

Fire effects on litter chemistry and early development of *Eucalyptus globulus*

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Abstract

Background and aim Fires affect what happens to litter in ecosystems. Biological and chemical effects of burnt litter on plants are not as of yet fully understood. We aimed to assess the effects of heat-treated leaf litter on germination and seedling root growth of *Eucalyptus globulus*.

Methods Litter from *E. globulus*, *Acacia dealbata*, *Pinus pinaster*, and *Quercus suber* was collected in Portugal, on Humic Cambisol, and heated between 25 °C and 600 °C.

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Those materials were then characterized by: ^{13}C CPMAS NMR spectroscopy, proximate lignin and cellulose, and elemental analyses. Afterwards, they were used as substrate in bioassays with *Eucalyptus* seeds.

Results Heating changed litter composition ($P < 0.05$), consistently across species: alkyl C, *O*-alkyl C, and methoxyl + *N*-alkyl C decreased more than 50%; and aromatic C increased more than 5-fold. Unheated and lightly heated litters inhibited germination and growth, with maximum inhibition by *Quercus* and *Eucalyptus* litters, down to 17% of the control ($P < 0.05$). Severely charred materials had neutral or stimulatory effects, up to 191% of the control ($P < 0.05$). These responses were associated with concentrations of dominant C types in litters ($P < 0.05$).

Conclusion Litter charring, as it occurs during wildfires, is potentially critical for the success of *E. globulus*'s regeneration from seeds.

Keywords Allelopathy · Char · ^{13}C CPMAS NMR · Plant-soil feedback · Phytotoxicity · *Eucalyptus globulus*

Introduction

Wildland fires are common in many regions in the world. They strongly influence ecosystem dynamics (Naveh 1974; Chandler et al. 1983; Bond and van Wilgen 2012). As a result, many plant *taxa*, which have evolved in fire-prone environments, have reproductive traits that allow for regeneration after fires have occurred (Keeley et al. 2011; Pausas and Keeley 2014). These

traits may be vital for plants, not only in their native range but also in other territories. Exotic species face new challenges to thrive outside their native ranges and may challenge other species for survival (Hallett 2006; Mitchell et al. 2006; Alpert 2006). Some of these challenges are related to allelopathy (Callaway and Aschehoug 2000; Cummings et al. 2012). Fire, which can cause profound chemical changes in organic matter (González-Pérez et al. 2004), has the potential to alter the chemical interference amongst plants (Bonanomi et al. 2006), if allelochemicals are affected. Consequently, fire may bring about a shift on the probability of plant establishment. This point is especially relevant with reference to the increasing cultivation of exotic species worldwide and in regions where fire regimes are changing.

Plants may release chemical compounds that affect other plants, either negatively or positively (Rice 1984). Such contrasting effects have been documented as far back as ancient Greece (Willis 1985), and are included in the concept of allelopathy, as expressed by Rice (1984). Although allelopathy is a phenomenon which has been known for centuries, especially in agriculture, the understanding of its impact on natural plant populations and communities is in its early stages (Meiners et al. 2012). Nevertheless, evidence shows that allelopathy affects species' capabilities of self-perpetuation and colonization, which influence succession dynamics (e.g. Bazzaz 1979), plant diversity (Bonanomi et al. 2005), and vegetation spatial-patterns (Carteni et al. 2012).

When plant species are introduced outside their native ranges, they interact with native species of recipient communities. In the medium- to long-term, new interactions can have different outcomes for the exotic species, ranging from introduction failure to invasion (Sakai et al. 2001; Hallett 2006; Mitchell et al. 2006; Blackburn et al. 2011). Allelopathy is considered in some of the hypotheses that explain these different outcomes (Catford et al. 2009). In his seminal work, Rabotnov (1974) postulated that species which have coevolved are less likely to chemically interfere with each other, than those from different biogeographical regions. In fact, some decades later, this idea was used to formulate two complementary hypotheses: the 'novel weapons hypothesis' (Callaway and Aschehoug 2000; Callaway and Ridenour 2004) and the 'homeland security hypothesis' (Cummings et al. 2012). Some authors claim that the use of a biogeographical approach is important in understanding allelopathy's role on the level of success

of exotic species (Hierro et al. 2005; Inderjit et al. 2008). This approach has been mostly used to test the allelopathic potential of exotic species on other species in the recipient communities (e.g. Callaway and Aschehoug 2000; Loydi et al. 2015). However, as there have been few studies testing the effects of recipient communities on exotic species (e.g. Christina et al. 2015; Ning et al. 2016), there is a need for further research on this topic.

Plant litter is an important source of allelochemicals in terrestrial ecosystems. In fact, inhibitory effects of litter have often been reported in agriculture, forestry (Rice 1984; Souto et al. 2001), and natural ecosystems (Rice 1984). The chemical substances of litter and their degradation products, affect plant-soil feedbacks in varied ways (Mazzoleni et al. 2015). Litter degradation can be caused by soil biological activity (Schlesinger 1977), photo-degradation (Austin and Vivanco 2006), or combustion caused by fire (Raison 1979; Pyne et al. 1996). As a result, litter can release organic compounds and mineral nutrients (Schlesinger 1977; Attiwill and Adams 1993). The effects of litter dynamics are observed at different scales: seed germination and seedling establishment; population demography and community structuring; and species evolution (Facelli and Pickett 1991; Stinchcombe and Schmitt 2006). The effects of litter decomposition on living plants have received a great deal of attention (Facelli and Pickett 1991; Reigosa et al. 2006), while the effects of litter combustion on plants have not.

Fire alters the chemical properties of organic matter, as thermally-induced modifications occur during combustion (González-Pérez et al. 2004). The effects of fire on soil organic molecules are complex and depend on fire-exposure time, fire temperature, and post-fire biological activity (reviews in González-Pérez et al. 2004; Certini 2005; Knicker 2007). Fire can directly neutralize phytotoxicity, through: direct alteration of allelochemicals (McPherson and Muller 1969); insolubilisation by condensation reactions, or sorption by pyrogenic char (Wardle et al. 1998; Hille and den Ouden 2005). In addition, some combustion products can break seed dormancy (Keeley and Fotheringham 2000), stimulate germination, and regulate seedling growth (Nelson et al. 2012). Conversely, others can inhibit germination (Nelson et al. 2012). Fire-derived allelochemicals are present in either smoke or combustion residues in soil, where they can come in contact with plants (Nelson et al. 2012). Additionally, the quality and magnitude of fire effects on allelopathic interactions can

be highly variable, as they depend on heating temperature (Johnson 1919; Bonanomi et al. 2016) and are species specific (Johnson 1919; Keeley et al. 1985; Nelson et al. 2012). Therefore, litter combustion can induce changes in the chemical environment of plants, thereby influencing their development.

The idea that fire can affect allelopathic interactions in ecosystems has been around for a long time (Johnson 1919; Muller et al. 1968), however, little is still known. Subsequent studies focused mainly on Californian chaparral species (e.g. McPherson and Muller 1969; Christensen and Muller 1975), and rarely on other systems (e.g. Razanamandranto et al. 2005). For instance, no relevant studies were conducted on *Eucalyptus*, a tree genus that deserves to be studied in this context for various reasons. Firstly, it has evolved in a fire-prone environment (Mount 1969). Secondly, its regeneration from seeds seems to be fire-dependent (Mount 1964; Kirkpatrick 1975). Indeed, spurts of regeneration have often been associated with the occurrence of severe fires (e.g. Mount 1964; Florence 1996). Thirdly, several species of this genus have allelopathic potential (May and Ash 1990; Willis 1999). Moreover, Mount (1969) suggests that the regeneration success of this *taxon* in burnt areas may be linked to the removal of chemical inhibitors from the soil's surface. Nevertheless, only Florence and Crocker (1962) have tested the effect of soil heating on eucalypt development, but their test temperatures were low ($T \leq 160$ °C) compared to those reached on soil surface during a fire. Finally, *Eucalyptus* has a large distribution area both as a native and as an exotic genus. It is almost exclusively native to Australia, existing abundantly throughout the country (Mount 1969; Rejmánek and Richardson 2011), and is the second most cultivated tree genus in the world ($\approx 20 \times 10^6$ ha, Rejmánek and Richardson 2011). Furthermore, its naturalisation and spread from cultivation has been documented in several countries (Rejmánek and Richardson 2011). As a result, *Eucalyptus* is a relevant plant genus to study the changes in allelopathic interactions that may underlie post-fire plant establishment.

Despite the numerous studies that have been conducted, allelopathic interactions remain difficult to disentangle from other ecological processes. Field-based investigations face a multitude of confounding factors, while bioassays, under controlled conditions, produce results whose extrapolation to field conditions is somewhat limited (May and Ash 1990; Inderjit and Nilsen 2003). However, if the latter are complemented by

chemical analyses, not only will they allow for the separation of the effects of several influencing factors, but also for the identification of potentially involved substances (Inderjit and Dakshini 1995; Inderjit and Nilsen 2003). A useful analytic technique for this purpose is solid-state ^{13}C nuclear magnetic resonance spectroscopy (^{13}C NMR), as it is a powerful tool for studying the molecular composition of complex organic materials (Baldock and Smernik 2002). Moreover, this technique has been successfully used for tracking organic-matter changes caused by heat and fire (e.g. Freitas et al. 1999; Almendros et al. 2003; Knicker et al. 2005). Another important strategy employed to improve ecological relevance of bioassay results is the careful selection of the species to be tested (donors and targets). Besides model species, species that coexist in the field need to be used. However, most laboratorial studies only use model species as target species (Inderjit and Nilsen 2003). As interspecific differences in sensitivity to allelochemicals are common (Inderjit and Nilsen 2003; Meiners et al. 2012), the ecological relevance of those studies is lessened. Furthermore, some studies have analysed the ecological impact of chemical changes caused by fire on litter (e.g. Guinto et al. 1999; Campos et al. 2012; Reyes et al. 2015; Bonanomi et al. 2016). However, these were exploratory, very divergent in their aims, and rarely examined interactions between species that actually coexist. Hence, bioassay results can be more meaningful in ecological terms, if the bioassays use donor and target species that coexist in the field, and if they are coupled with appropriate chemical analyses of the substrates.

This article focuses on allelopathic interactions in wildland fire context. It aims to study the potential of a fire-adapted species to persist in self-dominated forests or to colonize heterospecific communities. In particular, we have used bioassays under optimal environmental conditions to study the early development of *Eucalyptus globulus* Labill.. Substrates were dry and charred leaf litter, from either conspecific (*E. globulus*) or heterospecific plants (*Acacia dealbata* Link, *Pinus pinaster* Aiton, and *Quercus suber* L.). These species coexist with the target species in its native range (*A. dealbata*) and/or outside it (*A. dealbata*, *P. pinaster*, and *Q. suber*). The allelopathic effects of the same substrates were also examined on a model species (*Lepidium sativum* L.). Litter biochemical quality was analysed by Cross Polarization Magic Angle Spinning ^{13}C NMR (^{13}C CPMAS NMR) and its relationship with plant development was assessed.

Materials and methods

Study area and plant species

Study sites were located in the central massif of Serra de Monchique, in the hinterland of Algarve, SW Portugal. Sites' latitude ranges between 37°18'N and 37°20'N, while longitude ranges between 8°29'W and 8°34'W. Altitude ranges between 293 m and 442 m a.s.l. The local climate is classified as type Csb type according to Köppen-Geiger (Faria et al. 1981; Peel et al. 2007). The mean annual temperature is 15.5 °C, and annual precipitation is 1348 mm (Faria et al. 1981). The bedrock is a sienitic intrusion (Rock 1983). Soil is a Humic Cambisol, with a sandy texture, a high water holding capacity, and a high base saturation (Kopp et al. 1989). Local forests are mainly plantations of *E. globulus*, but *P. pinaster* plantations and *Q. suber* woodlands are also of importance there. These forests tend to be pure, but different combinations of these species also exist. In addition, *A. dealbata* occurs both within these main forest types and in monospecific patches.

The four aforementioned species were selected for this study, not solely based on their importance for forest management and conservation in S Europe and their relevance in fire ecology (Silva et al. 2009; Lorenzo et al. 2010; ICNF 2013; Águas et al. 2014), but also based on their allelopathic potential (del Moral and Muller 1969; Gonçalves et al. 2008; Lorenzo et al. 2008; Amri et al. 2013). In addition, we selected *Lepidium sativum* as a model target species because of its recognized sensitivity to phytotoxicity (Macías et al. 2000; Bonanomi et al. 2006). Hereafter, plant species will be referred to by their genus names. Species names will only be used if clarification is required.

Plant material collection

In the study region, different forest patches were selected by a stratified random design, with strata classified according to different dominant species. In May 2015, leaf litter and seeds were sampled. Recently abscised leaves were collected from under the canopies of 20 trees of the dominant species. These were randomly selected within each patch. With reference to *E. globulus*, which shows leaf heteroblasty, only adult leaves were collected. *Eucalyptus* seeds and litter were collected on the same day, from the same trees. Thirty ripened capsules were collected from each tree and were air dried. At the laboratory, leaf

litter materials were also air dried at room temperature, until constant mass was reached. They were milled (particle size <1 mm), mixed to a single composite sample per species, and stored at room temperature. Although milling could increase allelochemical bioavailability in litter samples (May and Ash 1990; Inderjit and Nilsen 2003), it was deemed necessary for homogeneous burning to occur, due to the large inter-specific differences in both structural and morphological leaf traits. *Eucalyptus* seeds were gravitationally extracted from capsules and separated from the chaff. *Lepidium* seeds were obtained from a commercial seedlot.

Heat treatments

Treatments consisted of the heating of samples from each species at 6 different temperatures (100, 200, 300, 400, 500 and 600 °C), for 30 min. In addition, one unheated sample from each litter species was used as the control for temperature. The crucible was a metal tray (21 × 19 × 4 cm). Litter samples were 100 g dry-mass, prior to heating. These samples were heated uncovered, in single batches, one litter species at a time, inside a pre-heated closed muffle furnace. As a result, 28 different litter types were obtained (4 litter species × 7 temperature levels).

Chemical characterization of litter materials

The 28 litter types were characterized by ¹³C CPMAS NMR obtained in solid state, under the same conditions, thus enabling quantitative comparisons among spectra. The method followed Bonanomi et al. (2011). A Bruker AV-300 spectrometer (Bruker Instrumental Inc., Billerica, MA, USA), equipped with a 4-mm wide bore MAS (magic angle spinning) probe, was used for these analyses. NMR spectra were obtained with a MAS of 13,000 Hz of rotor spin; a recycle time of 1 s; a contact time of 1 ms; an acquisition time of 20 ms; and 2000 scans. Samples were packed in 4-mm zirconium rotors with Kel-F caps (Wilma/Lab Glass, Buena, NJ, USA). The pulse sequence was applied with a ¹H ramp to account for non-homogeneity of the Hartmann–Hahn condition at high spin rotor rates. Pre-processing consisted of an exponential multiplication, with a line broadening of 50.0 Hz, that was applied to the free-induction decay, prior to Fourier transformation. All spectra were processed using the AMIX program (www.bruker.com), phased, and the baselines corrected

manually, to calculate the area of the peaks in selected regions. The relationship between spectral regions and C types was drawn from reference studies (Almendros et al. 2000; Kögel-Knabner 2002; Bonanomi et al. 2011): 0–45 ppm = alkyl C; 46–60 ppm = methoxyl + *N*-alkyl C; 61–90 ppm = *O*-alkyl C; 91–110 ppm = di-*O*-alkyl C; 111–140 ppm = *H*- and *C*-substituted aromatic C; 141–160 ppm = *O,N*-substituted aromatic C (phenolic C, *O*-aryl C, *N*-aryl C); and 161–190 ppm = carboxyl C.

All litter types were characterized for total C and N content in an elemental analyzer (Primac SCN100, Skalar, Netherlands). Flash combustion at 1100 °C of microsamples was followed by quantification of C by a near-infrared detector (NIRD) and quantification of N through thermal conductivity (Dumas method).

In addition, proximate cellulose and lignin content were quantified for unheated litter as the acid hydrolysable fraction and the acid non-hydrolysable materials, respectively (Gessner 2005). In short, proximate cellulose was determined as the hydrolysable fraction following a sulphuric acid treatment (loss upon 3 h in H₂SO₄ at 72%). Proximate lignin was determined as the non-hydrolysable fraction (loss upon ignition after the aforementioned H₂SO₄ treatment). All carbon fractions were presented as ash-free dry mass.

Plant bioassays

Plant bioassays were aimed at assessing the effects of unheated and heated litters on *Eucalyptus* seed germination and root growth, compared to *Lepidium*'s. Ten seeds of either *Lepidium* or *Eucalyptus* were placed in 9-cm Petri dishes, over 2 sheets of sterile filter paper, with 0.2 g of litter, and wetted with 4 mL of distilled water. Subsequently, dishes were wrapped with plastic film to ensure air tightness. Controls for each target species were Petri dishes with seeds placed on water-wetted paper. Each treatment combination was replicated 3 times. A total of 1740 seeds were used in the experiment ((28 litter types + control) × 2 target species × 10 seeds × 3 replicates). All Petri dishes were arranged following a fully randomized design at the laboratory, and kept at room temperature and under natural daylight (photoperiod of 12 h).

Counts of germinated seeds and measurements of seedling root length were recorded after incubations periods of 3 and 5 days for *Lepidium* and *Eucalyptus*, respectively, based on the germination time of each species (ISTA 2003). For both target species, seedlings were

counted and root measurements were taken when the longest roots in the control assays reached a length of 2 cm. In order to ensure that statistical analyses could be run without missing data, the few replicates which had no germinations were considered as having a root growth equal to zero. This approach is unlikely to produce biased inference, considering that in the absence of germination, there is no seedling growth, nor plant establishment.

Data analysis

One-way ANOVAs were used to test the effect of heating temperature (7 levels) on litter chemical quality. The concentrations of different C types, assessed by ¹³C NMR, were considered as the dependent variables. The tested null-hypotheses stated that litters heated at different temperatures contained an equal relative amount of each C type revealed in the respective spectrograms.

Species responses from the bioassays were expressed as percentage of the respective control and submitted to Generalized Linear Models (GLM) analysis. Separate GLMs were used for modelling germination and root growth, taking into consideration main and interactive fixed effects of target species (2 levels), litter species (4 levels), and heating temperature (7 levels). Pairwise differences among treatments were tested using Tukey's HSD post-hoc test. The occurrence of either inhibitory or stimulatory effects of treatments on the target-species responses were assessed, using two-tailed *t*-tests which compared each treatment combination and the respective control. For these tests, $\alpha = 0.05/N$, with *N* being the number of multiple comparisons, according to the Bonferroni's method.

To analyze the relationship between the litter chemical composition and the bioassay results, seed germination and seedling root growth of each target species were tested for correlation with litter N and C contents, C/N ratio, and amounts of the different C types assessed by ¹³C NMR. Following this, the relationship between litter C and plant responses was assessed in greater detail. For that purpose, linear correlations were calculated between the peak of each ¹³C NMR signal (width = 1 ppm; *n* = 200), recorded for the 28 litter types, and plant responses of the target species incubated on the same litters. Correlations were calculated separately for the two target species, as well as for germination and root growth. In order to control for type I statistical error, correlation was tested at $\alpha = 0.001$. This value was obtained using the false discovery rate method (Benjamini and Hochberg 1995), for multiple comparisons correction.

Finally, litter spectral data were submitted to multivariate analyses, including Cluster Analysis (CA) and Principal Component Analysis (PCA), in order to provide a synthetic representation of litter chemistry changes across heating treatments and their effects on the early development of the target species. Ward's aggregation rule and Euclidean distance were used in CA to minimize the within-cluster variance (Podani 2000). In the PCA, several supplementary variables were included (i.e. plotted in the multivariate space, but not used to calculate the principal components). This procedure followed the approach suggested by Legendre and Legendre (1998). The supplementary variables were: litter C and N contents; C/N ratio; and germination and root growth of *Eucalyptus* and *Lepidium* on the litter samples).

Statistica 7 software (StatSoft Inc., USA) was used for all statistical analyses.

Results

Molecular changes in heated litters

The ^{13}C NMR spectra revealed consistent changes of litter quality defined by C types across the four species, with major chemical changes progressively occurring at increasing heating temperatures (Fig. 1, Fig. S1). In general, these spectra only showed significant changes in litter chemical composition at $T \geq 300\text{ }^{\circ}\text{C}$ (Fig. 1). Within this temperature range, concentrations of both

methoxyl + *N*-alkyl C and *O*-alkyl-C fell, the latter experiencing a much more pronounced decline (Fig. 1). The concentration of alkyl C increased slightly between $200\text{ }^{\circ}\text{C}$ and $300\text{ }^{\circ}\text{C}$, and decreased substantially at higher temperatures (Fig. 1). In contrast, aromatic C (111–140 ppm) showed a steep increase when samples were burnt at $T \geq 300\text{ }^{\circ}\text{C}$ (Fig. 1).

Aside from the general pattern, interspecific differences among litter species were observed along the temperature gradient (Fig. 2 and Fig. S1). Among these, alkyl C was relatively more abundant in unheated litters of *Acacia* and even more so with respect to *Eucalyptus* litters. Above $300\text{ }^{\circ}\text{C}$, the relative amount of this C type dropped more gradually in *Acacia* litter compared with the other litter species (Fig. 2). The relative amount of *O,N*-substituted aromatic C showed a significant increase when litters from *Eucalyptus*, *Pinus* and *Quercus* were heated at $T \geq 300\text{ }^{\circ}\text{C}$ (Fig. 2). However, this did not occur with *Acacia* litter. Finally, changes in the relative amounts of carbonyl C and di-*O*-alkyl presented a species-specific pattern at $T \geq 300\text{ }^{\circ}\text{C}$ (Fig. 2).

Effects of heated litter on plant early development

Considering the overall effects of treatments in the bioassays (Table 1), results were consistent between germination and root growth response. Firstly, the main effects of temperature (T), and litter species (L) were highly significant (Table 1). Secondly, the thermal impact of temperature on litter was also highly significant

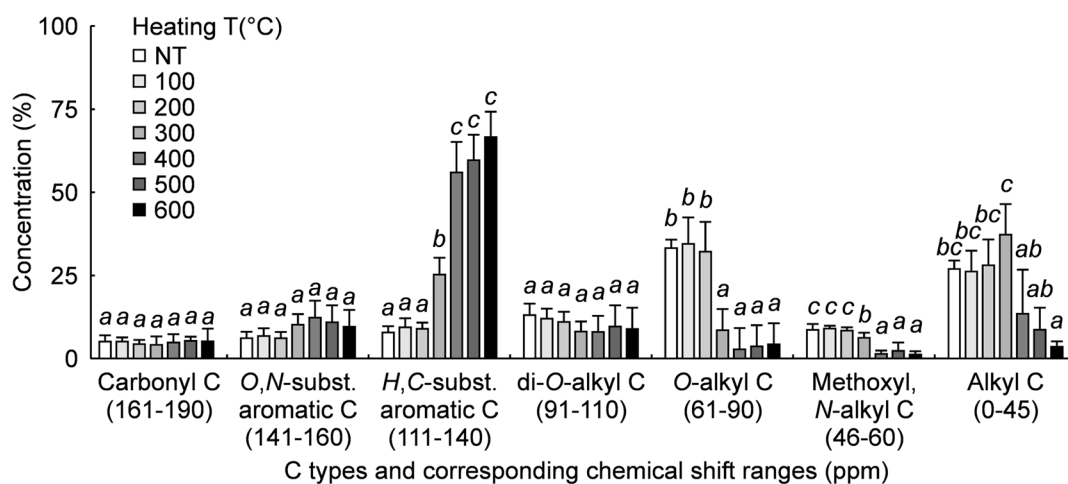


Fig. 1 Concentrations of seven classes of organic C types and corresponding ^{13}C CPMAS NMR spectral regions in litter materials treated at different temperatures for 30 min. Data refer to mean \pm SD

of litter species ($n = 4$). Different letters within each organic C class indicate temperature-dependent significant differences (Tukey's HSD post-hoc test from one-way ANOVA, $P < 0.05$). NT: unheated

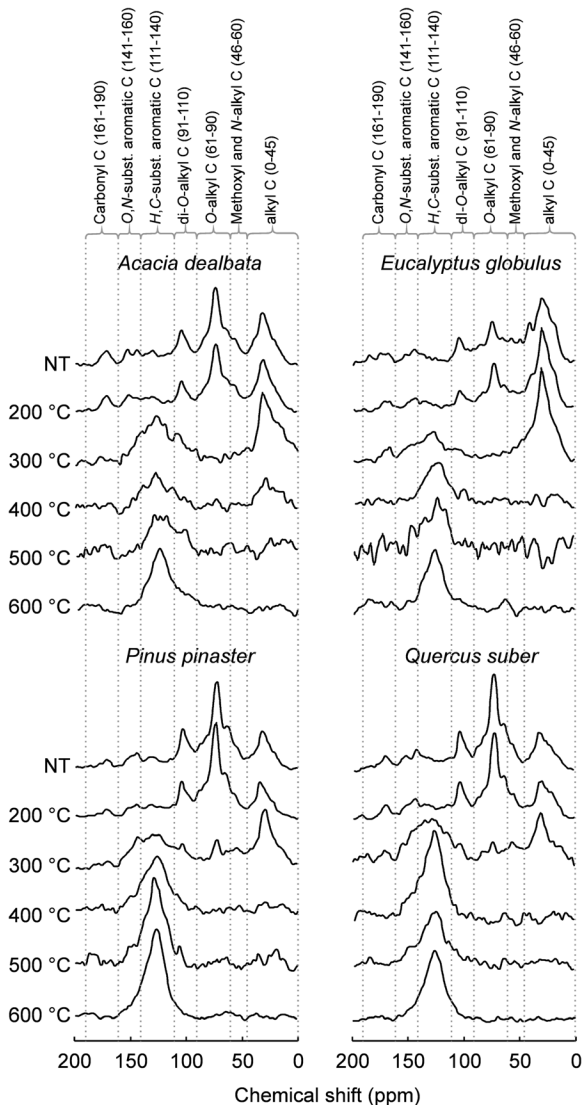


Fig. 2 ^{13}C CPMAS NMR spectra of *Acacia dealbata*, *Eucalyptus globulus*, *Pinus pinaster* and *Quercus suber* litters, heated at different temperatures for 30 min. Spectra obtained for 100 °C heated materials are not shown because they were not different from those of unheated materials (NT, 25 °C). Reference spectral regions and corresponding C types are reported on top of the panels, with chemical shift ranges indicated in brackets and by vertical dotted lines

for both responses, that is, species-specific differences induced by different litter species also varied with heating temperature (interactions $L \times T$, Table 1). Thirdly, the effect of target species (S) was also significant on both germination and root growth (Table 1), indicating a remarkable species-specific sensitivity of seeds and roots to treatments. Such a trend was consistent across litter materials, as indicated by the non-significant interaction of target species and litter species for both

response metrics (interaction $S \times L$, Table 1). Finally, the effect of heating at particular temperatures differed between *Lepidium* and *Eucalyptus* both for germination ($P < 0.001$) and root growth ($P = 0.007$) (interactions $S \times T$, Table 1). Although this effect had the same significance as the one of $L \times T$ interaction for germination, it was less significant for root growth.

Upon examining significant heating effects in greater detail, interesting patterns became evident (Fig. 3, Table S4). On the one hand, *Lepidium* germination was more sensitive to litter treatments than *Eucalyptus* (Fig. 3a–b, Table S4). The former was consistently inhibited by litter heated at $T \leq 200$ °C (Fig. 3a, Table S4), but the magnitude of inhibition depended on both temperature and litter species. In addition, litters heated at $T \geq 300$ °C had no effect on the germination of this species (Fig. 3a, Table S2). Meanwhile, *Eucalyptus* was inhibited only by litter of *Acacia*, *Eucalyptus* or *Quercus*, either unheated or heated at 100 °C (Table S4). Remarkably, conspecific litter heated at 100 °C was the substrate where *Eucalyptus* seeds experienced the worst performance (Fig. 3b). On the other hand, *Eucalyptus* performed better than *Lepidium* also in terms of root growth (in 19 out of 28 treatments, Table S4). However, in the case of root growth, differences were due not only to inhibition, but also to stimulation of plant development. Similarly to the germination results, *Acacia* and *Pinus* litters tended to inhibit less root growth than those of *Eucalyptus* and *Quercus*, when they were unheated or moderately heated. Conspecific litter heated at 100 °C was the most inhibiting litter for *Eucalyptus* root growth (Fig. 3d), just as it was for germination (Fig. 3b, Table S4). Litters heated at $T \geq 300$ °C did not cause root growth inhibition (Fig. 3c–d, Table S4). In particular, *Lepidium* root growth was not significantly affected by those treatments (Fig. 3c, Table S4), whereas several types of charred litter stimulated *Eucalyptus* root growth (Fig. 3d, Table S4). Thus, there is a recurring pattern. Despite target species respond differently to different litter types, they usually develop better and more homogeneously on litters which were severely charred than on those which were not.

Litter quality and target species sensitivity

Target species sensitivity to litter quality had a specific pattern, which was evidenced by correlations between plant responses on litter samples and biochemical quality of the same litter materials, as expressed by ^{13}C

Table 1 Summary of the General Linear Models (GLM) testing for main and interactive effects of target species (fixed factor, two levels: *Eucalyptus globulus* and *Lepidium sativum*), litter species (fixed factor, four levels: *Acacia dealbata*, *E. globulus*, *Pinus pinaster*, and *Quercus suber*), and litter heating temperature (fixed factor, seven levels: 25, 100, 200, 300, 400, 500, and 600 °C) on target species germination and root growth. For each effect, sum of squares (SS), degrees of freedom (df), mean of squares (MS), Fisher's statistic (*F*) and associated *P*-value are shown

	SS	df	MS	<i>F</i>	<i>P</i>
Germination					
Target species (S)	28,273.7	1	28,273.7	83.0	< 0.001
Litter type (L)	9997.1	3	3332.4	9.8	< 0.001
Temperature (T)	82,245.8	6	13,707.6	40.3	< 0.001
S × L	1239.4	3	413.1	1.2	n.s.
S × T	35,479.8	6	5913.3	17.4	< 0.001
L × T	28,740.5	18	1596.7	4.7	< 0.001
S × L × T	25,647.6	18	1424.9	1.6	n.s.
Residual	38,137.8	112	340.5		
Root growth					
Target species (S)	41,476.7	1	41,476.7	46.9	< 0.001
Litter type (L)	65,841.3	3	21,947.1	24.8	< 0.001
Temperature (T)	133,804.1	6	22,300.7	25.2	< 0.001
S × L	3855.1	3	1285.0	1.5	n.s.
S × T	16,674.0	6	2779.0	3.1	0.007
L × T	102,154.8	18	5675.3	6.4	< 0.001
S × L × T	12,033.9	18	668.5	1.9	0.018
Residual	99,036.1	112	884.3		

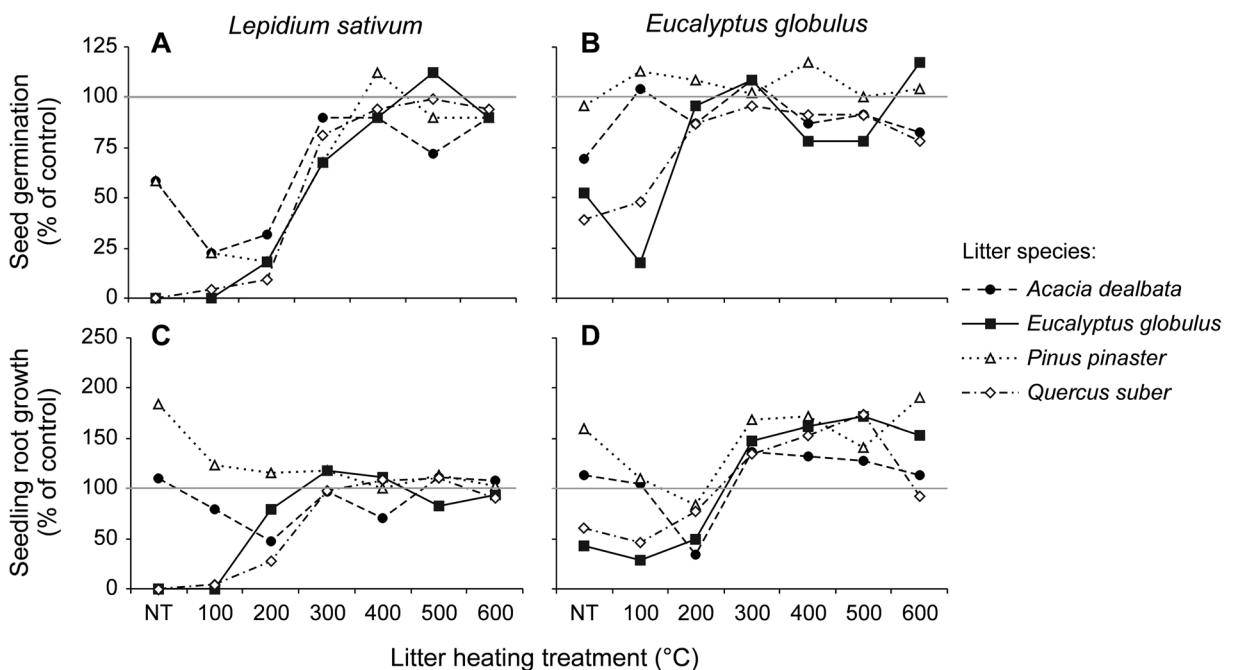


Fig. 3 Responses of *Lepidium sativum* (a, c) and *Eucalyptus globulus* (b, d) exposed to plant litters either unheated (NT) or heated at six different temperatures for 30 min. Data refer to germination and seedling root growth, expressed as percentage of unexposed controls (=100). Values are means of 3 replicates;

error bars are omitted to improve readability (results of testing for main and interactive effects of treatments are in Table 1; means, standard deviations, and results of pair-wise post-hoc comparisons among treatment combinations are in Table S4)

NMR data from reference spectral regions (Table 2). Indeed, seed germination of *Lepidium* was positively associated with aromatic C regions and negatively with *O*-alkyl C and methoxyl + *N*-alkyl C regions, while in the case of *Eucalyptus* the correlation values presented an identical pattern but were not statistically significant (Table 2). The same pattern, in terms of correlation magnitude and direction, was also found in seedling root growth, but, contrarily to germination, the associations with ^{13}C NMR data were significant for *Eucalyptus*, but not for *Lepidium* (Table 2). Interestingly, plant responses were unrelated to litter total C and N contents, as well as to litter C/N ratio (Table 2).

The detailed correlation profiles of plant responses along the NMR spectrum provided further insight into the relationships between the molecular composition of litters and the early development of plants (Fig. 4). These profiles were qualitatively similar between the two target species, as well as between the two response metrics. However, different restricted spectral regions were significantly related with either inhibitory or stimulatory effects (Fig. 4). On the one hand, correlation profiles of germination vs. NMR spectral data were similar for both target species but significant correlations existed only in the case of *Lepidium*. Germination of this species (Fig. 4a) was negatively associated with the whole *O*-alkyl C region, as well as with different restricted intervals within the carboxyl C (169–172 ppm), di-*O*-

alkyl C (102–104 ppm), methoxyl + *N*-alkyl C (51–60 ppm), and alkyl C (38–44 ppm and 31–34 ppm) regions. However, it was positively correlated with several parts of the spectrum, including the whole *H*- and *C*-substituted aromatic C region (111–140 ppm), and two adjacent restricted intervals within the di-*O*-alkyl C (108–110 ppm) and *O,N*-substituted aromatic C (141–150 ppm) regions, plus a restricted interval resonating at 193–195 ppm. Meanwhile, *Eucalyptus* germination followed a similar pattern of qualitative response in relation to spectral data but correlations were not significant (Fig. 4b). On the other hand, correlation profiles of root growth vs. NMR spectral data were qualitatively similar to those of germination but, in this case, significant correlations existed only for *Eucalyptus*. Root growth of this species was significantly correlated with several spectral regions (Fig. 4b). It was negatively correlated with most of the *O*-alkyl C region (66–87 ppm) and with a restricted interval of methoxyl + *N*-alkyl C region (57–59 ppm), while it was positively correlated with most of the *H*- and *C*-substituted aromatic C region (114–140 ppm) and with an adjacent restricted interval within the *O,N*-substituted aromatic C region (141–146 ppm). Meanwhile, *Lepidium* root growth had a similar qualitative correlation pattern with NMR spectral data but they were not significant (Fig. 4a). Thus, the pattern of correlations between plant development and chemical quality of litter was qualitatively similar for the

Table 2 Linear correlation (Pearson's r) between ^{13}C -CPMAS NMR spectral data, describing biochemical quality of 28 litter types, and target species responses on those litter types. Pearson's r was separately calculated for germination and root growth of each target species (*Eucalyptus globulus* and *Lepidium sativum*).

Correlations with litter content of N and C, and with C/N ratio are also reported. Bold indicates statistically significant r values ($P < 0.001$, after correction for multiple comparisons according to the false discovery rate method, Benjamini and Hochberg 1995)

Litter quality parameter	Germination		Root growth	
	<i>Lepidium</i>	<i>Eucalyptus</i>	<i>Lepidium</i>	<i>Eucalyptus</i>
Carbonyl C (161–200 ppm)	0.168	0.131	-0.058	0.012
<i>O,N</i> -substituted aromatic C (141–160 ppm)	0.599	0.221	0.216	0.578
<i>H, C</i> -substituted aromatic C (111–140 ppm)	0.809	0.312	0.298	0.622
Di- <i>O</i> -alkyl C (91–110 ppm)	-0.322	-0.241	0.008	-0.281
<i>O</i> -alkyl C (61–90 ppm)	-0.837	-0.345	-0.262	-0.618
Methoxyl + <i>N</i> -alkyl C (46–60 ppm)	-0.735	-0.374	-0.310	-0.571
Alkyl C (0–45 ppm)	-0.529	-0.138	-0.243	-0.439
C content (%)	0.083	0.168	0.137	0.174
N content (%)	0.255	0.085	-0.010	-0.010
C/N ratio	-0.273	0.073	0.212	0.042

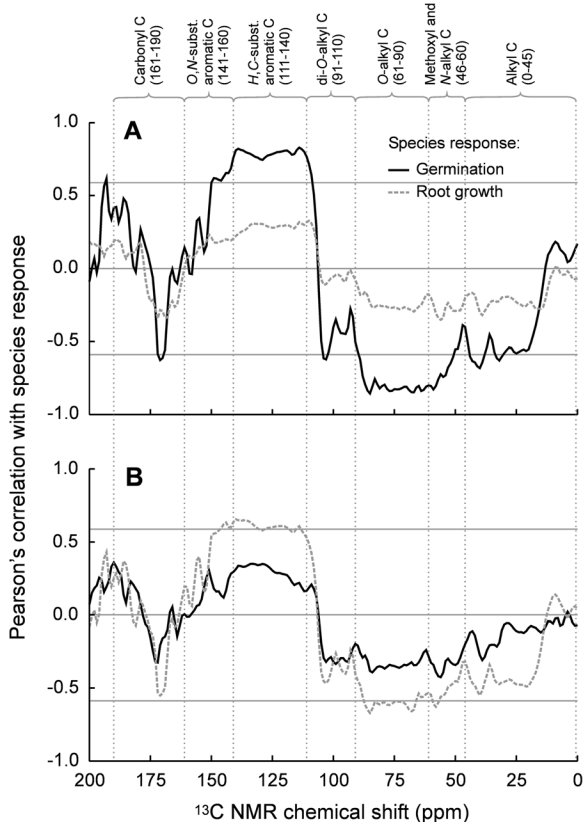


Fig. 4 Profiles of correlation (Pearson's r) between ^{13}C CPMAS NMR signals and plant responses (either germination or root growth) when sowed on burnt litter materials, reported separately for *Lepidium sativum* (a) and *Eucalyptus globulus* (b). Grey horizontal lines in each panel indicate threshold values of statistical significance for r according to sample size ($n = 28$, $P = 0.001$), after correction for multiple comparisons according to false discovery rate method (Benjamini and Hochberg 1995). Organic C types corresponding to reference spectral regions are indicated on the top of the panel

two development indicators and the two target species. Nevertheless, the litter quality had the strongest correlations with *Lepidium* germination and *Eucalyptus* root growth.

The CA results highlighted species-specific differences among unheated or lightly heated litters (up to 200 °C) and confirmed that litters of different species treated at higher temperatures were chemically more similar (Fig. 5). Indeed, the dendrogram of litter samples, based on ^{13}C NMR spectra, presented two main clusters segregated at high Euclidean distance and including treatments either at $T \leq 300$ °C or at higher temperatures (Fig. 5a). Moreover, within the low-temperature cluster, samples heated below 300 °C and belonging to the same litter species were consistently

aggregated at the lowest distance levels, while within the high-temperature cluster sample aggregation was independent from litter species, and more related to temperature (Fig. 5a).

The PCA provided a synthetic picture of the chemistry-dependent effects on the target species responses (Fig. 5b), confirming the above-described relationships between the proportions of organic C types in the litters and *Eucalyptus* and *Lepidium* germination and root growth over such materials.

Discussion

We assessed the allelopathic potential of unheated and heated litter, from both conspecific and heterospecific trees coexisting with *E. globulus* in the field. Burning, aside from altering litter molecular composition, generally affected the early stages of plant development on litter materials. The magnitude and direction of the effect decisively depended on the heating temperature, the litter species and the target-species sensitivity.

Burning effects on litter chemistry

Original litter materials were chemically diverse among species. Those differences were kept in litters that were moderately heated. Cluster analysis showed that interspecific differences in the chemical composition of litter clearly prevailed over temperature effects on litter chemistry at $T \leq 200$ °C. However, heat treatments above 200 °C homogenized the chemical composition of litter across species. In keeping with this, Gundale and DeLuca (2006) found that temperature has greater influence on chemical properties of severely burnt plant materials than do plant species. Therefore, the influence of heating temperature is more important than litter species, for determining the chemical composition of charred litter. Nevertheless, the opposite is true for non-charred litter.

The spectral regions of *O*-alkyl C, di-*O*-alkyl C, and alkyl C had a greater contribution to the molecular composition of unheated litters than the other regions. Firstly, *O*-alkyl C and di-*O*-alkyl C are related to polysaccharides (Kögel-Knabner 2002), such as cellulose and hemicelluloses (Freitas et al. 1999; Czimeczik et al. 2002). In fact, proximate cellulose content in litter samples ranged from 14.9% to 27.0%, and was consistently higher in samples with higher concentration of

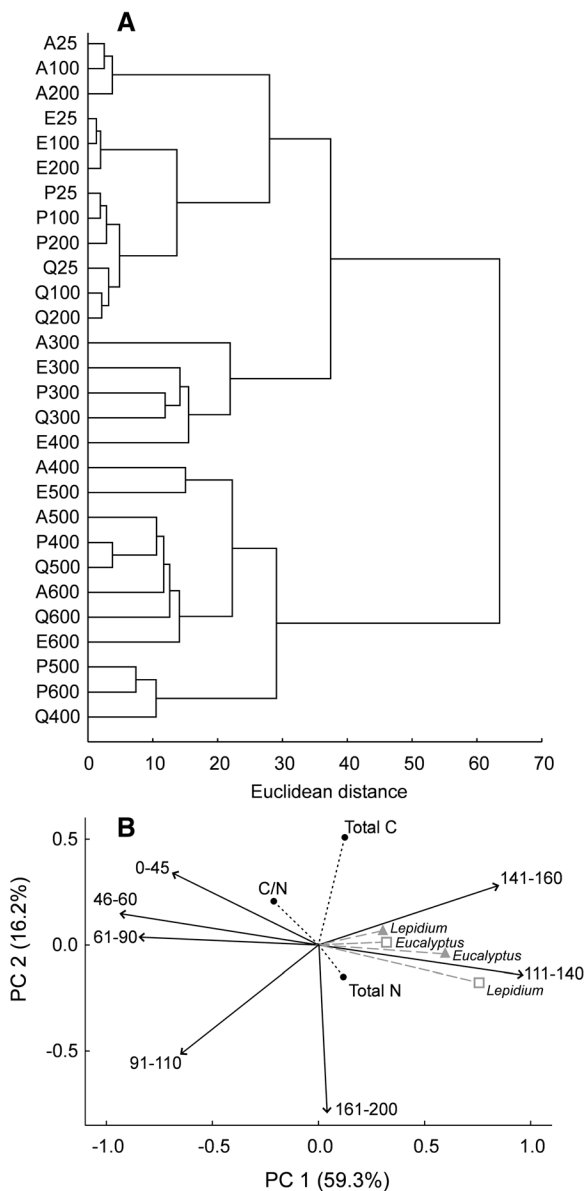


Fig. 5 **a** Dendrogram of litter materials, labelled by species initial (A, *Acacia dealbata*; E, *Eucalyptus globulus*; P, *Pinus pinaster*; and Q, *Quercus suber*) and temperature of heating treatment (25, 100, 200, 300, 400, 500, or 600 °C). **b** PCA ordination of the spectral regions in the litter materials. Data refer to loading vectors of the spectral regions (black arrows), labelled by chemical shift ranges (0–45 ppm: alkyl C; 46–60 ppm: methoxyl + *N*-alkyl C; 61–90 ppm: *O*-alkyl C; 91–110 ppm: di-*O*-alkyl C; 111–140 ppm: *H*- and *C*-substituted aromatic C; 141–160 ppm: *O,N*-substituted aromatic C; and 161–190 ppm: carbonyl C). Supplementary variables are also plotted, following Legendre and Legendre (1998): germination (grey dashed vectors with open squares); root growth (grey dashed vectors with solid triangles); C and N content, and C/N ratio in litter (dotted vectors)

corresponding C types. Secondly, as expected, the amount of alkyl C present in unheated litters was also high, corresponding to the spectral region centred at 30 ppm. This region is associated with: polymethylene C; cutin (Kögel-Knabner 2002); cutan (McKinney et al. 1996; Knicker et al. 2005); aminoacid residues and protein-like structures (Tinoco et al. 2004; Knicker et al. 2005); dehydroxylated lignin side-chains (Knicker et al. 2005); and probably with other not yet described aliphatic biomacromolecules. Usually, this signal presents high intensity in cutin- and wax-rich litter (Preston et al. 2009). Thus, peaks in this spectral region indicate a high content of waxes in *Eucalyptus* and *Acacia* samples, which ensure leaf surface hydrophobicity (Li et al. 1997; Neinhuis and Barthlott 1997). Regarding *Eucalyptus* samples, the peak could also indicate the existence of leaf essential oils such as cineole and eudesmol (Jacobs 1979). As for the *Acacia* samples, the peak likely corresponded to peptides, as this litter is very rich in nitrogen. Finally, it is worth mentioning that, although lignin shows relevant levels in litter samples (26.4–37.0%), it cannot be easily detected in ^{13}C NMR spectra. This difficulty arises from the fact that lignin carbon atoms resonate in several different spectral regions, overlapping with signals of other chemical compounds (Kögel-Knabner 2002).

According to ^{13}C NMR data, all litter samples were chemically stable when heated up to 200 °C. On the contrary, litter chemical changes were recorded at $T > 200$ °C, namely: the decrease of *O*-alkyl C and methoxyl + *N*-alkyl C, and the increase of *H*- and *C*-substituted aromatic C. These changes are consistent with recent observations on other litter species (Bonanomi et al. 2016) and are further supported by literature concerning burning effects on specific molecular classes. Indeed, the decomposition of lignin and hemicelluloses accelerates at 200 °C, and cellulose chemically dehydrates between 200 °C and 280 °C (Chandler et al. 1983). Organic matter charring starts above 200 °C (González-Pérez et al. 2004) and progressively alters *O*-alkyl structures forming aromatic compounds (Baldock and Smernik 2002; Czimeczik et al. 2002; Certini 2005), which replace carbohydrates in the range of 250 °C to 310 °C (Knicker 2007), while most destructive distillation occurs between 280 °C and 320 °C (Chandler et al. 1983). Temperatures exceeding 300 °C cause further structural changes, promoting an additional increase of the aromatic fraction by: dehydration; dealkylation; decarboxylation of six- and five-

membered rings, alkyl chains and aminoacids; and, to a lesser extent, selective persistence of heat-resistant aromatics (Freitas et al. 1999; González-Pérez et al. 2004; Knicker et al. 2005). In particular, signals of mono- and polycyclic aromatic hydrocarbons appear between 300 °C and 600 °C, while the methoxyl C signal of lignin disappears at $T \geq 400$ °C (Knicker 2007). Notably, the chemical shifts that occurred in the litter materials, between 200 °C and 400 °C, corresponded to clearly observable variations in litter colour, ranging from brown to black shades, as heating temperature increased. Therefore, litter composition changes dramatically, when heated between 200 °C and 400 °C.

Alkyl C showed a unimodal pattern along the temperature gradient, with the maximum at 300 °C followed by an abrupt depletion. The spectral peak may have emerged from several concomitant processes. For example, thermal distillation is known to release lipidic compounds, at early heating stages of litter (González-Pérez et al. 2004), and lead to the accumulation of additional aliphatic C above 270 °C (Knicker 2007). Long-chain residual paraffins and methyl, methylene or alicyclic structures can remain embedded in the matrix of newly-formed aromatics, while alkyl groups can be subsequently removed by distillation (Almendros et al. 2003). In addition, during browning, the sugars react with proteins to produce melanoidins and other Maillard-type substances (Brands 2002). A further decrease of alkyl C, at $T > 300$ °C, can be explained by progressive polymerization, demethylation and volatilization of hydrocarbons and hydrophobic products (Savage 1974; Freitas et al. 1999). Interestingly, the temperature-dependent pattern observed for alkyl C is consistent with water repellency changes, which are reported for experimentally heated soils (DeBano and Krammes 1966) and are often observed in burnt soils (DeBano 1981; Doerr et al. 2000).

Carbonyl C content showed species-specific changes at $T \geq 300$ °C. Different chemical compounds in the litter could contribute to this signal, namely: hemicelluloses; uronic acids; biopolyesters; wax esters, possessing free carboxylic or ester groups; and also lignoproteins and melanoidins, possessing amide groups. In such conditions, organic molecules can undergo decarboxylation (Freitas et al. 1999; González-Pérez et al. 2004), often leading to the loss of carboxyl C in charred litter (Bonanomi et al. 2016). This is consistent with our results for *Pinus* litter, but not for the remaining litter species, nor for all pooled data. These

results might be explained by species-specific net effects, emerging from the balance of several chemical transformations related to litter charring. For instance, carboxyl C may persist at high temperatures, bonded to aromatic rings which are formed after the breakdown of aliphatic and polycyclic structures (Almendros et al. 2003; González-Pérez et al. 2004). This is confirmed by the relatively high amount of carboxyl C in the soluble fraction of char (Knicker 2007). Furthermore, the non-association of carbonyl C content variations with heating temperature, in *Q. suber* samples, is consistent with a recent report by Bonanomi et al. (2016) on *Quercus ilex*, an oak with leaf traits that are closely related. An increase of carbonyl C with temperature, as we observed for *Eucalyptus* litter, was also previously reported. Knicker et al. (2005) observed higher content of carboxyl C in burnt forest soils than in unburnt forest. As changes of carbonyl C content were very diverse in our litter samples at $T > 300$ °C, no generalizations can be inferred.

PCA results outlined the general chemical composition of litter samples and its changes with temperature quite well, though they concealed interspecific differences of litter composition. PCA highlighted two opposing groups of C types: substituted and unsubstituted alkyl C vs. substituted and unsubstituted aromatic C. The former dominated the composition of uncharred litters, while the latter dominated that of charred litters. Chemical processes responsible for this shift mainly occurred at temperatures between 200 °C and 400 °C.

Effects of litter chemistry on germination and root growth

The associations of germination and root growth with ^{13}C NMR data were qualitatively similar in the two target species. These species showed a consistent pattern of inhibition or stimulation. However, the magnitude of the effects was different in the two target species. In particular, both germination and root growth were negatively correlated with the litter content of *O*-alkyl C and methoxyl + *N*-alkyl C, and positively correlated with the content of aromatic C. These correlations confirm recent observations made by Bonanomi et al. (2016), and support an old idea by Johnson (1919), suggesting that the same property can negatively affect both germination and growth. This pattern may be explained by the phylogenetically widespread sensitivity to bioactive

combustion products (Nelson et al. 2012). Developmental mechanisms in angiosperms have emerged early in their evolution (Taiz and Zeiger 2002). Germination and early root growth, whose inhibition is often mutually associated (Evenari 1949), share similar basic processes of morphogenesis and size increase in higher plants. Cell division and inherently intense metabolic activity may be affected by known phytotoxins, targeting respiration, protein synthesis, and mitosis (Rice 1984). On the other hand, species-specific differences in the magnitude of litter effects clearly indicate target-species differ in their sensitivity to litter quality. This because *Lepidium* germination and *Eucalyptus* root growth were the most affected by the same interfering C types.

Finally, the species responses were not related with either litter total C or N contents or with C/N ratio. Available N in differently heated litters most likely represented different fractions of the total N because combustion has a mineralizing effect. Nevertheless, our results suggest that the early development was independent from external nutrient supply, being sustained by seed resources (Ingestad and Lund 1986).

Unheated litter and allelopathic interactions

Unheated litter had species-specific effects. Differences in molecular composition among the tested materials could contribute to explain these results.

Eucalyptus globulus litter prevented *Lepidium* germination and strongly inhibited conspecific root growth. Its allelopathic potential, as well as that of its congeners, has been known for a long time (del Moral and Muller 1969; May and Ash 1990; Espinosa-García 1996; Willis 1999). It is more intense in spring (Souto et al. 2001) and relies on several leaf compounds, such as: essential oils, mainly terpenes; phenolics; flavonoids; and ellagitannins (Hillis 1966; del Moral and Muller 1969; Rice 1984; Souto et al. 1994). Allelopathic effects of this species on germination and/or root growth were previously assessed using: different plant parts, either fresh or abscised; their leachates or macerates; throughfall; stemflow; litter; and beneath-canopy soils (del Moral and Muller 1969; May and Ash 1990; Molina et al. 1991; Souto et al. 2001). Consistent with our findings, these studies found that interspecific effects range from strong inhibition to neutral. Hence, phytotoxicity might contribute to explain the reduced biodiversity and the low densities of herbs in plantations of this species, observed by other authors outside its

native range (del Moral and Muller 1969; Basanta et al. 1989). Actually, phytotoxicity involving species from different biogeographical regions is an important mechanism to enhance the success of species outside their native ranges (novel weapons hypothesis) (Rabotnov 1974; Hierro et al. 2005). In addition, *Eucalyptus* autotoxic effects were also reported by del Moral and Muller (1969). Autotoxicity might also help to explain why *E. globulus* wildlings are usually absent or sparse in unburnt conspecific plantations (Larcombe et al. 2013; Fernandes et al. 2016; Águas et al. 2017). Furthermore, considering that other eucalypt species have autotoxic potential (Willis 1999), it is likely that similar phenomena happen in unburnt natural forests, where the failure of natural regeneration is apparently related to litter presence (Mount 1964). Autotoxicity has ecological significance in the self-regulation of populations in space and time, inhibiting plant development where and when conspecific plants coexist (Singh et al. 1999). Therefore, *Eucalyptus* populations might be self-controlled through allelopathy.

Quercus suber litter prevented *Lepidium* germination and inhibited *Eucalyptus* development, affecting the germination of the latter more than its root growth. Previous tests on *Lactuca sativa* reported low phytotoxic potential on both seeds (Gonçalves et al. 2008) and roots (Domínguez 1994). Nevertheless, *Q. suber* contains significant amounts of allelochemicals in phellogen, cork and leaves, including: flavonoids; gallic; ellagic, *p*-coumaric, caffeic, and ferulic acids; and juglone (Carvalho 1992). Therefore, species-specific sensitivity of plants to different allelochemicals might explain our results on different target species, as well as differences between our results and those from the aforementioned studies. Meiners et al. (2012) postulate that interspecific differences in sensitivity to phytotoxic substances might result from selective pressures. Indeed, they are common, even among standard target species or related species (Perez 1990; Haugland and Brandsaeter 1996; Macías et al. 2000). In addition, differences in results among studies may also come from differences in the methods used in those studies (Haugland and Brandsaeter 1996). It is noteworthy that *Eucalyptus* germination was inhibited by *Quercus*. This result is consistent with field observations (pers. obs.) and supports the ‘homeland security hypothesis’ (Cummings et al. 2012). In fact, it is a very relevant result as it points to the potential resistance of native forests, as

Q. suber forests, to invasion by exotic species, as *E. globulus*.

Acacia dealbata litter showed phytotoxic effects on germination but apparently did not affect root growth. Its leaves contain at least three allelochemicals (resorcinol, moretenone, and maculosin, Aguilera et al. 2015). Interestingly, our sampling period is compatible with the seasonal peak of phytotoxicity previously observed in Spain (Carballeira and Reigosa 1999). Our results are similar to those from other studies, as both germination inhibition (Carballeira and Reigosa 1999; Lorenzo et al. 2008) and the neutral effects on root growth (Lorenzo et al. 2010) have already been observed in other species. The absence of inhibition on root growth could be related to short-lived phytotoxic compounds (up to few days; Rice 1984), which may have been lost through decomposition.

Although *P. pinaster* litter inhibited germination of *Lepidium*, but not *Eucalyptus*'s, it stimulated root growth of both target species. Essential oils of *P. pinaster* leaves are well-known weedicides. They have high contents of important phytotoxics, as α -pinene (31.4%) and β -pinene (4.2%), as well as the antifungal (*Z*)-caryophyllene (28.1%) (Amri et al. 2013). Furthermore, plantations of this species lay over soils rich in phenol derivatives, including vanillic, benzoic, salicylic, syringic, *p*-coumaric, and gentisic acids (Muscolo et al. 2005), which can act as plant development inhibitors (Evenari 1949; Rice 1984). Such soils also contain protocatechuic acid, which protects plants from fungal infection (Rice 1984). On the other hand, some allelochemicals are known for changing effect magnitude and direction along a gradient of concentration, being either inhibitory or stimulatory, at high or low concentrations, respectively (Evenari 1949; Leather and Einhellig 1988). Souto et al. (1994) observed an effect shift from inhibition to stimulation of plant development by *Pinus radiata* and *Acacia melanoxylon* litters, after 7 days of decomposition. Therefore, if the concentrations of phytotoxic substances decreased with incubation time due to litter decay, those substances could have firstly inhibited germination and then stimulated growth. Moreover, antifungal substances were probably more relevant for plant development as time went by. Notably, fungi were not observed in any assay with *Pinus* litter, however they were observed in several assays with unheated or slightly heated litters of either *Eucalyptus* or *Quercus*. Fungi control might have contributed for root growth on *Pinus*

litter, the strongest among the four litter species. These results are consistent with the occurrence of *E. globulus* in at least 13.7% of the forests dominated by *P. pinaster* in Portugal (AFN 2010), a country where such a species combination does not usually result from human decision.

Heated litter and allelopathic interactions

Interestingly, both target species had very poor performances on moderately heated materials, either at 100 °C or at 200 °C. Most of these performances were even poorer than those on the correspondent unheated litters. This result is consistent with those from previous studies. Several explanations were previously proposed for inhibition of plant development by soils heated at similar temperatures: an unknown water-soluble substance (Attiwill 1962; Wilkinson et al. 1993), ammonia (Johnson 1919), or manganese released during combustion (Chambers and Attiwill 1994). In our bioassays, the inhibition of germination and root growth, for both target species, was not due to ammonia (Britto and Kronzucker 2002) nor manganese (Reichman 2002) toxicity, as no other typical symptoms of such toxicities were present. Nevertheless, the apparent chemical stability of litter heated up to 200 °C, revealed by ¹³C NMR, may hide some physiological changes. For example, the break-up of biological tissues can start at temperatures between 40 °C and 70 °C (Knicker 2007), and leaf cells left intact by the grinding alone may then release their content. This release would include phytotoxic compounds, which would then increase their concentrations around both seeds and seedling roots. In line with this, Chou and Muller (1972) observed that phytotoxic substances from *Arctostaphylos glandulosa* became more leachable after litter heating up to 140 °C. Moreover, those concentration differences among aqueous extracts had a close correspondence to root growth inhibition in *Bromus rigidus* (Chou and Muller 1972). It is noteworthy that leaf litter of *A. glandulosa* shares several phenolic allelochemicals with the *Eucalyptus*, *Pinus*, and *Quercus* we have studied (Hillis 1966; del Moral and Muller 1969; Chou and Muller 1972; Carvalho 1992; Muscolo et al. 2005).

Remarkably, the strongest inhibition of *Eucalyptus* development was on conspecific litter heated at 100 °C. The specificity of these effects might indicate another underlying phenomenon, possibly related to the ability of self-DNA to inhibit plant development, similar to

what Mazzoleni et al. (2015) have found in bioassays with decomposed conspecific litter. Indeed, we can speculate that litter heating might cause an increased concentration of degraded fragments of DNA, which would be able to induce the observed self-inhibition. This hypothesis will require further investigation.

However, the inhibition of plant development completely disappeared at either $T \geq 200$ °C or $T \geq 300$ °C, and plant responses were much more similar across litter species at those heating temperatures than at lower ones. Our results are consistent with previous findings by Bonanomi et al. (2016) and Chou and Muller (1972), who observed that 200 °C is the temperature turning point above which litter heating results in the complete loss of root growth inhibition. Moreover, the disappearance of inhibitory capacity occurred across all litter species, somewhat in parallel with the deep chemical changes caused by severe heating in those litters.

The results, originating from our severely charred litter bioassays, generally suggest that molecular transformations have occurred at high temperatures, causing not only the disappearance or the neutralization of inhibitory compounds, but also the production of new stimulatory substances. Data from ^{13}C NMR summarized by PCA support such inference. In fact, charring of litters significantly increased concentrations of substances which were negatively correlated with plant development. This was evident for methoxyl + *N*-alkyl C (46–60 ppm) and even more so for O-alkyl C (61–90 ppm). Conversely, the same charring strongly increased aromatic C concentration (111–140 ppm) in litters, as revealed by a very sharp and typical peak at 128 ppm (Almendros et al. 2003; Knicker 2007). And this was precisely the C type that had the strongest positive correlation with plant development. Research from Bonanomi et al. (2016) seems to corroborate these ideas. In addition, Chou and Muller (1972) found that phytotoxic phenolics substantially reduce their concentrations in litter aqueous extracts, after heating litter at $T \approx 180$ °C, and completely disappear, when litter is heated at $T > 200$ °C. Therefore, our results and literature suggest three complementary hypotheses to explain the observed inhibition release.

First, bioactive compounds which existed in unheated litter have been affected by important physicochemical changes, which occurred in organic matter. Severe heating may have destroyed them (McPherson and Muller 1969, Chou and Muller 1972), or turned them insoluble, condensed, or chemically bonded to other macromolecules (Brodowski et al. 2005).

Second, as char is able to adsorb and deactivate phytotoxic compounds (Bonanomi et al. 2015), it might have enabled seed germination and seedling growth that otherwise would be inhibited (Wardle et al. 1998; Hille and den Ouden 2005). This way, combustion-derived inhibitory compounds, as phenolics (Campos et al. 2012; Nelson et al. 2012), might have been neutralized by char, which kept their concentration low in the bioassay.

Third, litter combustion produced new substances, as discussed before, and some of them might have stimulatory effects on plants. Residues of plant combustion can stimulate germination and seedling growth, as shown for shrub species of chaparral (Nelson et al. 2012). Few are known, as cyanohydrins and karrikins (Kochanek et al. 2016, Flematti et al. 2011, 2015), but there are others whose identity is still unknown (Downes et al. 2013, Baldos et al. 2015). Each of these substances is produced at a particular temperature range and becomes unstable when heated at a higher range (Flematti et al. 2015).

In addition, allelochemicals can interfere differently with the several components of plant development, because they have specific targets in plant physiology (Lotina-Hennsen et al. 2006). Similarly, interspecific differences exist in plant sensitivity to the various combustion-derived compounds (Downes et al. 2013, Baldos et al. 2015). These facts may explain why *Eucalyptus* germination was the only response variable that turned up at 200 °C and not at 300 °C. Therefore, plant development on heated litters is influenced by the balance between active stimulants and inhibitors that result from combustion (Baldos et al. 2015).

Bioassay results also suggest that heating litter, at $T \geq 300$ °C, substantially homogenized the litter-species effects on the early development of plants. The dramatic chemical changes shared by all litter species, between 200 °C and 400 °C, resulted in a chemical homogenisation across all litter species, as demonstrated by CA. Aromatic C (111–160 ppm) became absolutely dominant in all litters after charring. Heat directly homogenised chemical composition across litter species, as it promoted chemical reactions which destroyed or inactivated different substances and produced new ones. Char is a direct product of combustion and is very rich in aromatic C (Certini 2005), which positively correlated with plant development of both target species. Since char is able to sorb and inactivate bioactive compounds, it can make the several litter species

functionally similar, even if they contain different arrays of stimulants and inhibitors. Therefore, severe heating of different litter species nullifies many chemical differences among them, through both direct and indirect processes. As a consequence, their capacity to interfere with plant development becomes similar.

Our study bears great important and significant implications for fire ecology. *E. globulus* saplings frequently occur in burnt areas, as in the cases of forests dominated by *P. pinaster* or *E. globulus* or mixed forests of both species in Portugal (Águas et al. 2014). Similarly, saplings from many eucalypt species abound in burnt Australian forests, but are not nearly as prevalent in the unburnt forests (Mount 1964, 1969; Florence 1996). Our results suggest that the depletion or deactivation of chemical inhibitors caused by fire passage, as well as the production of stimulatory molecules by combustion, could play a role in *E. globulus* success in recently burnt areas, along with other fire-related factors reported in literature. Importantly, inhibition caused by *Quercus* litter, a species from *Eucalyptus* exotic range, was lost by severe combustion. This fact might ease the spread of *Eucalyptus* into forests dominated by *Quercus*.

Conclusion

Burning litter of tree species coexisting with *E. globulus* may affect the early development of this species. The potential effects are species-specific, ranging from prevention of seed germination to stimulation of seedling growth, and greatly depend on the severity of litter burning. Differences in plant responses are associated with litter molecular composition. In *E. globulus*, early root growth is more sensitive than germination to thermally induced changes of litter quality. Mild heating exacerbates the inhibition of plant development by litter, especially in the case of conspecific material. Conversely, severe litter burning results in the general disappearance of inhibition of both germination and root growth and, in some cases, in the appearance of stimulation of root growth. This effect shift corresponds to litter chemical transformations related to combustion, namely a decrease of *O*-alkyl C and methoxyl + *N*-alkyl C contents, along with an increase of aromatic C content. Severe heating of litter releases *E. globulus* germination and allows roots to grow longer in the resultant seedlings. Therefore, burning litter seems to improve recruitment and establishment of plants of this species. Hence,

a window of opportunity may open for (re)colonization of forests by this species right after fire. Since *E. globulus* is widely planted in fire-prone regions outside its native range, our results are extremely relevant for forest management and conservation.

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