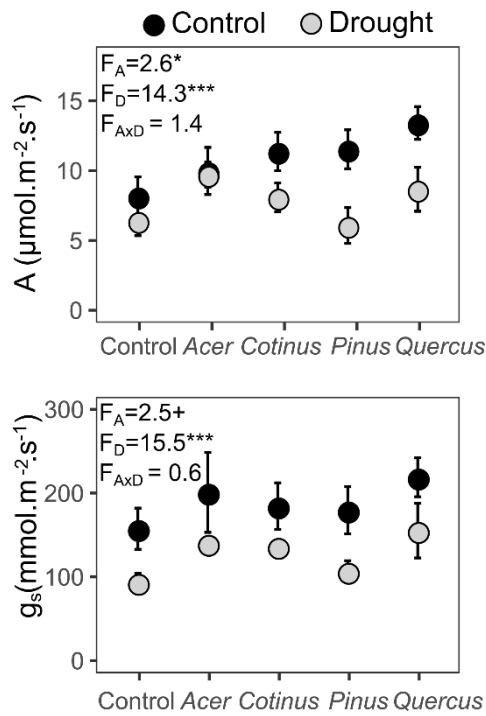
Drought and allelopathy affected all biomass and biomass allocation parameters, except fine roots biomass (Figure 4). No significant interaction was detected. Over the total duration of the experiment, the drought treatment caused an average decrease of 27% of total biomass, 39% of aerial biomass, and 20% of root biomass compared to seedlings under non-stressed conditions. Drought also caused a 48% decrease in the number of leaves, which is the most significant morphological effect of this treatment (data not shown). Litter and leachates of *Cotinus* led to a decrease in the number of leaves, coarse and fine root biomass, total seedling biomass and root/shoot ratio compared to the control. Root biomass and root/shoot ratio were also reduced by the *Pinus* treatment (Figure 4).



**Fig 4:** Drought and allelopathy effects on seedling biomass and root/shoot ratio. Allelopathy has been applied during 2 growing seasons and drought during the last growing season. Data are means  $\pm$  standard errors ( $n=11-17$  seedlings per treatment). Results of two-ways ANOVA testing the effect of allelopathy ( $F_A$ ,  $DF = 4$ ), drought ( $F_D$ ,  $DF = 1$ ) and their interaction ( $F_A \times F_D$ ,  $DF = 4$ ) are indicated (with the symbols \* for  $P < 0.05$ , \*\* for  $P < 0.01$  and \*\*\* for  $P < 0.001$ ).

### 3.3 Allelopathy and drought effects on *Q. pubescens* physiology and mycorrhization rate

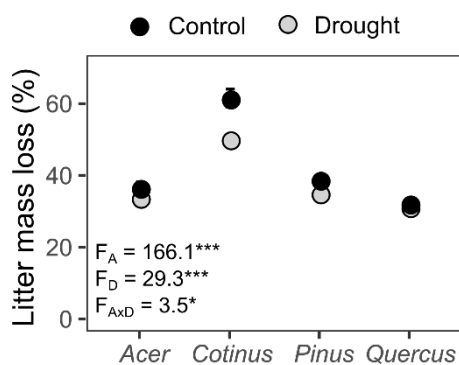
Drought reduced stomatal conductance ( $g_s$ ) and photosynthesis ( $A$ ) by 33.5 and 28.5% respectively compared to the unstressed condition (Figure 5). Allelopathy marginally influenced stomatal conductance ( $P = 0.05$ ) and influenced seedling photosynthetic rate ( $P = 0.02$ ), with in both cases seedlings under *Quercus* treatment having higher values than the control (Figure 5). Mycorrhization rates of *Q. pubescens* seedlings were generally high, ranging from about 70% to 75%, with no significant differences of mycorrhization rates between drought treatments ( $P = 0.92$ ), allelopathy treatments ( $P = 0.96$ ) or their interaction ( $P = 0.84$ ) (data not shown).



**Fig 5:** Allelopathy and drought effects on seedling leaf photosynthetic rate (A) and stomatal conductance ( $g_s$ ) during the summer of the second growing season. Data are means  $\pm$  standard errors ( $n=10$  seedlings per treatment). Results of two-ways ANOVA testing the effect of allelopathy ( $F_A$ ,  $DF = 4$ ), drought ( $F_D$ ,  $DF = 1$ ) and their interaction ( $F_{A \times D}$ ,  $DF = 4$ ) are indicated (with the symbols + for  $P < 0.1$ , \* for  $P < 0.05$ , \*\* for  $P < 0.01$  and \*\*\* for  $P < 0.001$ ).

### 3.4 Allelopathy and drought effects on litter decomposition

Drought and allelopathy had an interactive effect on litter decomposition ( $F_{A \times D}=3.5$ ,  $P = 0.01$ ; Figure 6). Under well-watered conditions there was a difference in species decomposition rates according to the gradient *Quercus* < *Acer* <= *Pinus* < *Cotinus*, with the *Cotinus* litter decomposing particularly fast with a loss of 61% of its mass at the end of the experiment under control conditions. The drought treatment reduced only the decomposition of *Cotinus* and *Pinus* litter, leading to a decrease of the difference between species in the drought treatment (Figure 6).



**Fig 6:** Litter mass loss in the different allelopathic and drought treatments. Data are means  $\pm$  standard errors (n=11-17 pots of surviving seedlings per treatment). Results of two-ways ANOVA testing the effect of allelopathic source species ( $F_A$ , DF = 3), drought ( $F_D$ , DF = 1) and their interaction ( $F_A \times F_D$ , DF = 3) are indicated (with the symbols \* for  $P < 0.05$ , \*\* for  $P < 0.01$  and \*\*\* for  $P < 0.001$ ).

## 4. Discussion

### 4.1 Allelopathy from companion species but not autotoxicity affects *Q. pubescens* seedlings development

The present results confirm the allelopathic potential of *C. coggygria* and *P. halepensis*, as they decreased seedling biomass and in particular root biomass of *Q. pubescens*. Several studies have found that the root part of a target plant is more sensitive to allelochemicals than the aerial part (Ben-Hammouda et al. 2001; Turk and Tawaha 2003; Gatti et al. 2010; Zhang et al. 2010). This sensitivity could be related to the root uptake role implying a direct contact between roots and allelochemicals in the soil (Turk and Tawaha 2003). Inhibition of root growth by allelochemicals may be due to changes in DNA synthesis in cells of root apical meristem, alteration of mitochondrial metabolism (Abraham et al. 2000) or changes in cellular mitotic indices (Dayan et al. 1999; Romagni et al. 2000). As in this study, an inhibition of root development by *C. coggygria* leaf aqueous extracts has also been demonstrated on *Q. pubescens* 2-month-old seedling (Gavinet et al. 2019). However, Gavinet et al. (2018) found no effect of *C. coggygria* and *P. halepensis* litter on *Q. pubescens* seedlings development, which suggest that aqueous extracts may have a greater allelopathic effect than litter. The chemical composition and the quantity of allelochemicals in the leaves are highly variable between green leaves and litter leaves (Fernandez et al. 2009; Hashoum et al. 2017; Silva et al. 2014). Nektarios et al. (2005) also found that green needle extracts of *P. halepensis* had a more pronounced negative effects on herbaceous species growth than litter extracts. The supply of aqueous extracts and litter could also have cumulative effects as the concentration of available allelochemicals in soil water is a direct determinant of the phytotoxic activity of allelochemicals in soil (Kobayashi 2004). Litter decomposition allow to release nutrients and allelochemicals according to initial leaf litter quality (Chomel et al. 2014, 2016, Santona et al. 2015 a, b), and *Cotinus* and *Pinus* litter have a high concentration of phenolics and terpenoids, respectively (Santonja et al. 2015a; Gavinet et al. 2018).

We found no evidence of autotoxicity for *Q. pubescens*. The treatment with litter and aqueous extracts from *Q. pubescens* tended to inhibit seedling emergence but increased seedling length. These effects may be due to mechanical litter effects (Facelli and Pickett 1991; Kostel-Hughes et al. 2005). *Q. pubescens* litter had the largest and thickest leaves, which may constitute a greater physical barrier in line with its tendency to decrease seedling

emergence compared to the artificial litter treatment. Xiong et al (2001) also showed that the negative effects of litter increased with the size of the litter leaves. Similarly, after cutting oak litter leaves, Li and Ma (2003) observed an increase in seedling emergence. The increase in seedling length is consistent with our hypothesis of a physical effect of *Quercus* litter through light interception (Facelli and Pickett 1991), which can accelerate the elongation of the stem. Interestingly, *Quercus* treatment improved seedling photosynthesis and transpiration. As this species litter present the lowest decomposition rate (Figure 5) and is known to release few nutrients (Santonja et al. 2015b), the positive effects of its own litter may come from improved seedling water status thanks to a lower soil moisture evaporation, as also suggested by Gavinet et al. (2018).

#### 4.2 Drought affect *Q. pubescens* seedlings physiology and growth but do not interact with allelopathy

Water stress induced a progressive decrease in stomatal conductance and photosynthesis, a classic physiological response to drought already documented for different oak species (Chaves et al. 2002; Vaz et al. 2010, Arend et al. 2013). The reduced photosynthesis combined with a lower leaf biomass indicates a lower carbon gain for the plant, which participate to explain the lower seedling biomass under drought stress. Seedling biomass reduction by drought was particularly marked for leaves but not for roots, indicating a change in biomass allocation and an increased root/shoot ratio as expected (Poorter et al. 2012). However, contrary to our hypothesis, drought effects were not more important for seedlings submitted to allelopathic treatments. This is particularly surprising as regards the *Cotinus* and *Pinus* treatments which strongly reduced root development. These allelopathic treatments, however, caused no change in leaf carbon and water exchange rates, contrary to previous results showing that allelopathy can reduce plant water uptake, stomatal conductance or photosynthesis (Barkosky et al. 2000; Zhou and Yu 2006). In our study, seedling length and diameter relative growth during the second year did not differ between allelopathic treatments, whatever the drought stress. This suggest that i) allelopathic effects were restricted to the first phase of our experiment, ii) root biomass reduction that occurred in the first year was not strong enough to induce a reduction of water absorption by roots during subsequent drought. An element of explanation of this latter effect could be that *Cotinus* and *Pinus* treatments did not affect rooting depth, which is the main factor controlling seedling water absorption (Padilla and Pugnaire 2007). Finally, the absence of treatment effect on mycorrhization rate may hide differences in mycorrhization type : for instance, mycorrhizae with longer extrametrical mycelia may help seedlings to sustain their carbon and water exchange and growth rates (García de Jalón et al. 2020) despite lower root biomass.

Our results do not support the hypothesis of Pedrol et al. (2006) that sensitivity to allelochemicals is increased under drought stress. At the contrary, allelopathic effects tended to be less pronounced (although not significantly) under drought stress. A reduced allelopathic effect under drought stress is possible if the lower water availability

prevents the transport of soluble allelochemicals to the roots. The dry treatment also reduced litter decomposition rate for *Cotinus* and *Pinus*, possibly limiting the release of litters' allelochemicals through volatilization or solubilization. However, it must be highlighted that drought was applied during the second year of oak seedling development only, a period during which allelopathic effects were reduced as indicated by growth and physiological measurements. Similarly, Gavinet et al. (2019) showed that *Cotinus* inhibited root development of young seedling but not that of older saplings. Differences of root biomass measured at the end of the experiment may thus reflect only legacy effects of the first year allelopathic effects. Drought applied during the first year, or on more sensitive target species, may lead to different results. For instance, Hashoum et al. (2019) showed that drought and allelopathy from *Cotinus* interact to decrease the germination of the herbaceous *Linum perenne* L. In addition, under natural conditions drought can be more pronounced than the drought treatment applied here and reach lethal levels for seedlings, particularly under the expected climate change (Giorgi and Lionello 2008). In that case, an inhibition of oak seedling root development by neighboring allelopathic species such as *Cotinus* and *Pinus* could reduce survival probability in case of more pronounced water stress. Finally, we have not investigated whether drought could induce changes in the production of allelochemicals by the source species, which must be considered to assess if allelopathic interactions will change under increased drought stress. However, other studies contradict this view, evidencing at the contrary a lower total phenolics production under water stress (e.g. Karageorgou et al. 2002), meaning that this issue is far from being resolved.

#### 4.3 Consequences for oak regeneration in different forest microsites

In a previous study, Hashoum et al. (2017) showed that the dominant tree *Q. pubescens* and its companion species *A. monspessulanum* and *C. coggygria* may limit the growth of understory herbaceous plants in downy oak forests through the production and release of allelochemicals. Here, we evidenced no negative effects of *Q. pubescens* and *A. monspessulanum* on *Q. pubescens* seedlings, suggesting that herb inhibition by allelochemicals could be an advantage for oak regeneration since herbs are usually strong competitors for seedling establishment (Gordon et al. 1989; Coll et al. 2003; Rey Benayas et al. 2005; Gavinet et al. 2016b). In contrast, *C. coggygria* affects both herb and oak seedlings, pointing to a possible negative effect of this species on understory diversity and oak regeneration (Gavinet et al. 2019). Finally, *P. halepensis* had negative effect on oak regeneration, but strong autotoxicity have also been evidenced. Oak regeneration is often successful in pine habitats (Lookingbill and Zavala 2000), especially under moderate pine cover (Gavinet et al. 2015), indicating that allelopathy is not a strong limitation for oak regeneration under pine.

In conclusion, our results suggest a better potential for *Quercus pubescens* regeneration under its own stands, due to the positive effects of its litter and leachates, but in the absence of *Cotinus coggygria* in the understory.



Allelopathy does not appear to be stronger under drought stress, but further investigations are needed in particular to elucidate the consequences of the opposite effects of allelopathy and drought on the root/shoot ratio. Finally, the allelopathic treatments tested here were rather limited, particularly during their interaction with drought in the second year. Interactions with drought may be more evident in systems where allelopathy is a stronger driver of plant performance, particularly in the case of strategies adopted by Exotic Invasive Plants Species (e.g. Medina-Villar et al. 2020)

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