Phytotoxicity, not nitrogen immobilization, explains plant litter inhibitory effects: evidence from solid-state $^{13}$C NMR spectroscopy

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Summary

- Litter decomposition provides nutrients that sustain ecosystem productivity, but litter may also hamper root proliferation. The objectives of this work were to assess the inhibitory effect of litter decomposition on seedling growth and root proliferation; to study the role of nutrient immobilization and phytotoxicity; and to characterize decomposing litter by $^{13}$C NMR spectroscopy.
- A litter-bag experiment was carried out for 180 d with 16 litter types. Litter inhibitory effects were assessed by two bioassays: seed germination and root proliferation bioassays. Activated carbon (C) and nutrient solutions were used to evaluate the effects of phytotoxic factors and nutrient immobilization.
- An inhibitory effect was found for all species in the early phase of decomposition, followed by a decrease over time. The addition of activated C to litter removed this inhibition. No evidence of nutrient immobilization was found in the analysis of nitrogen dynamics. NMR revealed consistent chemical changes during decomposition, with a decrease in $\text{O-alkyl}$ and an increase in alkyl and methoxyl C.
- Significant correlations were found among inhibitory effects, the litter decay rate and indices derived from NMR. The results show that it is possible to predict litter inhibitory effects across a range of litter types on the basis of their chemical composition.

Introduction

Ecosystems can be characterized by either open or relatively closed nutrient cycles, with elements being continuously lost and replenished in the former systems and recycled in the latter (Jordan, 1982), where organic matter is crucial to sustain the nutrition of primary producers (Brearley et al., 2003). In this context, factors affecting litter decomposition rates and related nutrient dynamics have been thoroughly investigated in relation to environmental conditions and litter chemical characteristics (Vitousek & Sanford, 1986; Attiwill & Adams, 1993). The accumulation of plant litter has an important role in affecting plant community structure. Several studies have demonstrated that dense litter layers can limit the diversity of productive habitats by inhibiting species establishment through a variety of mechanisms, such as shading (Facelli & Pickett, 1991), mechanical impediment (Wedin & Tilman, 1993) and release of allelopathic compounds during litter decomposition (Bonanomi et al., 2006).

In addition to the recognized role of litter as a nutrient source (Vitousek & Sanford, 1986), many experimental studies have reported that the addition of plant litter and other types of organic matter to the soil can inhibit plant growth. This has been observed in the laboratory (Hodge et al., 1998), in natural ecosystems (Meier et al., 2009), and in many farming systems (Putnam, 1994). In a recent review of agricultural applications of organic matter, Bonanomi et al. (2007) reported that soil amendment with plant residues inhibited plant growth in 119 of 1728 experiments (6.9%). In addition, it has been demonstrated that root colonization of plant litter can require several weeks for
herbaceous species in controlled conditions (c. 35 d for various grasses; reviewed by Hodge, 2004), but up to months for temperate forest trees under field conditions (Conn & Dighton, 2000). This evidence is in striking contrast to that obtained in other studies reporting faster root proliferation in soils enriched with either mineral nutrients (Jackson & Caldwell, 1989) or water (North & Nobel, 1998).

Traditionally, two mutually nonexclusive hypotheses have been proposed to explain the inhibitory effect of plant litter on whole plant growth and root proliferation: nitrogen (N) immobilization by microbial competition (Michelsen et al., 1995) and the release of phytotoxic compounds from decomposing plant litter and microbes (Blum et al., 1999; Inderjit, 2005). First, when N is available at low concentrations, as in decaying plant tissues with a high carbon (C) : N ratio, saprophytic microbes compete with plants for this limiting resource (Hodge et al., 2000). Thus, nutrient immobilization can frequently occur in the early phases of litter decomposition for values of the organic matter C : N ratio > 30, while values < 30 usually correspond to good conditions of plant nutrition as a result of continuous N release during decomposition (Hodge et al., 2000; Bonanomi et al., 2010).

Experimental studies reporting inhibition of either seed germination or growth by litter extracts under laboratory conditions are very common (for reviews, see Patrick, 1971; Rice, 1984; Singh et al., 1999). A recent paper (Bonanomi et al., 2006) demonstrated that the inhibitory effect of plant litter is not limited to a few ‘allelopathic’ species but is rather widespread across different plant functional groups, with the highest phytotoxicity shown by N fixer species, followed by forbs and woody species, and with limited phytotoxicity shown by grasses and sedges. The same study showed that the inhibitory effect consistently changed with decomposition, with inhibition being greatest in the early stages of decomposition and then declining in aerobic conditions. Stimulation of growth may occur in the later stages of decomposition (Bonanomi et al., 2006). Allelopathy is one possible explanation for deleterious effects of plant litter, but a major criticism of this hypothesis is that, despite the widespread presence of inhibitory allelopathic compounds in most plant residues (Rice, 1984), these compounds are rapidly degraded by soil microbial activity into nontoxic molecules (Schmidt & Lipson, 2004; Kaur et al., 2009). However, the inhibitory effects of litter can last from a few days to weeks in laboratory conditions (Xuan et al., 2005; Bonanomi et al., 2006), although early experiments have been criticized because they were often carried out under unrealistic laboratory conditions (e.g. powdered litter, decomposition in water suspension conditions, or litter extracts obtained with organic solvent) that make comparisons with field observations difficult (Inderjit & Callaway, 2003).

A further concern about the allelopathic hypothesis is related to the identification of the phytotoxic compounds. Hundreds of phytotoxic organic molecules have been identified and quantified in plant litter, including short-chain organic acids (Armstrong & Armstrong, 1999), tannins (Kraus et al., 2003) and phenols (Blum et al., 1999), among many others. However, single molecules alone hardly explain the entirety of the inhibitory effect of plant litter during the decomposition process (An et al., 2001). For this reason, instead of focusing on specific organic compounds, we investigated the changes in the chemical composition of different organic litter types by 13C cross-polarization magic angle spinning (CPMAS) nuclear magnetic resonance (NMR) (Kögel-Knabner, 2002). This method, traditionally used in metabolomic studies, is very useful because it does not rely on extraction and separation of the analytes, enabling the detailed characterization of complex mixtures, such as soil organic matter and litter samples (Almendros et al., 2000; Preston et al., 2009; Ono et al., 2011). The method allows the resonance signals of all the carbons of the analyzed samples to be obtained by CPMAS. The chemical shifts of different C atoms are dependent on their molecular environment, thus providing important information about their chemical type and the nature and number of substituents, which allows the attribution of observed carbons to a particular class of organic compounds.

To address the relative roles of phytotoxicity and N immobilization in litter inhibitory effects, a litter-bag decomposition experiment was performed in which activated carbon (AC) was used to separate nutrient immobilization from phytotoxic effects. AC has been widely applied in allelopathic studies to adsorb and neutralize phytotoxic organic molecules while having a limited impact on mineral nutrients (Mahall & Callaway, 1992; Nilsson, 1994; Hille & den Ouden, 2005). The specific objectives of the study were to describe the dynamics of the inhibitory effect of litter leachates during the decomposition process; to assess the roles of nutrient immobilization and phytotoxic compounds in litter inhibitory effects; and to analyze the chemical composition dynamics related to the litter inhibitory effect by 13C-CPMAS NMR.

Materials and Methods

Collection of plant material

Sixteen different plant species were selected from vegetation types of both Mediterranean and temperate environments located in the Campania Region, southern Italy. The species pool, representing a wide range of litter quality (see the section ‘Litter chemical analyses and 13C-CPMAS NMR characterization’), included: two perennial grasses (Ampelodesmos mauritanicus (Poir.) T. Durand & Schinz, and Festuca drymeia M. et K.), one forb (Medicago sativa L.),
two evergreen shrubs (*Arbutus unedo* L. and *Coronilla emerus*), one vine (*Hedera helix* L.), three evergreen trees (*Picea excelsa* L., *Pinus halepensis* Mill. and *Quercus ilex* L.), and seven deciduous trees (*Castanea sativa* Mill., *Fagus sylvatica* L., *Fraxinus ornus* L., *Populus nigra* L., *Quercus pubescens* Willd., *Robinia pseudoacacia* L. and *Salix alba* L.). Three of these species are N-fixing (*C. emerus*, *M. sativa* and *R. pseudoacacia*) and are known to have a strong inhibitory effect (Bonanomi *et al.*, 2006). Freshly abscised leaves were collected by placing nets under plants (>20 randomly selected individuals for each species), dried at 40°C until a constant weight was reached and then stored at room temperature.

**Litter decomposition experiment**

Decomposition experiments were carried out in microcosms placed in a growth chamber to simulate field decomposition conditions. Litter decomposition in open fields mainly depends on organic matter quality, water availability and temperature (Gholz *et al.*, 2000). Therefore, to analyze only the effect of litter quality, we worked under controlled conditions, thus avoiding variations caused by changes in water availability and temperature.

Experiments were carried out using litter-bags (Berg & McClaugherty, 2008). Large (20 × 20 cm) terylene litter-bags (mesh size 2 mm) were filled with 6 g of dry leaf litter and placed inside square trays (30 cm deep; 100 cm wide). A microbial inoculum, obtained by mixing 10 g of soil taken from the fields from which litter was collected (top 10 cm) and 90 g of water, was added in order to improve the start up of the decomposition process. The microbial inoculum was sprayed over the litter-bag. Microcosms were kept in a growth chamber under controlled temperature (18 ± 2°C: 24 ± 2°C, night: day) and irrigation (watered every 7 d to holding capacity with distilled water) conditions. The litter water-holding capacity was determined by soaking 5 g of litter in distilled water for 24 h, shaking off excess water, weighing the saturated material, drying in an oven to a constant weight and reweighing.

Litter-bags were harvested after 30, 90 and 180 d of decomposition for a total of 384 litter-bags (16 species × 3 sampling dates × 8 replicates). Bags were dried in the laboratory (40°C until a constant weight was reached) and the remaining material was weighed. The litter negative exponential decay constant (k) was calculated according to Berg & McClaugherty (2008) as follows:

\[ M_t = M_0 e^{-kt} \]  
(Eqn 1)

\( M_0 \) the initial litter mass; \( M_t \) the mass remaining after a certain time \( t \); \( k \), the decay rate constant.) For each plant species, litter decay rate (k) was calculated for three decomposition periods (0–30, 30–90 and 90–180 d).

Litter inhibitory effect during decomposition

The first bioassay, hereafter called the ‘seed germination’ experiment, was based on *Lepidium sativum* as a target species because of its recognized sensitivity to phytotoxic compounds (Bonanomi *et al.*, 2006). The use of one test plant has the advantage of standardizing the results for different litter types.

The ‘seed germination’ bioassay, described by Bonanomi *et al.* (2006), was carried out to assess how decomposition influences the inhibitory effects of different plant litter types. Twenty seeds were placed in 9-cm Petri dishes over sterile filter papers with 4 ml of test solution. To obtain the water extract for the bioassay, dried litter material was mixed with distilled water in a beaker at 5% of dry weight (50 g l\(^{-1}\)) and shaken for 5 h. The aqueous suspensions were then centrifuged (2395 g for 10 min), sterilized (microfiltration with a 0.22-μm pore filter), diluted with distilled water to three concentrations (50, 17 and 5 g l\(^{-1}\)) and stored at −20°C until used in the bioassay.

Every solution plus the control with distilled water was replicated five times for a total of 1200 seeds for each plant litter species. Petri dishes were arranged in a growing room in a completely randomized design and seedling root length was measured 36 h after germination. A total of 20 800 seedlings were measured for all the experiments. Data were expressed as inhibition of root growth (i.e. per cent difference in root length) compared with the control.

**Nutrient immobilization and phytotoxicity**

The second bioassay, referred to as the ‘root proliferation’ experiment, was conducted in root observation chambers (Mahall & Callaway, 1992) with the aim of determining the capability of *L. sativum* seedling roots to colonize the different litter types independently from the germination process. A further aim was to assess the cause of growth inhibition. We applied a mineral nutrient solution and/or AC to assess the roles of both nutrient immobilization and phytotoxic effects.

A 2-cm-wide sterile filter paper strip was placed immediately above a wider (5-cm) strip in square Petri dishes (size 12 × 12 × 1.5 cm). The two strips were separated by 5 mm of free space. *Lepidium sativum* seeds (five in each dish) were placed over the 2-cm strip, and the different nutrient solutions, AC and litter were applied to the lower 5-cm-wide strip (Supporting Information Fig. S1). This set-up was used to prevent these materials from coming into contact with the seeds during germination. Petri dishes were oriented at a 45° slope on a horizontal surface so that the positive geotropism of roots would allow growth down along the plate. Plates were covered with opaque sheets when roots were not under observation.

The experimental design included four treatments: the control, with sterile distilled water; the addition of a...
complete nutrient solution (half strength (2.15 g l\(^{-1}\)) Murashige and Skoog Basal Salt Mixture; Sigma-Aldrich Co.), to assess whether the inhibitory effect was attributable to nutrient immobilization; the addition of AC (Sigma-Aldrich Co.) at 0.2 g per dish, to assess whether the inhibitory effect was attributable to phytotoxic compounds; and the combined application of the nutrient solution and AC. These four treatments were applied without (controls) and with litter on the lower strip (Fig. S1). The upper strip with the seeds was treated only with distilled water. Dry, powdered litter (separately for each of the 16 species at four decomposition stages, for a total of 64 samples) was applied at two rates (0.2–0.02 g per dish) to the lower strip. Experimental values of litter addition were consistent with the levels of litterfall observed in natural ecosystems (Vogt et al., 1986). Each treatment was replicated six times. Petri dishes were arranged in a growing room according to a totally randomized design and seedling shoot and root lengths were measured 10 d after germination. Data were expressed as root and shoot growth (i.e. per cent difference in root length) compared with the control. The seedling shoot : root ratio was calculated based on shoot and root length measurements.

Litter chemical analyses and \(^{13}\)C-CPMAS NMR characterization

Litter total C and N contents were determined by flash combustion of microsamples (5 mg of litter) in an Elemental Analyser NA 1500 (Carlo Erba Strumentazione, Milan, Italy).

Litter types from the litter-bag experiment were characterized by \(^{13}\)C-CPMAS NMR in solid state under the same conditions in order to allow a quantitative comparison to be performed among spectra. Samples were analyzed at four different times, corresponding to leaf decomposition for 0, 30, 90 and 180 d (total of 64 samples). The spectrometer used was a Bruker AV-300 (Bruker Instrumental Inc, Billerica, MA, USA) equipped with a 4 mm wide-bore MAS (magic angle spinning) probe. 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 (Wilmad/Lab Glass, Buena, NJ, USA). The pulse sequence was applied with a \(^1\)H ramp to account for nonhomogeneity of the Hartmann–Hahn condition at high spin rotor rates. Each \(^{13}\)C-CPMAS NMR spectrum was automatically integrated to calculate the area of the peaks which appeared in the chosen region. Spectral regions have been selected and C-types identified in previous reference studies (Lorenz et al., 2000; Kögel-Knabner, 2002; Mathers et al., 2007; Pane et al., 2011: 0–45 ppm = alkyl C; 46–60 ppm = methoxyl and 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-substituted aromatic C (phenolic and O-aryl C); 161–190 ppm = carboxyl C). The degree of hydrophobicity (hydrophobic (HB) : hydrophilic (HH)), the alkyl C : O-alkyl C ratio (0–45 : 61–110) and the O-alkyl C : methoxyl and N-alkyl C ratio (61–90 : 46–60; thereafter termed CC : MC), which are considered robust indicators of the degree of litter decomposition, were calculated following Spaccini et al. (2000), Almendros et al. (2000) and Mathers et al. (2007). Moreover, a new index was calculated as the ratio between two restricted regions of O-alkyl C and methoxyl and N alky C (i.e. 70–75 : 52–57). Major C-type contributions to region 70–75 derive from the C2, C3, and C5 of carbohydrates, although it also comprises a side-chain of phenylpropanoids (lignin, cutin, and condensed tannins). The 52–57 region mainly corresponds to the methoxyl C of lignin, but its signal could also arise from the C2 of amino acids.

Data analysis

Generalized linear models (GLMs) were used to analyze the results of the seed germination and root proliferation bioassays. Main and second-order interactive effects of litter type (16 species), extract concentration (three concentrations) and decomposition time (treated as a continuous variable) on L. sativum root elongation were determined in the seed germination experiment. In the case of the root proliferation bioassay, further GLMs were applied to test the effects of litter type (16 species), decomposition time (continuous variable), and the application of nutrient solution (two levels) and AC (two levels) on root and shoot growth of L. sativum. Litter mass loss and changes in N content were statistically evaluated by one-way ANOVA. Decomposition data were arc-sine transformed to satisfy the assumption of normality. Significance was evaluated in all cases at \(P < 0.05\) and 0.01. To address the relationship between the inhibitory effect and litter characteristics (i.e. decay rate, N content, N release, C : N ratio, and \(^{13}\)C-CPMAS NMR spectra), three approaches were used. (1) A simple correlation analysis was used to identify parameters and regions of the \(^{13}\)C-CPMAS NMR spectra that were significantly related (either positively or negatively) to the litter inhibitory effect. This was achieved by testing either two sets of \(^{13}\)C-CPMAS NMR spectral regions selected from the literature (Almendros et al., 2000; Spaccini et al., 2000; Kögel-Knabner, 2002) or clusters of consecutive signals along the spectrum showing a significant linear correlation with the litter inhibitory effect. (2) A linear regression analysis was used to determine the association between the litter inhibitory effect and indexes based on the spectral regions identified in analysis (1). (3) A principal component regression (PCR), a multiple linear regression in which principal component analysis (PCA) is used to calculate independent variables (Jolliffe, 2002) to avoid multicollinearity (i.e. a significant correlation between
two or more predictor variables in a multiregressive model), was performed.

About the analysis described in point (3), two models were tested, in which the previously identified spectral regions were considered as predictive variables for litter inhibitory effects. Independent linear combinations of the predictors were calculated for each model by principal component ordination of a matrix of the 64 litter types and the relative abundances of the spectral regions. For each PCA axis, the correlation with the litter inhibitory effect was calculated using the factorial scores and root growth in the litter samples. Following the approach suggested by Legendre & Legendre (1998) for supplementary variables, root growth was also plotted as a loading vector on the bi-dimensional space even if it was not used to compute the eigenvalues of the same ordination space. Finally, the four orthogonal PCA axes most correlated with litter inhibitory effects were used as independent predictive variables in multiple regression models.

After linear regression analysis of the whole NMR spectra, a simple empirical index was calculated as the direct correlation with the litter inhibitory effect. Independent linear combinations of the predictors, whereas litter decomposition time was considered as an independent continuous variable.

Results

Litter inhibitory effect during decomposition

In the seed germination bioassay, litter extracts had a significant effect on the growth of L. sativum (Table 1) and higher extract concentrations of litter from all species increased the inhibition of root growth (Figs 1, S2). Undecomposed litter of all species showed an inhibitory effect, which was close to 100% at the highest concentrations (Figs 1, S2). However, all litter types showed a significant and rapid decrease in their inhibitory effect during decomposition, with a sharp reduction in the first 30 d of decomposition (Figs 1, S2). After 180 d of decomposition, the inhibitory effect disappeared at the lower extract concentrations, while a slight but significant inhibition still could be detected at the highest concentration (Figs 1, S2). The progressive reduction in the inhibitory effect was rapid for some species (e.g. A. unedo, F. drymeia and F. sylvatica) and slow for others (e.g. C. emerus, H. helix and P. halepensis), as indicated by the highly significant interaction between litter type and decomposition time (Table 1).

Nutrient immobilization and phytotoxicity

In the root proliferation bioassay, the effect of litter on root and shoot growth of L. sativum was significantly affected by decomposition time, extract concentration and the addition of AC (Table 1; Fig. 2). In the GLM, the application of the mineral solution had no significant effect (Table 1). In the control without litter, the addition of AC (treatment AC+...
N+) significantly increased (+21%) root growth compared with the water control (treatment AC– N–), whereas the addition of mineral solution and the combined application of AC and nutrients had no significant effect.

All undecomposed litter inhibited L. sativum root and shoot growth, although the effect was much more severe on root growth (Figs 2, S3). However, the inhibitory effect of litter disappeared after 30 d of decomposition. In contrast, root growth after the application of more decomposed (30, 90 and 180 d) litter was significantly higher compared with the water control (Fig. 2). The stimulating effect of litter was most pronounced for P. excelsa and A. mauritanicus (Fig. S3). Application of the mineral solution to the undecomposed litter plates had no significant effects on root and shoot growth (Fig. 2). In contrast, the inhibitory effect of undecomposed litter was substantially relieved when AC was added to the medium at a level comparable to that of the water control (Figs 2, S3). However, the application of AC had the opposite effect on more decomposed litter (30, 90 and 180 d), as indicated by the significant interaction between decomposition time and AC application (Table 1).

In these cases, root growth was reduced to values similar to those observed for the water control (Fig. 2). The combined application of mineral solution and AC did not show any interactive effect, with root growth similar to that observed for AC alone (Fig. 2). The shoot : root ratio was significantly affected by litter and AC applications. Shoot : root values increased with the application of undecomposed litter and decreased, to levels comparable to those of the control, after the application of AC (Fig. 2).

Litter decomposition and associated chemical changes

The litter mass loss of all species displayed a negative exponential relationship with decomposition time (Fig. S4). However, the litter decay rate varied greatly among the 16 species, being very rapid for C. emerus, E. helix and F. ornus, and relatively slow for P. excelsa, Q. ilex and F. sylvatica, while the remaining species showed intermediate values (Fig. S4). Initial litter C : N ratios also varied greatly among plant species, ranging from 40 (A. mauritanicus) to 12 (M. sativa), with a mean (± standard error (SE)) of 23.4 ± 7.0 (Table S1). The C : N ratios significantly decreased for all species (ANOVA; P < 0.05) during the decomposition process, reaching a mean (± SE) of 13.2 ± 2.3 after 180 d (Table S1). The amount of N retained in litter during decomposition changed in relation to the plant species (Fig. S5). Two species (S. alba and P. excelsa) did not release N during the first 30 d of decomposition, and only a limited release (> 90% of the initial N amount was retained) was recorded for Q. ilex, P. alba and M. sativa. In contrast, in the first 30 d of decomposition, three species (C. emerus, E. helix and M. sativa) retained < 40% of the initial N (Fig. S5). As decomposition proceeded, the amount of N retained decreased for all litter types, ranging from 81% for Q. ilex to 8% for C. emerus after 180 d (Fig. S5).

The 13C-CPMAS NMR spectra revealed significant and consistent changes in litter C types for all the species, and the major chemical changes occurred during the first 30 d of decomposition (Figs 3, S6; Table S2). The O-alkyl-C region (61–90 ppm), which is mainly associated with sugars and polysaccharides, decreased greatly in the first 30 d of decomposition and then showed a less marked but continuous reduction (Figs 3, S6; Table S2). The aliphatic alkyl-C region (0–45 ppm; characteristic of lipid waxes, cutins and microbial products) increased sharply in the first 30 d of decomposition and slowly in the later stages of decomposition. Both the reduction in the O-alkyl-C region and the increase in the aliphatic alkyl-C region were more pronounced for rapidly decomposing litter types (e.g. H. helix, C. emerus and F. ornus) than for slowly decomposing...
As the data for chemical quality relationships between inhibitory effects and litter decomposition in controlled conditions. Values are the average ± SD of 16 different species. CC/MC and HB/HF indicate the carbohydrate C : methoxyl C ratio and the degree of hydrophobicity, respectively.

Several highly significant correlations were found between inhibitory characteristics and root growth inhibition. The strongest positive correlation occurred with litter decay rate; that is, the faster the litter decomposition, the more inhibitory was its effect (Fig. 4). The C : N ratio and the amount of N released during decomposition showed weak but statistically significant positive correlations with root inhibition (Table 2). In this regard, we found that the C : N ratio and the amount of N released during decomposition were negatively correlated only during the first 30 d of decomposition ($P = 0.006; \ r = -0.66$).

Concerning the results obtained in $^{13}$C-CPMAS NMR analyses, most of the spectral regions and indexes derived from the literature were not significantly or only weakly correlated with root growth inhibition (Table 2). However, the O-alkyl C region and CC : MC ratio were positively correlated with inhibitory effects, while methoxyl C and the hydrophobic ratio were significantly negatively correlated with inhibitory effects. To search for indexes of litter quality more predictive of the inhibitory effect, all regions of the $^{13}$C-CPMAS NMR spectra were extensively tested. Two restricted bands of O-alkyl C (70–75 ppm) and methoxyl C (52–57 ppm; Table S2) were slightly more predictive than the corresponding wide regions, showing positive and negative significant correlations with root growth inhibition, respectively (Table 2). Based on these selected regions, a simple empirical index was calculated as the ratio 70–75 : 52–57, providing the maximum correlation with root growth inhibition (Fig. 4; Tables 2, S2). This index showed a sharp decline during litter decomposition for all plant species (Fig. 3).

PCA provided a satisfactory ordination of the $^{13}$C-CPMAS NMR data across litter types, both for reference regions derived from the literature (Almendros et al., 2000; Spaccini et al., 2000; Ono et al., 2007), and then showed a further progressive reduction (Fig. 3). Finally, the CC : MC ratio rapidly decreased in the first 30 d, consistent with previous results (Mathers et al., 2007), and then showed a further progressive reduction (Fig. 3). The CC : MC ratio steadily declined with advancing decomposition, because the relative amounts of their components (i.e. carbohydrate C and methoxyl C) decreased and increased, respectively.

Relationships between inhibitory effects and litter chemical quality

As the data for L. sativum root growth inhibition assessed using the two bioassays were highly correlated ($r = 0.86; \ P < 0.001$), in order to discriminate among litter types we used only data from the seed germination experiment, which was more sensitive than the root proliferation experiment.
Spaccini et al., 2000; Kögel-Knabner, 2002) and for sets of consecutive signals statistically selected (Figs 4, 5; Table S3), with the first four eigenvalues accounting for 98.3% (52.1, 26.7, 12.1, and 7.4%) and 90.6% (44.8, 31.6, 9.1, and 5.1%) of the total variance, respectively. In Fig. 5 are reported the loading vectors (i.e. relative abundance of each $^{13}$C-NMR region measured on each sample and how they relate to the PC axes), and the factorial scores of the 64 litter samples on the bi-dimensional space. The first two components show the individual litter sample spreading accordingly to the NMR spectral regions during the decomposition process, and the related trajectories of the different species in the multivariate ordination space. The multiple regression analysis based on the results of PCA, the aim of which was to assess the overall predictivity of $^{13}$C-CPMAS NMR data for litter inhibitory effects, provided two highly significant models for root growth inhibition of $L$. sativum (Table 3; Fig. 4). In both models, the relative contributions of three out of the four considered predictors were statistically significant (Table 3). The intercept term of the models was also significant, thus indicating a residual variability of the litter inhibitory effects not explained by the $^{13}$C-CPMAS NMR spectra. Both models, based on a training subset of data for 10 species, were significantly predictive once applied to a validation data set of a further six species in terms of both fitting (multiple $r$ = 0.69 and 0.77 for models 1 and 2, respectively) and predictivity ($r$ of predicted vs observed values 0.66 and 0.68, respectively) for root growth inhibition (Fig. 4).

Discussion

In this work, we found that all litter caused a strong inhibition of $L$. sativum root growth, thus confirming the findings of a previous study (Bonanomi et al., 2006) which reported inhibitory effects of undecomposed litter from 22 species out of 25 tested. Thus, the inhibitory effect of undecomposed litter, at least for Mediterranean and temperate species, seems to be a rather general phenomenon not restricted to a few ‘allelopathic’ plants. Further studies are needed to extend these results to other ecosystems (e.g. grassland and tropical forest).

![Graphs](image-url)
The initial phase of decomposition basically consists of plant tissue breakdown and subsequent release of cell contents. At this stage, the inhibitory effect showed a certain amount of variability, being high for some N-fixing species such as *C. emerus* and *M. sativa*, but also for *H. helix* and *P. excelsa*, and low for others (e.g. *Q. ilex*, *Q. pubescens* and *F. sylvatica*). The inhibitory effect rapidly decreased during the first 30 d of decomposition for all tested litter types. However, it is interesting to note that the litter of several species strongly promoted root proliferation after 30, 90 and 180 d of decomposition. A quantitative assessment of the inhibition ‘window’, that is, the temporal phase in which litter has an inhibitory effect (Bonanomi et al., 2006), could be based on the observation of root capability to proliferate into the litter. In fact, after 30 d of decomposition, all litter types were readily colonized by the *L. sativum* roots. The observed time span of the inhibitory

<table>
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<tr>
<th>Elemental chemical parameters</th>
<th>Pearson coefficient and P-value (n = 64)</th>
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<tbody>
<tr>
<td>C : N ratio</td>
<td>+0.53 (P &lt; 0.001)</td>
</tr>
<tr>
<td>N concentration (g g⁻¹)</td>
<td>−0.13 (P = 0.28)</td>
</tr>
<tr>
<td>N release (g g⁻¹ d⁻¹)</td>
<td>+0.39 (P = 0.005)</td>
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**13C-CPMAS NMR-derived chemical parameters**

- Carboxylic C: 161–190 ppm: −0.15 (P = 0.24)
- O-substituted aromatic: +0.02 (P = 0.86)
- C: 141–160 ppm: −0.14 (P = 0.23)
- H-C-substituted aromatic: −0.14 (P = 0.23)
- C: 111–140 ppm:
  - di-O-alkyl: 91–110 ppm: +0.29 (P = 0.001)
  - O-alkyl C: 61–90 ppm: +0.44 (P < 0.001)
  - Methoxyl C: 46–60 ppm: −0.60 (P < 0.001)
  - Alkyl C: 0–45 ppm: −0.29 (P = 0.03)
  - Hydrophobic ratio: HB : HI: −0.38 (P = 0.002)
  - Alkyl C/O-alkyl C: −0.25 (P = 0.04)
  - CC/MC: +0.61 (P < 0.001)
  - Region between 70 and 75 ppm: +0.53 (P < 0.001)
  - Region between 52 and 57 ppm: −0.61 (P < 0.001)
  - 70–75/52–57 ppm: +0.71 (P < 0.001)

Values in bold are significant at P < 0.01.

*Calculated between measured root inhibition and N release or litter decay rate in the following decomposition phase.

**Region between 70 and 75 ppm**

**Region between 52 and 57 ppm**

**70–75/52–57 ppm**

The initial phase of decomposition basically consists of plant tissue breakdown and subsequent release of cell contents. At this stage, the inhibitory effect showed a certain amount of variability, being high for some N-fixing species such as *C. emerus* and *M. sativa*, but also for *H. helix* and *P. excelsa*, and low for others (e.g. *Q. ilex*, *Q. pubescens* and *F. sylvatica*). The inhibitory effect rapidly decreased during the first 30 d of decomposition for all tested litter types. However, it is interesting to note that the litter of several species strongly promoted root proliferation after 30, 90 and 180 d of decomposition. A quantitative assessment of the inhibition ‘window’, that is, the temporal phase in which litter has an inhibitory effect (Bonanomi et al., 2006), could be based on the observation of root capability

**Fig. 5** Principal component analysis (PCA) ordination of eight selected *13C NMR spectral regions in 64 litter samples (16 plant species at four stages of decomposition) tested for inhibition of root growth of *Lepidium sativum*. (a) Loading vectors of NMR regions (labelled by ppm ranges). Root growth is also plotted as a supplementary variable following Legendre & Legendre (1998). (b) Factorial scores of litter samples represented according to decomposition time (closed circles, undecomposed material; open circles, 30 d; squares, 90 d; triangles, 180 d). (c) Decomposition trajectories of plant litter between 0 and 180 d.
The inhibitory effect of plant litter has been attributed to litter N immobilization (Hodge et al., 2000) or, alternatively, to the phytotoxic activity of an allelopathic factor(s) (Rice, 1984). In this work, the almost complete recovery after AC addition of plant growth indirectly supports the allelopathic hypothesis. The recovery of root proliferation was almost complete for all species, with the sole exception of *M. sativa* (Fig. S3), a species reported to be very phytotoxic when undecomposed (Miller, 1996). Previous investigations on the inhibitory effect mechanisms of plant litter have been carried out using AC (Nilsson, 1994). For instance, Hille & den Ouden (2005) demonstrated that both AC and natural pine charcoal reduced the phytotoxic effects of litter from *Calluna vulgaris* and *Vaccinium myrtillus* on germination of *Pinus sylvestris* seeds. In this study, consistent with the general trend observed by Lau et al. (2008), AC addition promoted *L. sativum* root growth compared with the water control. The use of AC has recently been criticized, as its direct effect on plant growth could alter the species competitive balance (Lau et al., 2008). However, our study did not include experiments with competitive interactions between plants. A direct promoting effect of AC has been previously attributed to a fertilizing input (AC contains small amounts of N and phosphorus (P)). Such an explanation probably does not apply in our case, because applications of mineral solution did not have promoting effects. Alternatively, we suggest that AC may neutralize an autotoxic factor(s) released in the root exudates (Webb et al., 1967; Singh et al., 1999; Perry et al., 2005), or may act as a cue for germination and seedling growth, as reported for charred wood and smoke (Keeley & Fotheringham, 1998). Further, although indirect, support for the phytotoxic hypothesis derives from the response of seedling shoot : root ratios to litter addition. The nutrient immobilization hypothesis predicts that litter addition, by reducing the available nutrients, will reduce the shoot : root ratio because of more energy being allocated to the root system to forage for the limiting resources (Schmidt et al., 1997). We observed the opposite response, with a sharp increase in the shoot : root ratio after the addition of undecomposed litter and recovery after AC applications. Such observations may be interpreted as reflecting higher sensitivity to inhibitory litter of root growth compared with shoot growth.

Unlike the allelopathic hypothesis, the N immobilization hypothesis was not supported by the results of the present study. First, the simple addition of mineral nutrient solutions to undecomposed litter did not produce any recovery of *L. sativum* root growth. In addition, three of the most inhibitory litter types (*C. emerus*, *H. helix* and *M. sativa*) released > 60% of their initial N content during the first 30 d of decomposition, thus excluding N immobilization as a cause of the observed inhibitory effects. The inconsistence of the N immobilization hypothesis was also evident by the lack of an expected consistent negative relationship between N release and inhibitory effect (Table 2). In contrast, plant litter retaining more N during decomposition showed a fas-

### Table 3  Multiple regression between root growth inhibition of *Lepidum sativum* and linear combinations of selected regions of 13C cross-polarization magic angle spinning (13C-CPMAS) NMR spectra for 40 litter types (10 plant species at four stages of decomposition)

<table>
<thead>
<tr>
<th>13C-CPMAS NMR data</th>
<th>$\beta^a$</th>
<th>$\beta$ SE</th>
<th>B</th>
<th>B SE</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 1</td>
<td>Intercept</td>
<td>-0.407</td>
<td>0.129</td>
<td>-7.346</td>
<td>2.345</td>
<td>-3.13</td>
</tr>
<tr>
<td>PCA axis 1</td>
<td></td>
<td>-0.283</td>
<td>0.123</td>
<td>-7.568</td>
<td>3.295</td>
<td>-2.29</td>
</tr>
<tr>
<td>PCA axis 2</td>
<td></td>
<td>0.182</td>
<td>0.131</td>
<td>7.220</td>
<td>5.211</td>
<td>1.38</td>
</tr>
<tr>
<td>PCA axis 3</td>
<td></td>
<td>0.444</td>
<td>0.125</td>
<td>20.258</td>
<td>5.740</td>
<td>3.52</td>
</tr>
<tr>
<td>Model 2</td>
<td>Intercept</td>
<td>-0.644</td>
<td>0.111</td>
<td>-11.406</td>
<td>1.971</td>
<td>-5.786</td>
</tr>
<tr>
<td>PCA axis 1</td>
<td></td>
<td>-0.224</td>
<td>0.111</td>
<td>-11.792</td>
<td>5.856</td>
<td>-2.014</td>
</tr>
<tr>
<td>PCA axis 2</td>
<td></td>
<td>-0.070</td>
<td>0.110</td>
<td>-3.689</td>
<td>5.771</td>
<td>-0.639</td>
</tr>
<tr>
<td>PCA axis 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>PCA axis 4</td>
<td></td>
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</tr>
</tbody>
</table>

Independent predictive factors in model 1 (multiple $R = 0.69$, $F_{4,35} = 8.07$, $P < 0.001$) correspond to the first four axes of principal component analysis (PCA) ordination of seven reference 13C-CPMAS NMR regions from the literature. In the case of model 2 (multiple $R = 0.77$, $F_{4,35} = 12.41$, $P < 0.001$), eight sequences of consecutive signals along the spectra, each showing a significant linear correlation with the dependent variable, were used in the PCA. For each predictive factor in the models, estimates for regression coefficient ($\beta$: standardized; B: raw value), associated standard errors (SEs), and statistical significance are reported.

*aThe $\beta$ coefficients are obtained by first standardizing all the variables, thus allowing comparison of the relative contribution of each independent variable in the prediction of the dependent variable.*
ter decrease in the inhibitory effect (e.g. *Q. ilex*, *Q. pubescens*, *P. nigra* and *P. excelsa*). In fact, we found a weak but significant positive correlation between N release and root growth inhibition, which is opposite to the expectations of the N immobilization hypothesis. These surprising results are probably attributable to the fact that litter types with a faster decay rate, releasing more N during decomposition (Table 2; Fig. S5), were also the most inhibitory to root growth (Fig. 4). Moreover, it is noteworthy that *C. emerus* and *H. helix* litter showed a rapid release of other major nutrients (potassium (K), calcium (Ca), magnesium (Mg) and iron (Fe)), in addition to N, during decomposition (Bonanomi et al. 2010). All these results indicate that, at least for the selected species in laboratory conditions, root proliferation is hampered in undecomposed litter, even in the presence of available mineral nutrients. Other factors are probably involved in the general inhibiting effect of undecomposed plant litter, although N limitation may remain important in specific ecological conditions (e.g. litter with a high C : N ratio), especially in resource-poor environments.

In fact, previous studies reported that the addition of labile C (e.g. plant extracts or glucose) to the soil can have negative effects on plant and root growth through immobilization of the available nutrients (Schmidt et al., 1997; Michelsen et al., 1999; Bowman et al., 2004). Further studies are required to determine the relative importance of phytotoxicity and nutrient immobilization during decomposition of leaf litter in plant–soil systems.

The study of litter allelopathy has been plagued by the oversimplified approach of searching for a putative phytotoxic compound(s). Hundreds of organic compounds with phytotoxic activity have been extracted from plant tissues, purified and identified (e.g. Rice, 1984; Rizvi & Rizvi, 1992; Reigosa et al., 2006), but the link between these single chemicals and the inhibitory effect of plant detritus has only been demonstrated in a limited number of cases (An et al., 2001; Trifonova et al., 2008). Recent studies have reported that plant litter is composed of a milieu of thousands of unique molecules that change during decomposition (Wallenstein et al., 2010). The great molecular diversity of plant litter suggests that just one or a few molecules can hardly explain the whole inhibitory effect of plant litter. In contrast to this approach, the use of 13C-CPMAS NMR allowed the monitoring of all chemical changes occurring during litter decomposition. We found, consistently with previous studies (Preston et al., 2009; Ono et al., 2011), a general loss of O-alkyl C, mainly from carbohydrate, and an increase in alkyl C and methoxyl C. Previous studies used 13C-CPMAS NMR to examine changes in litter chemicals during decomposition (Preston et al., 2009), to predict litter decay rates (Almendros et al., 2000), or to assess the ability of peat and compost to suppress the growth of soilborne pathogens (Pane et al., 2011). In this study, the inhibitory effect of litter decomposition was weakly correlated with single regions and indexes of 13C-CPMAS NMR spectra derived from the literature (Almendros et al., 2000; Spaccini et al., 2000; Kögler-Knabner, 2002). However, further analyses showed that the simple ratio between restricted spectral regions of O-alkyl C and methoxyl C (70–75 : 52–57 ppm) was positively correlated with litter inhibitory effect (Fig. 4). At this stage, we cannot speculate about the nature of the phytotoxic compound(s) contained in the litter, as this was not the purpose of the study. However, the significant positive correlation between litter decay rate and its inhibitory effect (Fig. 4) suggests that the source of phytotoxic compounds may be the rapidly decaying labile C fraction of litter. Early losses of condensed tannins in decomposition have been reported by Lorenz et al. (2000) and Preston et al. (2009) and thus there was no clear indication of which chemical compounds were associated with the litter inhibitory effect. It should be noted that the two models also differed in the source of the spectral regions used as predictive factors, with the source being the literature for model 1 and a statistically based internal selection for model 2 (i.e. sequences of consecutive signals along the spectrum each showing a significant linear correlation with the dependent variable). Thus, the underlying methodological approach of model 1, in contrast to model 2, was independent from the experimental data set of plant species litter. Comparing the performances of the two models, the slightly higher predictivity of model 2 compared with model 1 (Table 3; Fig. 4) was a consequence of the selection of predictive NMR regions on the basis of the data set used. However, model 1 has the advantage of a higher generality, being based on the whole NMR spectral range.

**Conclusions**

Our results demonstrate that the inhibitory effect of plant litter is a rather general phenomenon caused mostly by phytotoxicity, rather than N immobilization. As a consequence, plant litter does not act only as a nutrient source
and its dynamics should be taken into account in assessing inhibitory effects on plant growth. The possibility of monitoring, by $^{13}$C-CPMAS NMR, qualitative changes in organic C during the decomposition process allows the prediction of the occurrence of inhibitory effects. This study describes an improved method for defining litter quality using $^{13}$C-CPMAS NMR, and shows that it is possible, using this method, to obtain an objective prediction of litter inhibitory effects. This is relevant, in the context of natural plant community organization, for elucidation of the role of plant litter in plant regeneration, nutrient cycling, and plant–plant interactions, as species-specific litter effects could affect the balance between facilitation and competition (Callaway, 2007). These results are also of importance for practical applications in agriculture to assess the potential detrimental effects of organic matter as crop residues, cover crop and compost amendments.

Acknowledgements

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References


Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Experimental set-up of the ‘root proliferation’ bioassay.

Fig. S2 Results of the ‘seed germination’ bioassay.

Fig. S3 Results of the ‘root proliferation’ bioassay.

Fig. S4 Litter decay rates (k; year⁻¹) of 16 litter types calculated for three decomposition periods (0–30, 30–90 and 90–180 d of decomposition).

Fig. S5 Changes in nitrogen content during decomposition in litter-bags of litter from 16 different species.

Fig. S6 ¹³C cross-polarization magic angle spinning (¹³C-CPMAS) NMR spectra of undecomposed plant litter and after 180 d of decomposition in litter-bags.

Table S1 Carbon (C) and nitrogen (N) concentrations and C : N ratio of 64 litter types during the 180 d of decomposition.

Table S2 Relative percentages of carbon types assessed by ¹³C cross-polarization magic angle spinning (¹³C-CPMAS) NMR of 16 undecomposed plant leaves and after 30, 90 and 180 d of decomposition in litter-bags.

Table S3 Pearson’s correlation (r) and associated P-value (in bold; P < 0.05) for the relationship between *Lepidium sativum* root growth inhibition and principal component analysis (PCA) axes from ordination of selected regions of ¹³C cross-polarization magic angle spinning (¹³C-CPMAS) NMR spectra for 64 litter types.

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