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Research

Inhibitory and toxic effects of extracellular self-DNA in litter: a mechanism for negative plant–soil feedbacks?

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Summary

• Plant–soil negative feedback (NF) is recognized as an important factor affecting plant communities. The objectives of this work were to assess the effects of litter phytotoxicity and autotoxicity on root proliferation, and to test the hypothesis that DNA is a driver of litter autotoxicity and plant–soil NF.

• The inhibitory effect of decomposed litter was studied in different bioassays. Litter biochemical changes were evaluated with nuclear magnetic resonance (NMR) spectroscopy. DNA accumulation in litter and soil was measured and DNA toxicity was assessed in laboratory experiments.

• Undecomposed litter caused nonspecific inhibition of root growth, while autotoxicity was produced by aged litter. The addition of activated carbon (AC) removed phytotoxicity, but was ineffective against autotoxicity. Phytotoxicity was related to known labile allelopathic compounds. Restricted ^{13}C NMR signals related to nucleic acids were the only ones negatively correlated with root growth on conspecific substrates. DNA accumulation was observed in both litter decomposition and soil history experiments. Extracted total DNA showed evident species-specific toxicity.

• Results indicate a general occurrence of litter autotoxicity related to the exposure to fragmented self-DNA. The evidence also suggests the involvement of accumulated extracellular DNA in plant–soil NF. Further studies are needed to further investigate this unexpected function of extracellular DNA at the ecosystem level and related cellular and molecular mechanisms.

Introduction

Negative plant–soil feedback (NF) is the rise in soil of negative conditions for plant performance induced by the plants themselves (Klironomos, 2002). It is recognized as an important factor shaping natural plant communities (van der Putten *et al.*, 1993) and allowing species coexistence (Bever *et al.*, 1997; Bonanomi *et al.*, 2005). NF has been observed in a variety of environments, including grasslands (Reynolds *et al.*, 2003), temperate and tropical forests (Mangan *et al.*, 2010) and also anthropogenic agro-ecosystems (Singh *et al.*, 1999).

The large body of available data in the literature on species-specific NF (Kulmatisky *et al.*, 2008; van der Putten *et al.*, 2013) has been related to different non-mutually exclusive hypotheses of possible underlying mechanisms: soil nutrient depletion (Ehrenfeld *et al.*, 2005) and both the build-up (Packer & Clay, 2000) and changing composition (Kardol *et al.*, 2007) of soil-borne pathogen populations. However, observations of long-term 'soil sickness' and 'replant diseases' in agriculture (Zucconi, 1993; Singh *et al.*, 1999) are examples of species-specific inhibition unrelated to either nutrient availability or soil-borne pathogens. Similar findings have also been obtained for natural ecosystems (e.g. Bonanomi *et al.*, 2011a).

Another hypothesis proposed the release of phytotoxic compounds during litter and organic matter decomposition as a further possible mechanism to explain NF (Singh *et al.*, 1999). This was based on the observation that, in addition to the well-recognized role of litter as a nutrient source (Vitousek & Sanford, 1986), leaf and root litter can also inhibit plant growth by immobilizing nitrogen (N) (Hodge *et al.*, 2000) or by releasing phytotoxic compounds during decomposition (An *et al.*, 2001; Trifonova *et al.*, 2008). However, such toxins are known to be rapidly degraded by soil microbial activity. In fact, litter inhibitory effects have been found to be limited to short-term phases of early decomposition stages, usually lasting only a few weeks (Hodge, 2004; Bonanomi *et al.*, 2011b), and for this reason this hypothesis has been widely criticized (Fitter, 2003; Harper, 2010). Indeed, in traditional studies of litter allelopathy, hundreds of organic compounds, extracted from plant tissues, purified and identified (e.g. Rice, 1984; Rizvi & Rizvi, 1992; Reigosa

et al., 2006), only showed a general toxicity without species-specific effects. It seems evident that such phytotoxic compounds, given their short persistence in the soil and lack of specificity, can hardly explain species-specific plant–soil NF. On this basis, a recent review on the state of the art and research perspectives on plant–soil NF (van der Putten *et al.*, 2013) did not consider litter autotoxicity among the causal factors (Mazzoleni *et al.*, 2007, 2010).

In spite of such substantial evidence against the autotoxicity idea, some logical considerations suggest that the hypothesis of a chemical origin of NF should not be dismissed. First, the fact that plant–soil NF has been found to occur mainly in terrestrial systems, while it has rarely been observed in aquatic environments (Mazzoleni *et al.*, 2007), should be taken into account. Such indirect observations support the speculative idea that the inhibiting factor causing NF could be a water-soluble compound. Secondly, the spatial scale and patterns of many observed Janzen–Connell distributions (e.g. Wright, 2002) seemed consistent with diffusion processes of putative inhibitory substances related to litter accumulation, but less compatible with the involvement of pathogens whose mobility range is plausibly larger than the observed NF spatial scale. For example, the inhibition zone of seedling recruitment of *Prunus serotina* attributed to *Pythium* spp. by Packer & Clay (2000) extends for a few meters, but it should be noted that the invasion speed of this pathogen is *c.* 2–3 cm d^{-1} (Nelson, 2004). With such a colonization rate, the pathogens are able to cover an area larger than the plant root system and this reveals a mismatch between the area colonized by the pathogens and the observed pattern of seedlings affected by NF.

Consistent with such theoretical reasoning, modeling work demonstrated that the release of inhibitory factors by litter decomposition and their removal by water could indeed explain the latitudinal gradient of plant species diversity as well as the co-occurrence of monospecific stands of mangroves and riparian vegetation with highly diverse tropical forests (Mazzoleni *et al.*, 2010). In this context, the accumulation of autotoxicity in soil was also proposed as a driver of the spatial organization of vegetation (Cartenì *et al.*, 2012), with water also affecting the emergence of patterns and their dynamics (Marasco *et al.*, 2014). However, these models remained completely speculative without a demonstration of the actual existence of the supposed chemical compounds.

In this work, we sought definitively to verify or falsify our theoretical autotoxicity model through an in-depth investigation of the occurrence of conspecific litter inhibitory effects. We also searched for putative chemical compounds associated with such observations. We used an integrated approach including ecological, phytochemical, and biomolecular tools, on a large set of plant species. In detail, a throughput metabolomic characterization of plant litter at different decomposition stages was obtained by ^{1}H nuclear magnetic resonance (NMR) and ^{13}C-CPMAS (Cross Polarization Magic Angle Spinning) NMR (e.g. Kögel-Knabner, 2002; Verpoorte *et al.*, 2007; Preston *et al.*, 2009) and analyzed in relation to observed effects on root growth.

In theorizing about which chemical compounds could be responsible for litter autotoxicity, three main clues were taken into account: (1) long-lasting persistence in the environment; (2) specificity of effect and (3) water solubility. All these features suggested conspecific DNA as the target molecule of our hypothesis (from now on also referred to as self-DNA).

The specific aims of the study were: (1) to investigate species-specific inhibitory effects of decomposing plant litter and to assess the existence and significance of autotoxicity; (2) to relate toxicity effects to changes in biochemical litter quality during decomposition; (3) to assess species-specific inhibitory effects of extracellular DNA released during litter decomposition; (4) to test the species-specific toxicity of purified DNA in relation to its degradation level. Moreover, to assess the relevance of self-DNA toxicity under real ecological conditions and its possible association with NF, three complementary approaches were used: (1) evaluation of the growth of a test plant on different soil types treated with either self or heterologous DNA; (2) soil history experiments (Klironomos, 2002) assessing the accumulation of plant DNA in soil and its relationship to NF effects, and (3) measures of DNA accumulation in field conditions in relation to the observed Janzen–Connell distribution of seedling recruitment.

Materials and Methods

Litter collection and decomposition experiment

Twenty different plant species were selected from both Mediterranean and temperate environments. The species pool represented a wide range of litter quality (Table 1). Freshly abscised leaves were collected in natural communities by placing nets under randomly selected plants ($n > 20$), dried at room temperature in a ventilated chamber until a constant weight was reached and then stored at room temperature.

Under field conditions, litter decomposition depends on organic matter quality, water availability and temperature (Gholz *et al.*, 2000; Berg & McClaugherty, 2008). In order to focus only on litter quality effects, the experiment was carried out in a growth chamber with optimal water availability and temperature conditions: the litter was watered every 7 d to holding capacity with distilled water, and the temperature was $18 \pm 2°C$ at night and $24 \pm 2°C$ during the day. Dry leaf litter (100 g for each species in three replicates) was placed inside plastic trays (size $30 \times 50 \times 50$ cm). A microbial inoculum was prepared and distributed according to Bonanomi *et al.* (2011b).

Trays were harvested after 0, 30 and 120 d of decomposition for a total of 180 samples (20 species × 3 sampling dates × 3 replicates). Collected litter was dried in paper bags (at 40°C until a constant weight was reached) and weighed. In this way we produced 60 organic samples (20 species at three sampling dates) of different ages: fresh undecomposed litter (hereafter designated 0 d) and litter decomposed for 30 and 120 d.

Assessment of litter effects on target plant species

Bioassays were conducted in root observation plates (Mahall & Callaway, 1992) to assess the capability of plant seedling roots to colonize different litter types. Activated carbon (AC) was applied

Table 1 Design of main experiments on litter decomposition, litter toxicity, and effects of extracellular DNA on different species

1. Litter decomposition	Substrates	Experimental length	Parameters assessed
The experiment was carried out to produce organic material at different decomposition stages to be used for bioassay and chemical analyses	*Acanthus mollis* *Alnus cordata* *Ampelodesmos mauritanicus* *Arbutus unedo* *Castanea sativa* *Coronilla emerus* *Cupressus sempervirens* *Fagus sylvatica* *Festuca drymeia* *Fraxinus ornus* *Hedera helix* *Lepidium sativum* *Medicago sativa* *Picea excelsa* *Pinus halepensis* *Populus nigra* *Quercus ilex* *Quercus pubescens* *Robinia pseudoacacia* *Salix alba*	All litter types retrieved after 0, 30 and 120 d of decomposition	C and N content, litter quality assessed by ^{1}H NMR and ^{13}C NMR, and total DNA quantification
2. Litter toxicity	**Target plant**	**Experimental length**	**Parameters assessed**
This bioassay assessed general phytotoxic effects	*Lepidium sativum*	3 d	Root growth
These tests assessed the species-specific effects of litter at different decomposition stages on conspecific and heterospecific plants	*Acanthus mollis* *Ampelodesmos mauritanicus* *Cupressus sempervirens* *Festuca drymeia* *Hedera helix* *Pinus halepensis* *Quercus ilex* *Robinia pseudoacacia*	28 d 28 d 28 d 28 d 28 d 35 d 35 d 28 d	Root growth
3. DNA effect *in vitro*	**Target plant**	**Experimental length**	**Parameters assessed**
This experiment directly assessed the inhibitory effects of DNA on plants	*Acanthus mollis* *Ampelodesmos mauritanicus* *Arabidopsis thaliana* *Hedera helix* *Lepidium sativum* *Medicago sativa* *Pinus halepensis* *Quercus ilex* *Quercus pubescens*	14 d 14 d 7 d 21 d 3 d 3 d 28 d 28 d 28 d	Root growth
4. DNA accumulation and effects *in vivo*	**Target plant**	**Experimental length**	**Parameters assessed**
Test on two soil types Soil history experiment in glasshouse Janzen–Connell in field conditions	*Medicago sativa* *Medicago sativa* *Medicago marina* seeds and seedlings	60 d 360 d + 180 d 30 d	Root and shoot growth Mortality and shoot growth Seeds m^{-2}, seedlings m^{-2}, and soil DNA concentration

to evaluate relative toxicity and N immobilization effects as causes of observed growth inhibition (Mahall & Callaway, 1992; Hille & den Ouden, 2005). First, a bioassay for general phytotoxicity was carried out with *Lepidium sativum* on all litter material. Secondly, to assess species-specific effects, we tested 20 litter types at three different decomposition ages on eight target species (subset of the decomposition experiment), representatives of herbaceous, shrub and tree growth forms (Table 1). Such an experimental design enabled comparison of the litter effects on conspecific (autotoxicity) and heterospecific (phytotoxicity) plants.

The bioassay tests generally followed the methods described in a previous paper (Bonanomi *et al.*, 2011b). Briefly, an 8-cm-wide sterile filter paper strip was placed in square Petri dishes (size 12 × 12 × 1.5 cm). Pre-germinated seeds of each species (five for each dish) were placed at the top of the paper strip previously

amended with AC and different litter types (Supporting Information Fig. S1). Larger root observation plates (30 × 20 × 1.5 cm) were used for plants with large seeds (*Pinus halepensis* and *Quercus ilex*). Root observation plates were placed at 45° and covered with opaque sheets.

The experimental design included the following treatments: (1) a control with sterile distilled water; (2) addition of AC (Sigma-Aldrich Co.) at 0.2 g cm^{-2} of filter paper; applied both with and without litter (Fig. S1). The full methodology is reported in Methods S1.

^{1}H NMR and ^{13}C-CPMAS NMR litter characterization and relationships with phytotoxicity and autotoxicity

Litter total carbon (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).

The litter types were characterized by ^{1}H NMR using the following procedure. Litter samples at 0 and 30 d of decomposition (total of 40 samples, 0.3 g each) were treated separately with 10 ml of hydro-alcoholic solution (methanol:water 75 : 25). The organic soluble phase was filtered, taken to dryness and dissolved in CD_3OD, and the ^{1}H NMR spectra were recorded on a Bruker AV-400 spectrometer (Bruker AXS GmbH, Karlsruhe, Germany). Chemical shifts were referred to the residual solvent signal (CD_3OD: δ_H 3.31). For each sample, 128 scans were recorded with the following parameters: 0.126 Hz/point, pulse width = 4.0 μs (30°) and relations delay = 2.0 s. Free induction decays (FIDs) were Fourier-transformed with line broadening (LB) = 0.3 Hz. For quantitative analysis peak areas were used. The regions of the residual solvent signals were excluded from the analysis.

All litter types from the litterbag experiment were also characterized by ^{13}C-CPMAS NMR obtained in solid state under the same conditions in order to perform a quantitative comparison among spectra. Samples were analyzed at four different times, corresponding to leaves decomposed for 0, 30, and 120 d (total of 60 samples). The spectrometer used was a Bruker AV-300 equipped with a 4-mm wide-bore Magic Angle Spinning (MAS) probe. NMR spectra were obtained with MAS as follows: rotor spin, 13 000 Hz; recycle time, 1 s; contact time, 1 ms; acquisition time, 20 ms; and 2000 scans. Samples were packed in 4-mm zirconium rotors with Kel-F caps (Bruker). 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 signal peaks. Spectral regions were selected and C-types identified according to previous reference studies (Kögel-Knabner, 2002; Bonanomi *et al.*, 2013). Such regions were used to assess the chemical composition changes that occurred during the decomposition processes of the different litter types. For each spectral region, the signal variations among materials either undecomposed or after 30 and 120 d of decomposition were statistically analyzed using the Mann–Whitney–Wilcoxon test and Kruskal–Wallis ANOVA. The tested null hypothesis was that litter samples at different decomposition stages, showing equal signal intensities within each region, have equal relative contents of the corresponding C types. In a more detailed analysis, an extensive calculation was performed of the linear correlation between plant root growth over heterospecific and conspecific litter and ^{13}C NMR data recorded for the same material at each resonance signal (n = 190), providing a fine-resolution profile of the variation in C types in the tested material associated with the effect on seedling root growth. The correlation was tested for statistical significance controlling for multiple comparisons, according to the false discovery rate (FDR) approach (Benjamini & Hochberg, 1995).

Extraction and quantification of DNA from plant material

DNA was extracted from dried litter material using a DNeasy Plant Mini Kit as described by the manufacturer (Qiagen, Valencia, CA, USA). One hundred milligrams dry weight of starting material was eluted in 60 μl of sterile water. DNA extracts were spectrophotometrically quantified at 260 nm on a NanoDrop TM 1000 (Thermo Scientific, Wilmington, DE, USA) and visually verified on 1.5% agarose gel using Sybr® Safe (Invitrogen).

For plant leaves, a MAXI DNA extraction kit (Qiagen) was performed to prepare the treatment solution using the protocol of Fulton *et al.* (1995). One hundred grams fresh weight of starting material was harvested and frozen at −20°C. Then, the samples were blended and immersed in 200 ml of fresh microprep buffer, then filtered in cheese cloth. DNA was resuspended in pure sterile water. The extracted DNA from leaves was fragmented by sonication. This was performed with a Bandelin Sonopulse (Bandelin, Berlin, Germany) at 90% power with a 0.9-s pulse for 12 min. Verification of sonicated band sizes was performed on a 3% MetaPhor™ agarose gel (Lonza Scientific, Allendale, NJ, USA) using Sybr® Safe (Invitrogen).

Effects of purified DNA on target plants

Autotoxic and phytotoxic effects of purified DNA extracted from plant leaves were assessed on seven species selected from the litter toxicity experiment, with the addition of *Arabidopsis thaliana* which was used as a standard model plant. Autotoxicity was assessed using DNA extracted from the leaves of each target species; in contrast, phytotoxicity was assessed using a mixture of equal amounts of DNA from heterospecifics (Table 1). DNA was extracted as described in the previous paragraph.

The experimental design included the following treatments: (1) a control with sterile distilled water; (2) addition of total extracted self-DNA; (3) addition of total extracted and sonicated self-DNA; (4) addition of a mixture of DNA from heterospecifics; (5) addition of a sonicated mixture of heterospecific DNA; (6) addition of sonicated DNA from single heterospecifics (only in the cases of *A. thaliana, Acanthus mollis and L. sativum*). DNA was applied in both untreated and fragmented forms to represent its different decay levels observed during decomposition processes. DNA was applied at three concentrations (200, 20, and 2 μg ml^{-1}). Bioassays were performed *in vitro* using surface-sterile seeds (n = 20 in each plate) for each species (10 replicates)

placed in 9-cm Petri dishes over sterile filter paper imbibed with 4 ml of test solutions. Seedling root length was measured after a variable number of days since the start of the experiment depending on the target species (Table 1). Petri dishes were arranged in a growth chamber (temperature 22 ± 2°C; watered every 2 d with distilled water), according to a totally randomized design.

DNA autotoxicity was also assessed in a glasshouse experiment with two soils of contrasting texture (sandy and clay). DNA was extracted from leaves of the target species *Medicago sativa* (Table 1), a species that is reported to suffer strong NF by litter autotoxicity (Bonanomi *et al.*, 2011b) and which is of interest as the most important forage plant world-wide. DNA was extracted from fresh leaves as described in the previous section. The experimental design included the following treatments: (1) a control with sterile distilled water; (2) addition of total extracted self-DNA; (3) addition of total extracted and sonicated self-DNA. DNA was applied once at the beginning of the experiment in water solution at three concentrations (20, 200, and 2000 μg ml^{-1}) until field capacity (50 ml of each solution). Bioassays were performed in a glasshouse using seeds ($n = 5$ in each pot) placed in 12-cm-diameter pots. Two contrasting soil textures were used to evaluate their possible impact on DNA toxicity (Methods S2). Each treatment was replicated 20 times for a total of 120 pots. Plant shoot and root dry biomass was measured after 60 growing days since the start of the experiment (Table 1). Pots were arranged in a glasshouse (temperature 22 ± 2°C; watered every 2 d with distilled water), according to a fully randomized design.

Plant growth was statistically analyzed considering DNA origin (conspecific versus heterospecific), fragmentation level (untreated versus sonicated), and concentration (three levels) as independent factors. For each target species and for all species pooled, differences in root growth related to DNA origin at each concentration, or dependent on concentration within each DNA source, were tested for statistical significance using the Mann–Whitney–Wilcoxon test. In the glasshouse experiment, two-way ANOVA and the post hoc Duncan test were used to assess the effect of DNA treatment and soil type on plant growth.

Soil history experiment

In the context of plant–soil feedback studies, a soil history experimental approach has been widely used to investigate the effects and mechanisms of NF (e.g. Klironomos, 2002; Kulmatisky *et al.*, 2008; Petermann *et al.*, 2008). Similarly, in the present study two main questions were addressed: Does plant growth

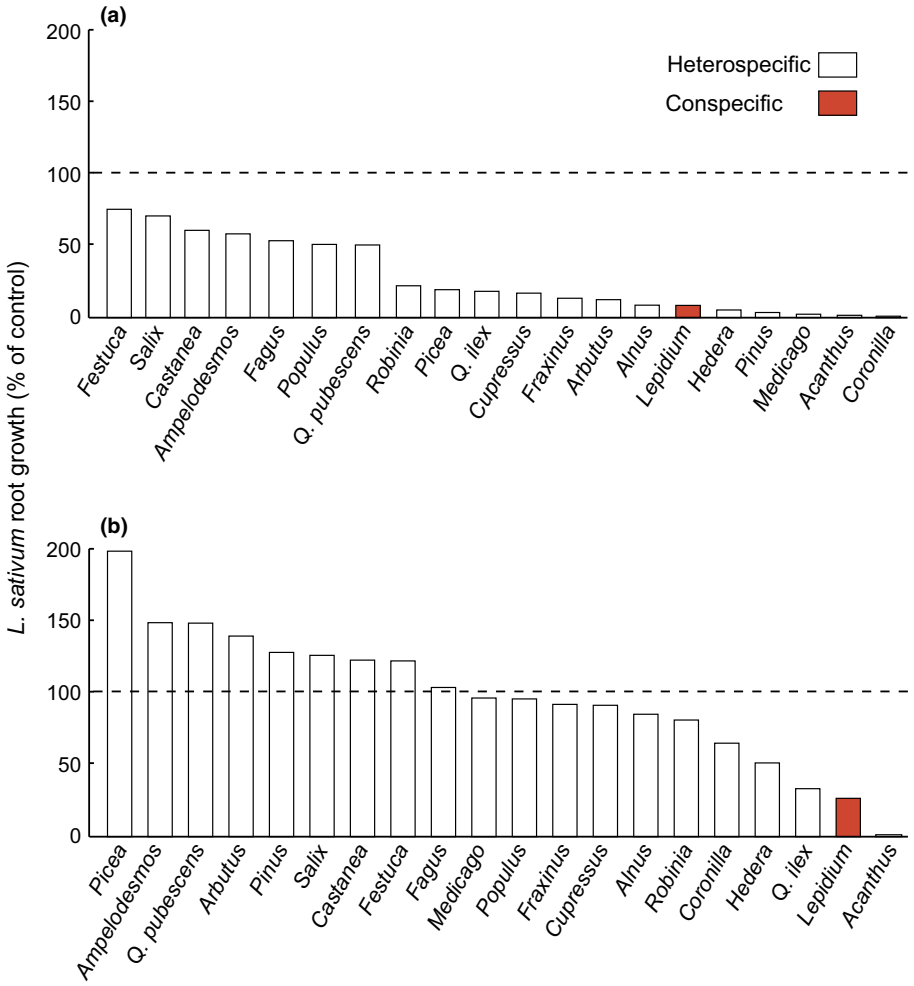

Fig. 1 (a, b) Effects of 20 litter types on *Lepidium sativum* root growth as percentages compared with the control (= 100%) on both undecomposed (a) and 120-d-old (b) material. Litter specific names are given in Table 1. White bars, heterospecific; red bars, conspecific. Data refer to the mean of five replicates within each litter type. Error bars are omitted to improve readability (standard deviations < 3 in all cases).

build up a plant–soil NF in the soil? Is the observed NF associated with changes in plant DNA accumulation in the soil? To address these questions, *M. sativa* was first cultivated on a soil for periods of different duration (soil histories) and then the seedling growth performance of the same species was tested on the same substrate. In the first experimental phase, a sandy agricultural soil was collected in the field from the first 20 cm depth after removal of surface litter. The soil was sieved in the laboratory (mesh size < 2 mm). Pots were filled with 1000 g of a mixture of dry soil and perlite (4 : 1 v/v). Three different soil treatments were set up by growing *M. sativa* as a monoculture for two different periods and using bare soil as a control. Seeds ($n = 10$) were sown in pots. In the first treatment, irrigation was maintained for 360 d and then stopped to dry the soil until plant death, which occurred within 15 d. The second treatment was set up later in the year and lasted for only 180 d. At the end of the first experimental phase dry above-ground biomass was removed by cutting at the soil surface without any soil disturbance. The pots containing the soil with the residual dead roots were used to establish the growth tests in the second experimental phase. Three new individual *M. sativa* plants were planted and grown in all soil types (three soil histories). At the end of the experiment, dry above-ground biomass was measured. Before the start of the second experimental phase, soil was sampled from pots of each soil history to measure DNA content. A full description of the soil history experiment methodology is given in Methods S3.

Table 2 Summary of the general linear mixed model (GLMM) testing for main and interactive effects of treatments on plant root growth in the litter toxicity experiment

	Effect type	SS	df	MS	*F*	*P*
Target plant species	Random	148.532	7	21.219	47.835	< 0.0001
Species specificity	Fixed	3.654	1	3.654	8.237	0.0041
Litter type	Random	1153.007	19	60.685	136.805	< 0.0001
Litter age	Fixed	1.710	1	1.710	3.856	0.0496
AC	Fixed	21.499	1	21.499	48.467	< 0.0001
Species specificity × litter age	Fixed	29.285	1	29.285	66.020	< 0.0001
Species specificity × AC	Fixed	5.393	1	5.393	12.159	0.0005
Litter age × AC	Fixed	2.121	1	2.121	4.782	0.0288
Species specificity × litter age × AC	Fixed	5.100	1	5.100	11.498	0.0007

The model includes the target plant species (eight levels) and litter species (20 levels) as random effects, and litter species specificity (either heterospecific or conspecific litter), litter age (treated as a continuous variable), addition of activated carbon (AC) (two levels), and their interactions as fixed factors.

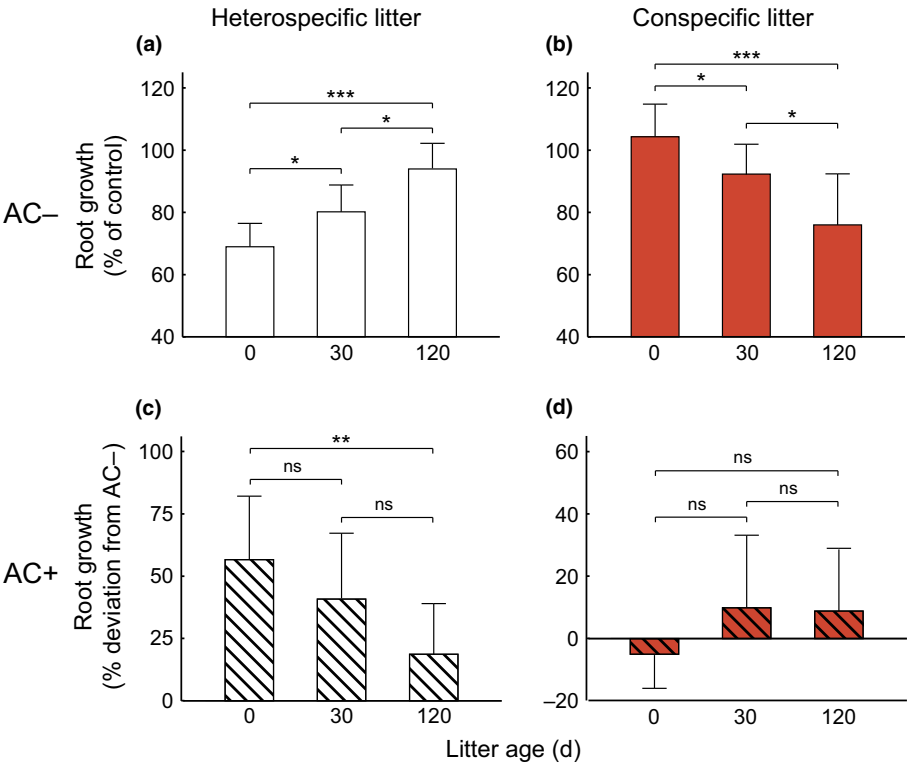

Fig. 2 (a, b) Root growth of eight target species (mean and 95% confidence interval) either on 19 different heterospecific litter samples (a) or on their own litter material (b) after 0, 30, and 120 d of decomposition. (c, d) Root growth changes (% deviation) when active carbon was added to the litter (AC+), compared with corresponding untreated samples (AC−). Age-dependent significant differences within each treatment combination: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, $P > 0.05$ (post hoc Duncan test from a Generalized Linear Mixed Model (GLMM) in Table 2; detailed statistics in Supporting Information Table S2).

DNA accumulation associated with Janzen–Connell distribution

The amount of DNA accumulated in the soil in field conditions was determined in a previously reported case of a Janzen–Connell distribution of seedling recruitment (Bonanomi *et al.*, 2008). The number of *Medicago marina* seedlings and their distance to the nearest adult *Medicago* crown were determined during the peak of seedling growth in April and May 2011. Data were collected within 30 randomly sampled plots (1 m^2). Soil samples from the top 10 cm were collected in July 2011 under and around adult *M. marina* shrubs (a detailed study site description is given in Bonanomi *et al.*, 2008). We defined three different areas of potential influence on seedling recruitment: the canopy crown area (IN; influenced by litter and roots), the area immediately adjacent to the crown (15–30 cm from the nearest shrub), and the area located far from shrubs (> 45 cm from the nearest shrub). *Medicago marina* seeds were counted by soil sieving (Bonanomi *et al.*, 2008). The total DNA content and presence of plant DNA in the soil were assessed as described in the previous section.

Significant differences of *M. marina* recruitment and soil DNA content among different sampling areas were assessed by one-way ANOVA on log-transformed data. The significance of pair-wise differences was determined using the Duncan post hoc test.

Results

Litter effects in laboratory conditions

Plant litter produced clear species-specific effects on root growth, depending on both litter type and decomposition time (Fig. 1). All undecomposed litter material showed an inhibitory effect on *L. sativum,* a reference plant commonly used in bioassay tests (Fig. 1a). Such inhibition varied greatly according to litter type, being very high in the case of litter from *Coronilla emerus, A. mollis*, *M. sativa*, *Pinus halepensis*, and *Hedera helix*, relatively low for grasses (*Festuca drymeia* and *Ampelodesmos mauritanicus*) and most broadleaved deciduous trees (*Salix alba, Castanea sativa, Fagus sylvatica, Populus nigra,* and *Quercus pubescens*), and intermediate for the remaining species (Fig. 1a). The test plant *L. sativum* was also strongly inhibited by its own litter. By contrast, litter decomposed for 120 d, with the exception only of *A. mollis*, showed a greatly reduced inhibitory effect, being strongly stimulative of root growth in nine cases (Fig. 1b). It should be noted, however, that *L. sativum* autotoxicity (i.e. growth inhibition on conspecific litter) was still very high in the case of decomposed litter.

Phytotoxic and autotoxic effects were further tested on eight different target species selected among the litter types as representative of different functional groups (herbaceous grasses, forbs, and evergreen and deciduous woody species). Plant root growth was significantly affected by all experimental treatments (Table 2). The target plant species and the litter type were, as expected, the major sources of variation (Table 2). In addition, a significant effect of litter species specificity, and its significant interactions with litter age and/or AC addition (Table 2), produced a completely different root growth pattern in seedlings grown on heterospecific compared with conspecific litter.

Root growth of target species was significantly inhibited on undecomposed heterospecific litter, while it always showed a better performance with increasing litter age (Fig. 2a). AC application showed a highly significant interaction with litter age (Tables S1, S2). The percentage deviation from root growth of untreated samples was high and significant when AC was added

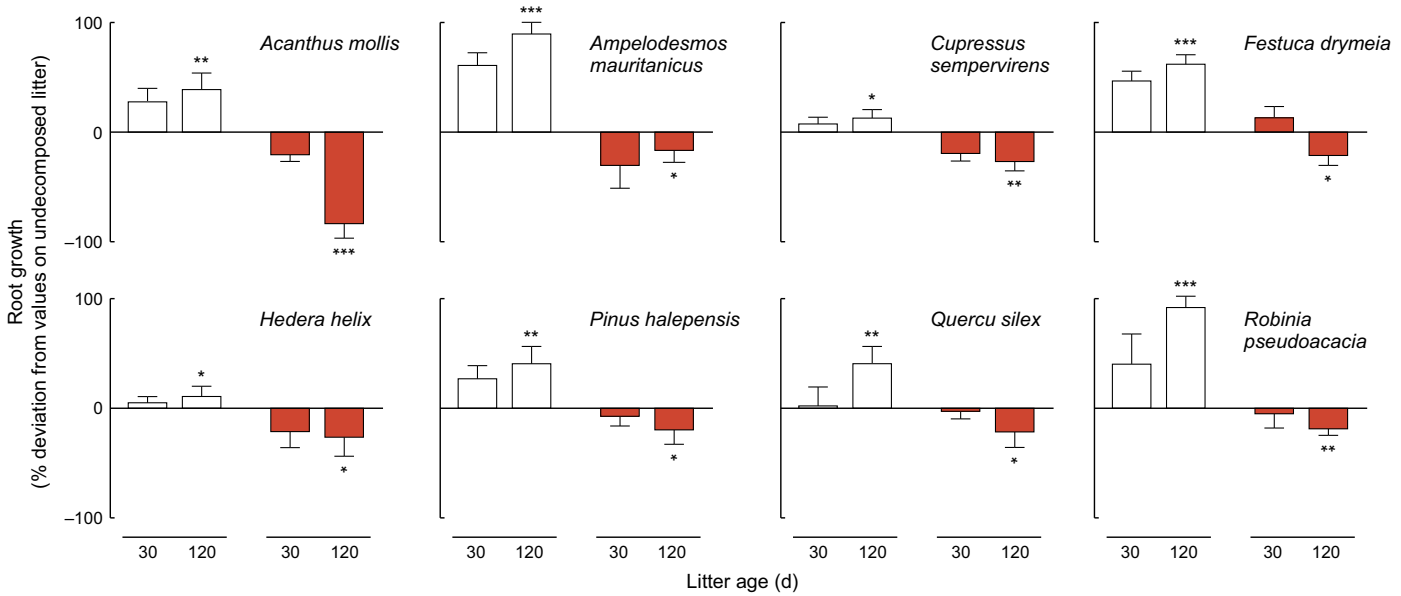

Fig. 3 Responses of eight target species to litter of heterospecifics and conspecifics of different ages (0, 30 and 120 d). Values (mean and SD of experimental replicates; $n \geq 5$ observation plates; five seedlings each) are expressed as percentage deviations compared with growth on undecomposed litter (= 0%). For each target species, data on litter at 120 d are marked with asterisks to indicate significant differences compared with undecomposed material (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; post hoc Duncan tests from a Generalized Linear Mixed Model (GLMM) in Table S1).

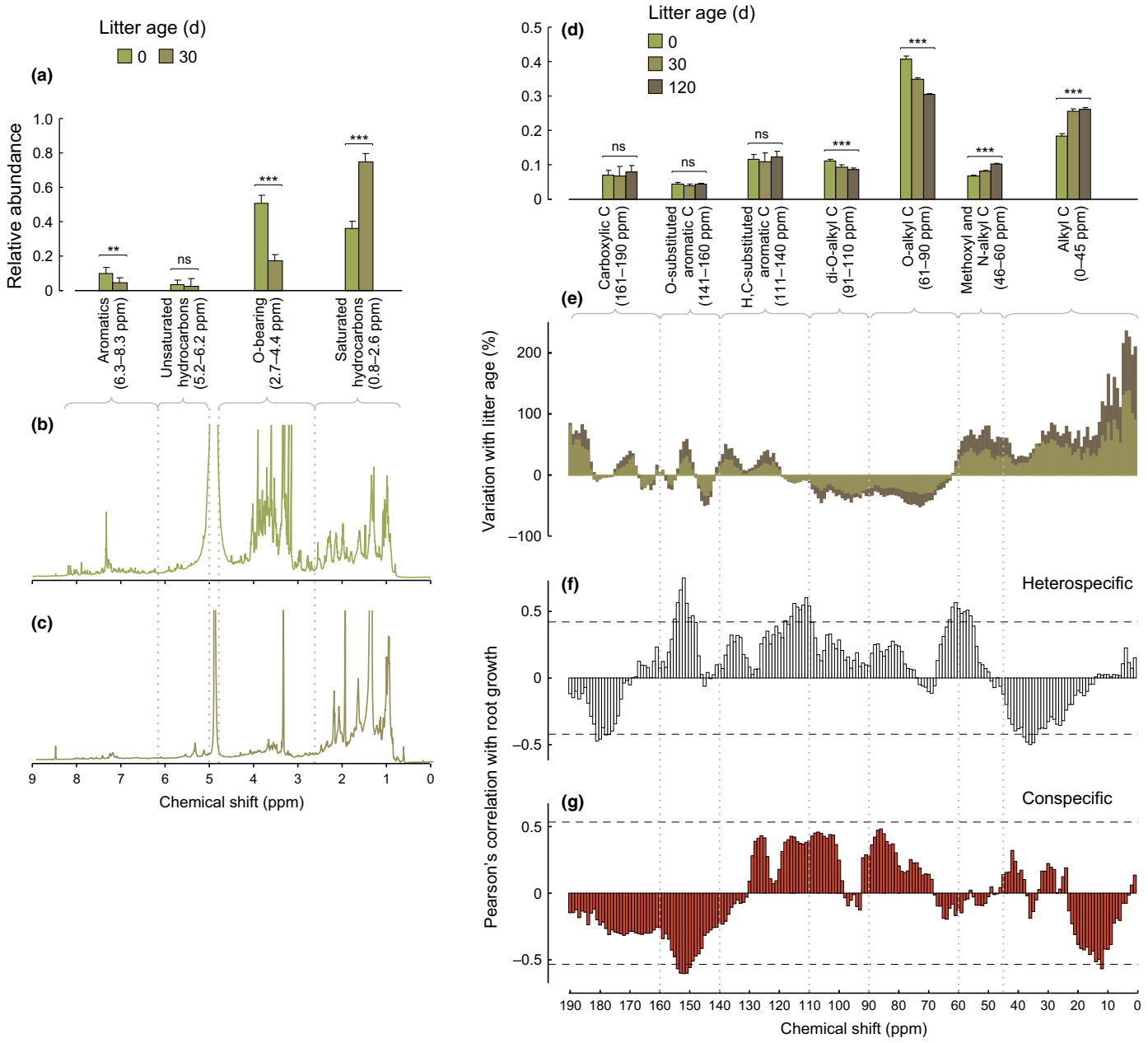

Fig. 4 ^{1}H and ^{13}C nuclear magnetic resonance (NMR) litter characterization and relationships with plant growth. (a) Variation of four main classes of organic compounds assessed by ^{1}H NMR spectroscopy in butanol extracts of litter at two ages (mean and SD; $n = 40$; ***, $P < 0.001$; **, $P < 0.01$; ns, $P > 0.05$ for significant variation between 0 and 30 d; Mann–Whitney–Wilcoxon test; detailed statistics in Table S3). (b, c) Examples of ^{1}H NMR spectra of butanol litter extracts from *Medicago sativa*, either undecomposed (b) or at 30 d (c). (d) Variation of seven main classes of organic carbon (C) assessed by ^{13}C-CPMAS NMR spectroscopy in plant litter at three ages (mean and SD; $n = 60$; ***, $P < 0.001$; ns, $P > 0.05$ for significant variation between 0 and 120 d; Mann–Whitney–Wilcoxon test; detailed statistics in Table S4) and (e) time-scale relative variation of all signals from ^{13}C-CPMAS NMR spectra ($n = 60$). (f, g) Correlation profiles (Pearson's r) between plant growth of eight target species over litter of heterospecifics (f) and conspecifics (g) and ^{13}C-CPMAS NMR spectral signals of the litter samples ($n = 60$ and $n = 24$ for heterospecifics and conspecifics, respectively). Dashed lines in (f) and (g) indicate threshold values of statistical significance for r ($P < 0.01$, after correction for multiple comparisons according to the false discovery rate method; Benjamini & Hochberg, 1995).

to undecomposed litter, and progressively decreased with increasing litter age (Fig. 2c). The opposite dynamics to those observed on heterospecific material were found when the analyses were restricted to conspecifics. In this case, root growth was inhibited by the aged litter material (Fig. 2b) and application of AC did not produce any significant effect on root growth at any time (Fig. 2d). The contrasting effects between heterospecific and conspecific litter were consistent and statistically significant for all target species (Fig. 3; Table S1), indicating an unknown general common pattern of species-specific inhibition.

Relationship between litter biochemical quality and effects on target plants

The litter C:N ratio and N release during decomposition were unrelated to root growth of the eight target species (Fig. S2), excluding in all cases N limitation as a possible causal factor for the observed root growth inhibition.

The ^{1}H NMR analysis showed a significant reduction in the concentrations of well-known toxic allelopathic compounds during early decomposition phases. These are known to be mainly phenols and aromatics of low molecular weight (e.g. see Rice, 1984) which are easily absorbed by AC. This was consistent with the observation of phytotoxicity removal by AC in the experiment reported in Fig. 2c. In detail, ^{1}H NMR spectra of crude organic extracts of litter material showed evident reductions in the concentrations of sugars (2.7–4.4 ppm) and labile phenolic compounds (6.3–8.3 ppm) during early decay phases (Fig. 4a–c). In contrast, the corresponding signals of saturated hydrocarbons (0.8–2.6 ppm) mainly related to alkyl chains of fatty acids increased significantly (Fig. 4a–c; Table S3).

Solid-state ^{13}C NMR spectroscopy of 0-, 30- and 120-d-old litter material showed significant changes in organic C components during decomposition (Fig. 4d; Table S4). The major patterns were an increase in alkyl-C (0–45 ppm) and methoxyl and N-alkyl C (46–60 ppm) regions, and a concomitant steep decrease in *O*-alkyl-C (61–90 ppm) and di-O-alkyl-C (91–110 ppm) regions (Fig. 4d). In contrast, the regions corresponding to H- and C-substituted aromatic C (111–140 ppm), O-substituted aromatic C (141–160 ppm), and carboxylic C (161–190 ppm) did not change significantly during litter decay (Fig. 4d). A more detailed observation of relative changes in each NMR signal compared with initial values (Fig. 4e) showed a major increase for all signals of the alkyl-C and methoxyl and N-alkyl C regions, and a consistent decrease for most signals of the O-alkyl-C and di-O-alkyl-C regions, whereas contrasting dynamics of the signals were observed within each of the three regions corresponding to aromatics and carbonyls (Fig. 4e).

An extensive analysis of the correlation between the root growth of the eight target species and variation in C signals in the ^{13}C NMR spectra showed strikingly different patterns in heterospecific and conspecific litter material (Fig. 4f,g). In the case of plants growing on heterospecific litter, positive correlations were found with carbons resonating at δ 56–63, δ 110–116, and δ 150–156 (Fig. 4f). Within the first region of C signals, the upfield resonances (δ 56–59) are diagnostic of amino acidic α-carbons. The other signals can be associated with hydroxymethylene (δ 60–63) and aromatic (δ 110–116, and δ 150–156) carbons, requiring further studies to elucidate their detailed chemical nature, as well as the physiological basis of the observed stimulatory effects. In contrast, root growth on heterospecific substrates was negatively associated with two NMR intervals corresponding to alkyl C and carboxylic C, with significant correlation at δ 177–181 and δ 34–37, respectively. These signals are related to well-known chemical compounds, such as terpenoids and carboxylic acids, among the class of molecules with allelopathic effects (e.g. Langenheim, 1994; Li *et al.*, 2010).

By contrast, the analysis limited to litter effects on conspecifics showed no significant positive correlation between root growth and NMR signals, whereas few signals corresponding to δ 12 and δ 151–154 were significantly correlated with autotoxic inhibitory effects (Figs 4g, S3). The signals resonating at lower fields (δ 151–154) can be attributable to unsaturated carbons bearing N

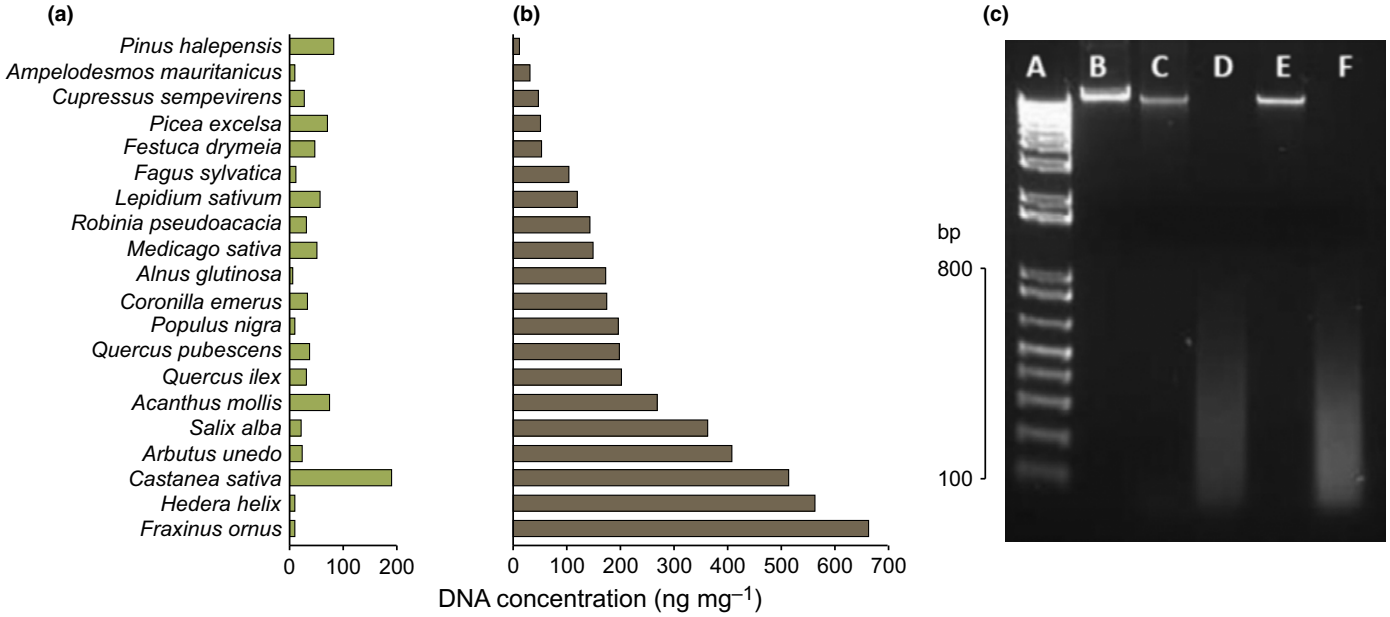

Fig. 5 DNA content in decomposing plant litter. (a) DNA concentration (ng mg^{-1}) assessed by UV spectrophotometry in undecomposed material. (b) DNA concentration after 120 d of decomposition. (c) Examples of DNA fragmentation patterns in *Acanthus mollis* (C and D, 0 and 120 d, respectively) and *Hedera helix* (E and F, 0 and 120 d, respectively). A and B refer to a 1-kb Plus Ladder (Qiagen) and lambda DNA, respectively. Gel electrophoresis showing fragmentation patterns of all litter materials is illustrated in Fig. S4(b).

that are part of the structure of the heterocyclic aromatic amines and are diagnostic of the N bases of nucleic acids. These values are characteristic of C2 and C4 of the purines adenine and guanine, and of C2 of the pyrimidines thymine and cytosine (Breitmaier & Voelter, 1987). The ^{13}C NMR signal resonating at higher field (δ 12) could be associated with the methyl group of thymine, and the other key signals of DNA, such as the carbons of 2-deoxyribose (δ 93–94 ($C1^I$), 61–65 ($C5^I$), 35–36 ($C2^I$)), were also negatively correlated to root growth in the case of autotoxicity by conspecific litter, although the correlation was not significant (Fig. 4g).

DNA quantification on plant litter

Direct plant litter DNA quantification by agarose gel electrophoresis showed a progressive increase of DNA concentration with decomposition time (Fig. 5a,b). An evident decrease in the size of DNA molecules was also observed in most litter material (Fig. 5c). In detail, only polynucleotides of < 500 base pairs were found in litter decomposed for 120 d. An increase of DNA content with litter age was also indirectly supported by the significant positive correlation with the variation in NMR signals resonating at δ 150–154 from the same material (Fig. S4a). The only exception to such a trend was the case of *Pinus halepensis* and *Picea excelsa*, with higher DNA concentrations in the undecomposed material, suggesting a rapid degradation of DNA with litter age in these coniferous species.

Effects of DNA on target species

In a first *in vitro* experiment, DNA from conspecifics and heterospecifics was supplied in two forms, either as total DNA obtained by total DNA extraction or as fragments produced by sonication of such extracted total DNA. The root growth of all target species was significantly inhibited by the sonicated self-DNA (Fig. 6a; Tables S5, S6), in a concentration-dependent manner (Fig. 6b;

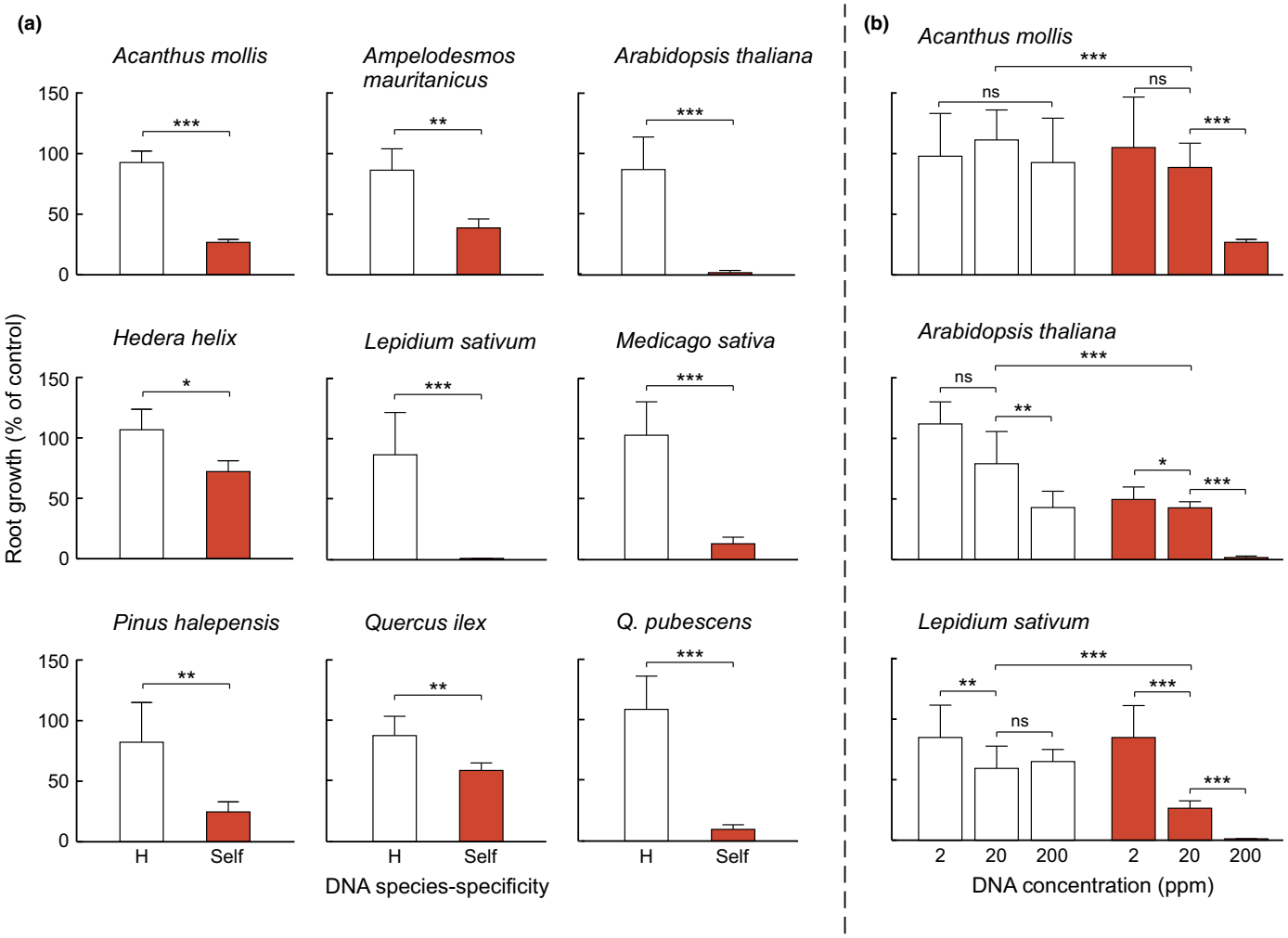

Fig. 6 Effects of heterologous and self-DNA on root growth of different plant species. (a) Root growth of nine plant species treated either with a mixture of heterologous (H) DNA or with their own (self) DNA at a high concentration (200 ppm) (detailed statistics in Table S5). (b) Examples of concentration-dependent effects of heterologous DNA (white bars) and self-DNA (red bars) on root growth of three target species. Heterologous DNA sources were *Arabidopsis thaliana* in the case of the *Lepidium sativum* target, and *L. sativum* for both treatments on *A. thaliana* and *Acanthus mollis*. Data are mean and SD; $n = 20$ for each bar; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, $P > 0.05$; Mann–Whitney–Wilcoxon test (detailed statistics in Tables S6 and S7).

Tables S7), while the mixtures of sonicated heterologous DNA, as well as intact genomic self-DNA (data not shown), did not affect root growth (Fig. 6a).

In a second experiment, DNA was added to two different soils in pots. Self-DNA clearly inhibited *M. sativa* seedling growth in a concentration-dependent manner. The inhibition at intermediate and high concentrations (200 and 2000 ppm) was significant in both soil types, but more evident in sand compared with clay soil (Fig. S5).

Soil history experiment

Medicago sativa plants grown on soils previously used by the same species showed a clear NF effect proportional to the duration of the previous growth period (growth inhibition, compared with bare soil control, was $22 \pm 4\%$ and $35 \pm 6\%$ in soils that had supported growth of *M. sativa* for 180 and 360 d, respectively). The NF effect could be associated with different levels of DNA accumulation in the soil (135 ± 25 and 240 ± 12 ng mg^{-1} in short and long soil histories, respectively). Effects of soil exposure to conspecifics were highly significant compared with the unexposed control and between the two soil histories, both for growth inhibition and for DNA content in the soil (one-way ANOVA, $F_{2,57} = 27.2$, $P < 0.0001$, and $F_{2,27} = 43.7$, $P < 0.0001$, respectively).

DNA accumulation associated with Janzen–Connell distribution

Consistently with our previous findings (Bonanomi *et al.*, 2008), the field survey of an *M. marina* stand showed a clear-cut Janzen–Connell distribution of seedling recruitment (Fig. 7). Such a spatial pattern of seedling inhibition under the crowns of conspecific adult plants was associated with significantly higher DNA accumulation in the soil compared with the surrounding area (Fig. 7; Table S8). Using a pair of *Medicago*-specific primers, unique bands of the expected sizes could be amplified only from DNA extracted from the soil sampled under the canopy (Fig. 7), while we could not detect any amplicons from DNA extracted from soil of the outer zone (data not shown).

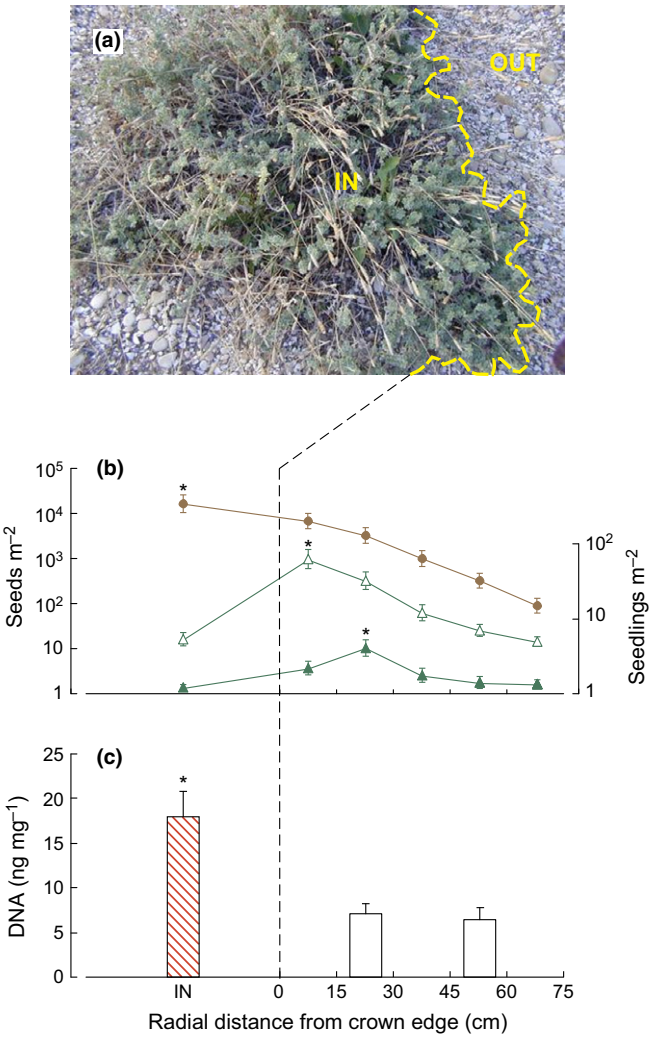

Fig. 7 Distribution of *Medicago marina* recruitment in field conditions and DNA accumulation in soil. (a) Photograph of *M. marina* at the experimental site with an indication of soil sampling zones within (IN) and outside (OUT) the canopy cover. (b) Seed dispersal (left axis) and seedling distribution (right axis) inside the canopy and at increasing distances from the plant edge. Brown circles, seeds; white triangles, seedlings < 1 yr; green triangles, seedlings > 1 yr. (c) DNA concentration in the topsoil (mean and SD of three replicates), inside the canopy and at increasing distance from the plant edge. The dashed bar indicates a positive result for *M. marina* DNA detection in DNA extracted from the soil. Data in all graphs are mean and SD; *, $P < 0.01$ for significantly highest values along transects; post hoc Duncan test (detailed statistics in Table S8).

Discussion

This work demonstrates that, while undecomposed litter is characterized by a general, but labile phytotoxicity, stable autotoxic effects become evident in aged plant litter. Evidence was obtained that self-DNA inhibitory effects may explain litter autotoxicity and also probably contribute to plant–soil NF. In detail, we found that extracellular DNA accumulated in both decayed litter and soil and that, despite the extent of its fragmentation as a result of decomposition processes, it was able to exert significant species-specific inhibitory effects. Moreover, experiments in laboratory conditions showed that purified total self-DNA had size- and concentration-dependent species-specific toxic effects on plant growth. Based on such findings, Fig. 8 represents a conceptual diagram of the autotoxicity model (Mazzoleni *et al.*, 2007, 2010; Cartenì *et al.*, 2012) in which the NF driving factor is proposed to be DNA.

In this study, the chemical changes in litter material observed by ^{13}C NMR were consistent with findings reported in previous studies (Preston *et al.*, 2009; Bonanomi *et al.*, 2011b). All undecomposed litter caused strong inhibition of root growth of different target species, consistent with the results of previous work (Bonanomi *et al.*, 2006, 2011b), showing this to be a general phenomenon not restricted to a few 'allelopathic' plants. The initial phase of decomposition basically consists of plant tissue breakdown and subsequent release of cell contents which may produce inhibitory effects related to the presence of different

toxic, allelopathic compounds (e.g. Rice, 1984). Such compounds, mainly phenols and aromatics of low molecular weight, show a rapid turnover during decomposition processes, with rapid disappearance of observed allelopathic effects. This was confirmed in this work by the analysis of ^{1}H NMR spectra of litter extracts during early decay phases. Moreover, the significant effect of AC addition to undecomposed litter also supported such a phytotoxic hypothesis because AC is known to absorb low-molecular-weight compounds, thus removing their inhibitory effect. Similar effects of AC were also reported in previous studies (Nilsson, 1994; Hille & den Ouden, 2005). As an alternative explanation, some studies reported N immobilization as a mechanism explaining litter inhibitory effects (Hodge, 2004). In our case, and consistent with previous observations (Bonanomi *et al.*, 2011b), the litter C-to-N ratio and N release during decomposition were unrelated to root growth inhibition (Fig. S2), thus excluding N limitation as a possible causal factor for the observed root growth inhibition, at least in the tested species.

In contrast to the phytotoxicity of fresh litter, the inhibitory effects of aged plant residues were highly species-specific, being limited to conspecific material. Decomposition for 120 d greatly reduced and in many cases reversed the inhibitory effect on heterospecific target plants. By contrast, inhibition of root growth on conspecific litter significantly increased with decomposition time. Furthermore, the application of AC did not produce any significant effect on the target species inhibited by their own aged litter (Fig. 2d), in contrast to its clear positive effect on fresh heterospecific litter. These results, reported here for the first time, clearly showed a common pattern of autotoxic inhibition of aged plant litter. The fact that such inhibition was species-specific (affecting only conspecifics), long-lasting (occurring on decomposed litter) and not removable by AC application indicates that the underlying toxic factor is different in nature from the phytotoxic compounds found in fresh litter material. An interesting point deserving further study is the reason for the different sensitivities of plant species to heterospecific and conspecific litter and whether these effects are related to phylogenetic distance.

The functional link between litter chemical composition and the occurrence of species-specific inhibitory effects was assessed by solid-state ^{13}C NMR spectroscopy. Such a method, traditionally used in metabolomic studies, is very useful because it does not rely on extraction and separation of the analytes, but enables detailed characterizations of complex mixtures in their intact solid state, such as soil organic matter and litter samples (Kögel-Knabner, 2002). Interestingly, the correlations between the changes in resonance signals and inhibitory effects were strikingly different for heterospecific and conspecific litter. Restricted NMR signals related to nucleic acids were the only ones negatively correlated with plant root growth over conspecific litter substrates (Fig. 4). This supported the hypothesis of self-DNA being the putative compound involved in litter autotoxicity. In contrast, the positive correlation of the same signals with growth on heterospecific litter could reflect the different role of heterologous DNA as a nutrient source, especially for phosphorous, which is consistent with the findings of previous studies (Paungfoo-Lonhienne *et al.*, 2010). Such a positive correlation with growth may also be related to the reported capability of plants to acquire amino acids as N sources (e.g. Lipson & Näsholm, 2001). Consistent with our results (Figs 5, 7, S3), significant DNA accumulation in litter and soil has been previously reported (e.g. Dilly *et al.*, 2004).

Large amounts of extracellular DNA have been reported to occur both in soil and in marine sediments (Steffan *et al.*, 1988). DNA is able to persist in soil because of its chemical stability and its protection against enzymatic degradation by absorption and binding onto soil minerals and organic matter components (Levy-Booth *et al.*, 2007). DNA persistence is also supported by reports on traces of DNA from transgenic plant sequences in soil, even when the presence of plant tissues is no longer detectable (Dale *et al.*, 2002). None of these studies associated DNA with observations of NF; however, such strong evidence of the ubiquitous persistence of DNA in the environment, coupled with our discovery of self-DNA toxicity, in contrast to reported cases of

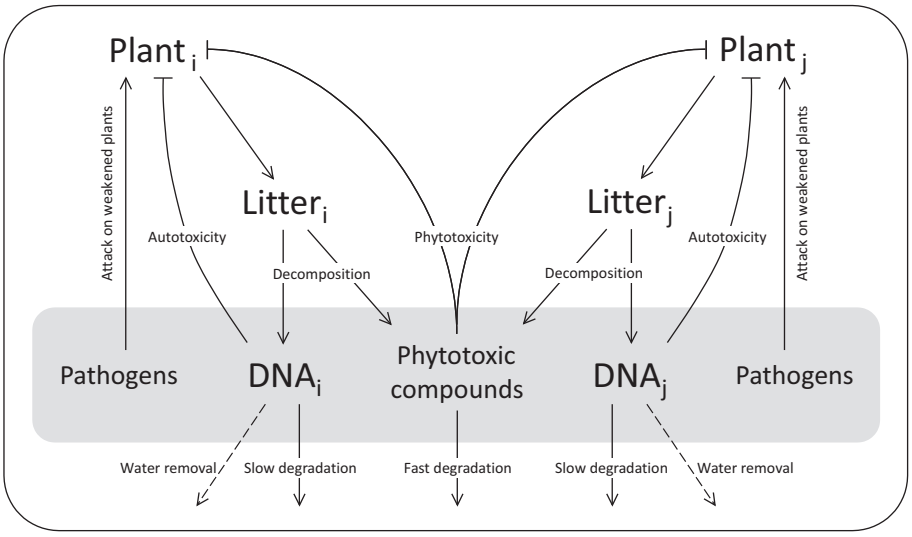

Fig. 8 Conceptual representation of plant–soil negative feedback according to the autotoxicity model. Phytotoxic (allelopathic) compounds are released during the first phases of litter decomposition and exert a generic inhibitory effect on plants. These compounds are quickly degraded and disappear from the soil (shaded area). In contrast, extracellular DNA accumulated during decomposition produces specific inhibitory effects, also increasing susceptibility to attack by soil-borne pathogens. DNA is slowly degraded unless removed by water.

labile allelopathic compounds, is consistent with the occurrence of NF in soils for months, and even years, observed in both natural vegetation (Hawkes *et al.*, 2013) and agro-ecosystems (Miller, 1996).

It is noteworthy that extracellular DNA is not preserved in the environment in its original genomic size, but is fragmented into a broad range of dimensional classes, from hundreds to some thousands of base pairs in length, as we observed in decaying litter. It has to be noted that the total DNA found in both litter and soil systems derives from both decomposing plant tissues and from microbial turnover (Levy-Booth *et al.*, 2007). Further studies are needed to clarify the relative quantities of these DNA sources in both litter and soil in natural conditions. Other research work will specifically address the quantitative discrimination of plant and microbial DNA associated with plant–soil NF.

Previous studies demonstrated an association between NF and the occurrences of species-specific pathogenic microbial communities (e.g. van der Putten *et al.*, 1993; Packer & Clay, 2000; Kardol *et al.*, 2007). In this study, in addition to the evidence of self-DNA toxicity in controlled *in vitro* tests, we have also reported a spatial correspondence between DNA accumulation and conspecific inhibition associated with a clear Janzen–Connell distribution in field conditions. The known capability of plant root cells to take up extracellular DNA (Paungfoo-Lonhienne *et al.*, 2010), together with the discovery here reported that self-DNA is toxic, indicates that self-DNA inhibitory effects should be considered as a further mechanism explaining species-specific NF.

Soil sterilization has been reported to reduce NF effects (e.g. Packer & Clay, 2000; Klironomos, 2002; Kardol *et al.*, 2007). This has typically been seen as evidence of the biotic origin of NF. However, it is well known that the usual sterilization methods (e.g. autoclaving and irradiation) also affect DNA molecules (see e.g. Gefrides *et al.*, 2010). We also observed (data not shown) that thermal treatments of extracted DNA samples affected their fragmentation level and toxic effect. This means that sterilization cannot be taken as conclusive proof of the microbial origin of NF. Indeed, it should be seriously considered that weakening of a plant as a result of exposure to extracellular self-DNA could eventually increase its susceptibility to pathogen attack. Future studies will be necessary to clarify the co-occurrence and interactions of different NF mechanisms in different environments (van der Putten *et al.*, 2013), including tropical forests, where NF is reported to play a crucial role in the maintenance of species diversity (Mangan *et al.*, 2010; Mazzoleni *et al.*, 2010). In this context, the observation of reduced DNA accumulation in the decomposed litter of coniferous species is also interesting because such a degradation pattern, possibly related to the acidic conditions of these litter types, might be related to the frequent occurrence of monospecific coniferous forests. In fact, according to our conceptual model (Fig. 8), the lack of accumulation of DNA in *Pinus halepensis* and *Picea excelsa* litter material should result in lower autotoxicity levels affecting the coexistence of the species. Furthermore, in relation to the occurrence of NF in different ecosystems, an interesting issue to be considered for future research is the relationship between the timings of litterfall, decomposition and germination.

Extracellular DNA has been found to serve several biological functions. These include the formation of neutrophil nets as protection from pathogens (Brinkmann *et al.*, 2004), roles as structural components of microbial biofilms (Whitchurch *et al.*, 2002), modulation of plant–pathogen interactions (Wen *et al.*, 2009), sources of nutrients for microbes and plants (Paungfoo-Lonhienne *et al.*, 2010), and sources of horizontal gene transfer (Doerfler *et al.*, 1995). Our discovery highlights an unexpected additional function of DNA in the litter–soil system where, if accumulated, it may have species-specific inhibitory effects. Clear toxic effects (e.g. root apex necrosis; data not shown) were found at high concentrations of self-DNA inducing death of both seeds and seedlings. However, recovery tests after exposure to low concentrations (data not shown) showed some capability to resume both germination and growth after inhibition. In the latter case, the observed reduction in root length may be associated with structural or chemical changes related to the possible signaling functions of extracellular self-DNA. For instance, there could be a signal to restrain growth in parts of the soil containing conspecific DNA because of the danger of pathogen attack. This is an interesting point deserving further investigation in the future, focused on morphological and biochemical changes induced by the exposure to extracellular self-DNA, to clarify such a double response mechanism, that is, toxicity at high concentrations and signaling at lower concentrations.

Notably, only fragmented DNA created negative responses; this is probably because only smaller fragments (< 500 bp) could enter the cells to produce their inhibitory effects. What can be the processes involved in such an inhibitory effect? In this work we have not yet addressed this issue at the molecular level. However, different cellular mechanisms may be hypothesized, such as the well-known processes of interference based on sequence-specific recognition of small-sized nucleotide molecules (Ecker & Davis, 1986). Such processes may cause inhibition of cell functionalities at multiple levels causing blocks to the transfer of genetic information from DNA to protein (Hannon, 2002) or affecting genome stability (Gruenert *et al.*, 2003). Similar processes have been reported as a general defense mechanism against pathogens (Obbard *et al.*, 2009) and are applied for specific gene silencing by antisense oligonucleotides (ASOs) in both medicine (Mansoor & Melendez, 2008; Zhigang *et al.*, 2010) and agriculture (Baum *et al.*, 2007; Tian *et al.*, 2009). On these bases, we propose the hypothesis that a mixture of random self-DNA fragments, such as those produced by the breakdown of the total genome during decomposition of litter and soil organic matter (simulated in our experiment by the sonication of total extracted DNA), may cause either complete interference or inhibition of the whole genome functionality. In this context, it is noteworthy that living cells evolved several defense and recognition mechanisms to protect them from extracellular DNA uptake (Stenglein *et al.*, 2010). However, little is known about the effective ability of cells to recognize extracellular self-DNA and eventually to activate specific defense responses against it. These are topics certainly deserving further in-depth studies. Finally, this unexpected function of extracellular DNA at the ecosystem level could also be related to the general issue of self/nonself recognition in plant

roots, the mechanisms of which are mostly unexplained (e.g. Falik *et al.*, 2003; Gruntman & Novoplansky, 2004; Biedrzycki *et al.*, 2010). Perhaps the sensing of the presence of conspecific DNA allows plants to adjust their root growth behavior.

Conclusions

Here, for the first time, we provided evidence that extracellular DNA produces species-specific inhibitory effects on plants by reducing conspecific root growth and seed germination without affecting heterospecifics. Such findings provide strong evidence of a chemical basis of autotoxicity which should be taken into account in mechanisms explaining plant–soil NF.

This discovery is consistent with previous modeling work on plant–soil NF which assumed a water-soluble inhibitor as the causal factor explaining the paradox of mangrove monospecific stands adjacent to extremely rich tropical forests (Mazzoleni *et al.*, 2010).

It is clear that the presented findings raise many issues deserving further investigation, especially to clarify the physiological and biochemical mechanisms underlying the inhibitory effect of extracellular self-DNA. Furthermore, the discovery reported poses some general questions with implications for the life sciences: Is this mechanism limited to plants or does it also occur in other biological systems? May the absence of accumulated DNA in the soil also explain the known strong invasiveness of some exotic species? May the exposure of plants to their own extracellular DNA accumulated in the substrate also act as a driver in evolutionary processes? In fact, the interactions between competing/coexisting species are *per se* driving factors of speciation and evolution. Therefore, an interesting point will be to clarify whether the exposure to extracellular self-DNA can act as a selective pressure factor leading the organisms either to colonize new environments or to modify their identity, that is, 'move or change'.

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Supporting Information

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

Fig. S1 Root observation plates from the litter toxicity experiment.

Fig. S2 Litter inhibitory effect unrelated to N immobilization.

Fig. S3 Litter inhibitory effect related to ^{13}C NMR spectral signals diagnostic of the nitrogen bases of nucleic acids.

Fig. S4 Correlation between NMR signals and DNA content in litter, and DNA fragmentation patterns of all litter material.

Fig. S5 Effect of self-DNA on *Medicago sativa* growth in a glasshouse experiment.

Table S1 Treatments affecting root growth on decomposing litter

Table S2 Root growth on either heterospecific or conspecific decomposing litter

Table S3 Variation of ^{1}H NMR regions and corresponding classes of organic compounds in decomposing litter

Table S4 Variation of ^{13}C NMR regions and corresponding C types in decomposing litter

Table S5 Root growth in seedlings exposed to either self-DNA or heterologous DNA

Table S6 Effects of purified DNA concentration on plant root growth

Table S7 Effects of purified DNA source on plant root growth

Table S8 Distribution of *Medicago marina* recruitment and DNA accumulation in soil

Methods S1 Assessment of litter effects on target plant species.

Methods S2 Impact of soil texture on DNA toxicity.

Methods S3 Soil history experiments.

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