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Litter Inhibitory Effects on Soil Microbial Biomass, Activity, and Catabolic Diversity in Two Paired Stands of *Robinia pseudoacacia* L. and *Pinus nigra* Arn.

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Abstract: Research Highlights: Plant cover drives the activity of the microbial decomposer community and affects carbon (C) sequestration in the soil. Despite the relationship between microbial activity and C sequestration in the soil, potential inhibition of soil microbial activity by plant cover has received little attention to date. Background and Objectives: Differences in soil microbial activity between two paired stands on soil at a very early stage of formation and a common story until afforestation, can be traced back to the plant cover. We hypothesized that in a black locust (*Robinia pseudoacacia* L.) stand the high-quality leaf litter of the tree, and that of the blackberry (*Rubus fruticosus* L.) understory had an inhibitory effect on soil microbial community resulting in lower mineralization of soil organic matter compared to the paired black pine (*Pinus nigra* Arn.) stand. Materials and Methods: We estimated potential mineralization rates (MR), microbial (MB), and active fungal biomass (AFB) of newly-shed litter, forest floor, and mineral soil. We tested the effects of litters' water extracts on soil MR, MB, AFB and its catabolic response profile (CRP). Results: Newly-shed litter of black locust had higher MR than that of blackberry and black pine; MR, MB, and AFB were higher in forest floor and in mineral soil under black pine than under black locust. Water extracts of black locust and blackberry litter had a negative effect on the amount, activity of microorganisms, and CRP. Conclusions: The results demonstrate the potential for black locust and blackberry litter to have a marked inhibitory effect on decomposer microorganisms that, in turn, reduce organic matter mineralization with possible consequences at the ecosystem level, by increasing C sequestration in mineral soil.

Keywords: black locust; blackberry; black pine; litter N; P; Mn; litter organic components; ^{13}CPMAS NMR; ^{1}H NMR

1. Introduction

Forest ecosystems store carbon (C) in aboveground biomass (trees and understorey plants) belowground biomass (e.g., roots), forest floor, and coarse woody debris, as well as in the mineral soil that is also a significant component in C sequestration. Plant cover drives the activity of soil microbial decomposer community [1] and affects total carbon stock and allocation in the forest floor and mineral soil [2,3]. The chemical composition of the litter species (e.g., nutrient and lignin concentrations,

C-to-nitrogen (N), and lignin-to-N ratios control litter degradability and the environmental conditions for soil microbes [4]. Some components of leaf litter such as polyphenols, including tannins, which are water-soluble polyphenolic compounds, have toxic effects on microbial metabolism [5] and inhibit fungal colonization [6]. Coté and Thibault [7] have reported a potential allelopathic activity of raspberry (*Rubus idaeus* L.) foliar leachates on the growth of ectomycorrhizal fungi associated with black spruce (*Picea mariana* (Mill.) BSP). Substances that suppress microbial activity in humus have been found in crowberry (*Empetrum nigrum* L.), a species common in boreal forests [8] and in walnut (*Juglans* sp.) leaves in temperate and subtropical forests [9,10].

There is a lack of studies with a focus on litter inhibitory effects that concurrently examine organic matter decomposability, microbial community, and organic matter mineralization in soil under different tree species. Indeed, the overall current knowledge of tree species effects on soil C stock dynamics is still poor [11] in spite of a growing need for guidelines in tree species selection for afforestation and forest management with the perspective of greenhouse gases and climate change mitigation.

The present paper is the third in a series reporting on our project "Carbon sequestration in the soil of paired plantations of black locust (*Robinia pseudoacacia* L.) and black pine (*Pinus nigra* Arn.)". The results of the two previous papers [12,13] have shown that C sequestration in the forest floor is higher under black pine and congruent with the higher C input with litter fall and the lower limit value for pine litter. Limit value is the level of accumulated mass loss at which the decomposition process either continues at a very low rate or possibly stops [14]. In contrast, C sequestration in the mineral soil was higher under black locust despite the lower C input with litter fall and the higher limit value for black locust litter [13]. Unlike the aboveground compartment we do not have estimates of C inputs from belowground litter and/or from root exudation; thus, their role in C sequestration in the mineral soil of the two stands is unknown.

Forest soil may function as a C sink if organic matter inputs to soil profile are not matched by losses due to leaching from the soil profile or to decomposition, e.g., if decomposition is inhibited by resistant humic substances or by physical protection of organic matter in soil aggregates. The reason why C accumulates to a greater extent in the black locust than in the black pine mineral soil is an open question. Vesterdal et al. [15] showed that tree species with lower C-to-N ratio in leaf litter had smaller C pools in the organic layers but larger C pools in mineral soil, like in the case of black locust and black pine. On the other hand, a higher concentration of water-soluble organic matter in the forest floor of the black locust stand [13] suggests that here a higher amount of C may move in the form of soluble compounds from organic to mineral layers, thus, accounting for the higher C sequestration in the mineral soil (a process named secondary sequestration by Berg et al. [16]). Finally, according to the MEMS (Microbial Efficiency-Matrix Stabilization) framework [17] substrate quality affects soil organic matter (SOM) formation because high-quality litter compared with low-quality litter is utilized more efficiently by microbes and proportionally more microbial-derived organic matter would be formed. This microorganism-derived organic matter needs to be protected by the soil matrix to be stabilized. The capacity for stabilization of microbial C is higher in fine-textured soils where microaggregates and clay-surfaces are abundant, and in soils with high allophane content [17].

A missing factor from the model above is a possible allelopathic effect of litter on the microbial decomposer community, a topic of great interest considering that this is a further driver of C sequestration in soil. The leaves of black locust are known to contain allelopathic compounds that inhibit the growth of other plants [18], thus, facilitating the colonization of new habitats [19]. Interestingly, the aromatic fraction of black locust litter extracts includes a substance, i.e., 4–hydroxyacetophenon [20], that is known for its allelopathic potential towards plants and microorganisms. Moreover, the invasion of native pitch pine (*Pinus rigida* Mill.) and oak (*Quercus velutina* Lam. and *Q. alba* L.) forests by black locust may alter the soil nitrification process and N availability [21]. Most interestingly, an increase in the density of black locust in Mediterranean evergreen mixed forests of stone pine (*Pinus pinea* L.) and holm oak (*Quercus ilex* L.) was found to be associated with a decrease of the microbial activity in the mineral soil [22]. Furthermore, the studied

black locust stand has a dense understory of blackberry bushes that contribute a C input to the soil system as high as that from the overstory foliage [13]. Blackberry (*Rubus fruticosus* L.) has well-known antibacterial and antifungal properties [23,24], but, so far, the effect of its litter on soil microbial community has not been tested.

According to Cserenyés and Támas [25], substances released from litter in black pine plantations are able to inhibit the germination and growth of various indigenous plant species. We found no data in the literature dealing with inhibitory effects of black pine litter on soil microorganisms. However, litter extracts of a related species of pine, i.e., Scots pine (*Pinus sylvestris* L.), have been found to inhibit the growth of fungal strains isolated from needles collected at other sites [26]. Tannin extracts from chir pine (*Pinus roxburghii* Sarg.) were found to be inhibitory to several microbes of agricultural importance [27].

The specific aim of the present study was to demonstrate the potential for leaf litter to have inhibitory effects on soil microbial community. We compare chemical properties and microbial biomass and activity of black locust, blackberry, and black pine litters. We concurrently assess chemical properties, microbial biomass, and activity as well as community-level catabolic diversity of mineral soil (0–5 cm) from the paired black locust and black pine stands. We test the inhibitory effects of litter extracts on soil microbial biomass and activity, and community-level catabolic diversity, by amendment of mineral soil with water extracts from newly-shed black locust, blackberry, and black pine litters, and from black locust forest-floor litter. We perform the inhibitory tests both on soil from the same stand as the litter and on soil from the other stand on the presumption that soil microbial community is adapted to the stand's own litter while a not adapted soil may respond clearly to allelopathic compounds. Ultimately, the present study tests the hypothesis that in the black locust stand the high-quality leaf litter of the tree, and that of the blackberry understory inhibit soil microbial community and reduces soil organic matter mineralization compared to the paired black pine stand.

2. Materials and Methods

2.1. Study Location and Site Description

The site (40°49′ N, 14°26′ E) was located within the Vesuvius National Park (Naples, South-Italy) at 810 m a. s. l. in an area called "Valle dell'Inferno". The paired stands, one with black locust and one with black pine were planted in 1970 at the same site, on fallout scoriaceous lapilli from the 1944 eruption. Before afforestation, the parent material had been colonized by soil biota, lichens, and herbaceous plants [28]. The two stands may be considered similar to a common garden [4], and as such, they allow us to specifically evaluate the influence of tree species on soil through the quality of litter being decomposed and the forest floor generated under the canopy. The basal areas were similar, i.e., 26.4 and 24.9 m^2ha^{-1}, in the black locust and in the black pine stand, respectively [12]. The black locust stand had a dense understory of blackberry with scattered herbs and grasses in the ground layer. The black pine forest had a very sparse understory of Mount Etna broom (*Genista aetnensis* (Biv.) DC.) and young holm oaks.

The soil, a Lepti-Vitric Andosol according to World Reference Base For Soil Resources. World Soil Resource (WRB-FAO) 1998, had a weak profile differentiation with no evidence of pedogenetic processes below 15 cm depth, a moderately coarse texture and good oxygen availability [29]. In both stands, the upper mineral soil (0–5 cm) had a very abundant (>50%) coarse fraction and sand accounted for >96% of the fine fraction (more details in De Marco et al. [13]).

The climate of the study area is typically Mediterranean, with a mean annual rainfall of 910 mm mostly restricted to autumn and winter, and a mean annual temperature of 14.2 °C (Gran Cono Meteorological Station, about 1000 m a. s. l., data collected from 2003 to 2009).

2.2. Newly-Shed Litter Sampling

Newly-shed litter (NL) from black locust and black pine was collected throughout the years 2006–2008, using 10 circular litter traps, each with a sampling area of 0.79 m^2, placed randomly at about 1 m above the ground. In the black locust stand, 20 rectangular litter traps, each of 0.15 m^2 were placed under the blackberry bushes to collect the understory litter. The collector was a polyester net with a mesh size of ca. 1 mm.

2.3. Forest Floor and Mineral Soil Sampling

In the years 2007–2008 (December, April, and June), bulk samples (all layers combined) of forest-floor litter (FF) were collected at 20 spots in each of the two stands using a quadratic sampler of 20 × 20 cm; after removing living grasses and weeds, the samples were immediately transported to the laboratory and stored at 4 °C. At the same spots, mineral soil was sampled, using a corer of 25 cm^2 to a maximum depth of 5 cm. The cores were sieved separately (2 mm mesh) and processed for chemical and biological analyses.

2.4. Chemical Analyses of Litter and Soil

Litter and soil samples were oven-dried at 75 °C to constant mass and ground to a fine powder using an agate mortar and pestle (Fritsch Analysette Spartan 3 Pulverisette 0, Fritsch, Berlin, Germany). A CNS analyzer-Thermo Finnigan, Flash 112 Series EA (Thermo Finnigan, Milan, Italy) was used to determine C and N content by gas chromatography. Manganese (Mn) content was measured by atomic absorption spectrometry via flame (AAS; SpectrAA 20 Varian) after digestion in a mixture of hydrofluoric and nitric acid (HF 50% v/v: HNO_3 65% v/v = 1:2) in a Milestone (mls 1200) Microwave Laboratory System. Phosphorus (P) content was measured with the molybdate colorimetric method [30] after digestion with sulfuric acid (5 M), hydrofluoric acid (50%) and hydrogen peroxide (H_2O_2) (30%), using a Hitachi U-1500 spectrophotometer (Hitachi, Tokyo, Japan).

To determine water-soluble C and N fractions, oven-dried samples of litter (0.5 g) or soil (20 g) were soaked in 70 mL and 200 mL, respectively, of distilled water and stirred for 24 h (Universal Table-Shaker 709, 130 rpm) with two sonications [31]. The water extracts, thus, obtained were centrifuged at 5000 rpm for 15 min, filtered (Whatman Ø15 µm) and lyophilized to determine C and N contents by gas-chromatography (CNS analyser-Thermo Finnigan, Flash 112 Series EA).

Soil pH-H_2O (1:2.5 soil:water) and cation exchange capacity (CEC) (replacement of exchangeable cations by ammonium acetate) were determined on the fine fraction following the protocol by Ministero delle Politiche Agricole e Forestali [32].

Samples of newly-shed litter of black locust, blackberry, and black pine, as well as samples of forest-floor litter from both stands, were analyzed for ^{13}C CPMAS NMR spectra, using a Bruker AV300 Spectrometer Solid-State ^{13}C Nuclear Magnetic Resonance (NMR) with cross polarization and magic angle spinning (CP/MAS). The mineral soil (MS) samples of the two stands contained considerable amounts of mineral and paramagnetic compounds, which decreased the sensitivity of solid-state ^{13}C NMR, giving poor resolution spectra and low signal-to-noise ratios; therefore, the NMR data for mineral soil were not considered. The ^{13}C CPMAS spectra showed peaks in the whole range of chemical shifts between 0 and 200 ppm, with overlapping peaks due to the presence of a great diversity of compounds in the SOM samples. The spectra were divided into six chemical shift regions representative of the major types of carbon present in the litter (Carboxyl-C: 200–160 ppm; Phenol-C: 160–145 ppm; Aryl-C: 145–110 ppm; O-Alkyl-C: 110–60 ppm; Methoxyl-C: 60–45 ppm, and Alkyl-C: 0–45 ppm). Carbon types have been identified in previous reference studies [33]. The mass of each C fraction per gram of litter/forest floor was calculated from peak integrations of NMR spectra and the total C per gram; moreover, the Methoxyl C-to-Phenol C ratio, aromaticity index (Phenol C + Aryl-C-to-O-Alkyl C), and hydrophobicity index (Aryl-C + Phenol C + Alkyl-C-to-Carboxyl-C + O-Alkyl-C) were calculated.

2.5. Microbiological Analyses

The mineralization rate (MR), total microbial biomass (MB), and active fungal biomass (AFB) were determined for newly-shed litters, forest floors' litter, and the mineral soil (0–5 cm depth) within a week after sampling.

MB was evaluated by the substrate-induced respiration (SIR) method [34]. CO_2 evolution was determined by gas chromatography (Gas Chromatograph 8000 series, Fisons Instruments, Dearborn, MI, USA), after addition of 2 mL D-glucose solution (75 mM) to a quantity of litter/soil corresponding to 1.0 g dry mass, and incubation for 4 h (25 °C in darkness). SIR rates were converted into MB using the equation: $\mu g\ C\ g^{-1} = 50.4 \times$ respiration rate ($\mu L\ CO_2\ g^{-1}$ litter/soil h^{-1}) [35].

MR was evaluated, by gas chromatography, as CO_2 evolved from samples incubated in microcosms for 4 h under standard conditions (25 °C, in darkness).

AFB was measured by the membrane filter technique [36], using fluorescein diacetate [37]. One gram of litter or soil sample was dispersed in 100 mL phosphate buffer (60 mM; pH 7.5) using a blender at 6000 rev min^{-1}. The suspension was transferred to a membrane filter (0.45 µm mesh size) and stained. After clearing by immersion oil, 20 microscopic fields were observed at a magnification of 400×. Fungal mycelium was estimated by the intersection method and mass calculated according to Berg and Söderström [38].

The catabolic response profile (CRP) was assessed by measuring the short-term respiration after addition of 25 low-molecular simple organic substrates normally present in the rhizosphere [34]. CO_2 evolution was measured as reported above after incubation for 4 h under standard conditions. The substrates used in the assay were: 7 amino acids (L-arginine, L-asparagine, L-glutamic acid, L-glutamine, L-histidine, L-lysine, L-serine), 3 carbohydrates (D-glucosamine, D-glucose, D-mannose), 15 carboxylic acids (L-ascorbic, citric, fumaric, gluconic, α-ketobutyric, α-ketoglutaric, α-ketovaleric, DL-malic, malonic, pantothenic, quinic, succinic, tartaric, uric, urocanic acids). The various substrates were added as 2 mL solutions (15 mM for amino acids; 75 mM for carbohydrates; 100 mM for carboxylic acids) to 1 g equivalent dry weight of soil in 25 mL glass bottles sealed with Vacutainer stoppers. Two hours after substrate addition, all bottles were shaken for 5 min and again before sampling head space gas with a syringe.

2.6. Effect of Leaf-Litter Water Extracts on Microbial Activity and Biomass In Mineral Soil

We tested the inhibitory effects of black locust, blackberry, and black pine newly-shed leaf litters on mineral soil of both stands. In contrast, only black locust forest-floor litter was tested for the potential to inhibit soil microbial community. Indeed, it was considered not necessary to test the inhibitory effect of the black pine forest floor to answer our research question. Moreover, the significantly higher potential mineralization of organic matter and the higher amount of microbial biomass of the black pine forest floor indicated the absence of constraining conditions compared to the paired black locust stand.

Leaf-litter water extracts equivalent to leachate from litter to soil in a field setting, were prepared taking into account the average daily precipitation at Mount Vesuvius (range from c. 2 to 5 mm, i.e., c. 2 to 5 L m^{-2}) and the amount of foliar litter in the forest floor (155 and 169 g m^{-2} in black locust and black pine stand, respectively [13]). The aqueous extract was obtained by mechanically shaking the samples with distilled water (solid-to-liquid ratio = 5 g /50 g L^{-1}) for 5 h [31]. After centrifuging at 4000 rpm for 10 min, the supernatants were filtered (0.22 µm mesh size) and diluted (10 g L^{-1}; 1 g L^{-1}; 0.01 g L^{-1}) with distilled water. Allelopathic effects of litter extracts were tested on mineral soil (0–5 cm) from both stands. Soil samples were amended with litter aqueous extracts to 55% of water-holding capacity (WHC). Control samples received distilled water. The samples were incubated in the dark at 25 °C for 10 days and analyzed for MR, MB, AFB, and CRP as described above.

The chemical composition of the water extracts was assessed by ^1H NMR spectra, using a Brucker AV 400 Spectrometer Liquid–state (frequency: 400.13 MHz; pulse delay: 1.0 s; acquisition time: 278 s; line broadening factor: 1 Hz). The ^1H NMR spectra indicated the presence of hydrogens belonging to different functional groups. Hence, for data analysis, the ^1H NMR spectra were divided into five

regions according to the functional groups [39]. The identified regions were those of Carboxylic acids/Aldehydes (12–8 ppm), Aromatic compounds (8–6 ppm), Carbohydrates (6–4 ppm), O-Alkyls (4–3 ppm), and Alkyls (3–0 ppm). The relative concentrations of the functional groups were calculated by integrating the areas of corresponding peaks in the ^{1}H NMR spectrum.

2.7. Statistical Analysis

The statistical analyses were performed using Systat_SigmaPlot_12.2 software (Jandel Scientific, San Jose, CA, USA). Data were reported as means of the different samplings and were checked for normality and heteroscedasticity and transformed when necessary. Student *t*-test was used to assess the significance of differences between stands. One way-ANOVA was used to assess the significance of differences for chemical parameters between litter species. Two-way ANOVA followed by Holm-Sidak test was used to assess the significance of differences between forest stands and among samples of NL, FF, and MS for C fractions (^{13}C CPMAS NMR) and for microbiological parameters as well as to assess differences among soil samples amended with litter extracts.

Principal component analysis (PCA) was performed on CPR data of black locust and black pine soil amended with water extracts of NL and FF black locust litter. A matrix of 6 columns (soils) and 25 lines (substrates listed in Figure 2) was processed using package Syn-tax 2000 [40].

3. Results

3.1. Litter and Soil Chemical Characteristics

The newly-shed litters of black locust and black pine somewhat differed for nutrient concentration and fractions of soluble C and N (Table 1). Blackberry NL had lower N and higher Mn concentrations compared to black locust ($p = 0.02$, $p = 0.03$ respectively) and black pine ($p = 0.04$, $p = 0.004$ respectively) NL; black pine NL was richer in P compared with those of black locust ($p = 0.04$) and blackberry ($p = 0.002$).

Forest floors of the two stands differed in pH, concentrations of ash, P, soluble C, and N, as well as in C-to-N and N-to-P ratios (Table 1). Differences in pH and chemical composition between the two stands also occurred in the mineral soil (Table 1). The black locust soil had higher pH ($p = 0.02$) and CEC and was richer in nutrients ($p < 0.05$) and soluble N ($p < 0.001$) than the soil of black pine. Soil water content was similar in the two stands and ranged between 33% and 60% of water holding capacity throughout the sampling season.

Differences in organic components between newly-shed litters of black locust, black pine, and blackberry, as well as between forest floors of the two stands, are shown by ^{13}C CPMAS NMR spectra (Table 2). The Methoxyl C-to-Phenol C (MC-to-PC) ratio was lowest in NL of black locust and highest in that of blackberry, with $p = 0.003$ and $p = 0.002$ respectively, for the comparison of black locust with blackberry and black pine, and $p = 0.01$ for the comparison black pine-blackberry. The degree of aromaticity and the hydrophobicity index (Table 2) were higher in the black locust plantation with $p = 0.001$ for the difference in aromaticity between black pine and black locust and between black pine and blackberry; hydrophobicity index differed significantly between black locust and blackberry ($p = 0.01$) and between black locust and black pine ($p < 0.001$). The degree of aromaticity and the hydrophobicity index increased from NL to FF ($p < 0.01$).

3.2. Mineralization Rate, Microbial Biomass, Active Fungal Biomass

The newly-shed litter of black locust had a significantly higher microbial respiration rate (Figure 1) compared to those of blackberry ($p = 0.002$) and black pine ($p = 0.002$). In contrast, MR of forest floor and mineral soil were significantly higher ($p < 0.001$) in the pine stand (Figure 1).

The three types of NL did not differ for microbial biomass, whereas active fungal biomass was significantly higher ($p = 0.005$) in black pine litter than in that of black locust and blackberry. Forest

floor and mineral soil had significantly higher MB ($p < 0.01$) and AFB ($p < 0.001$) in the pine stand than in the black locust stand (Figure 1).

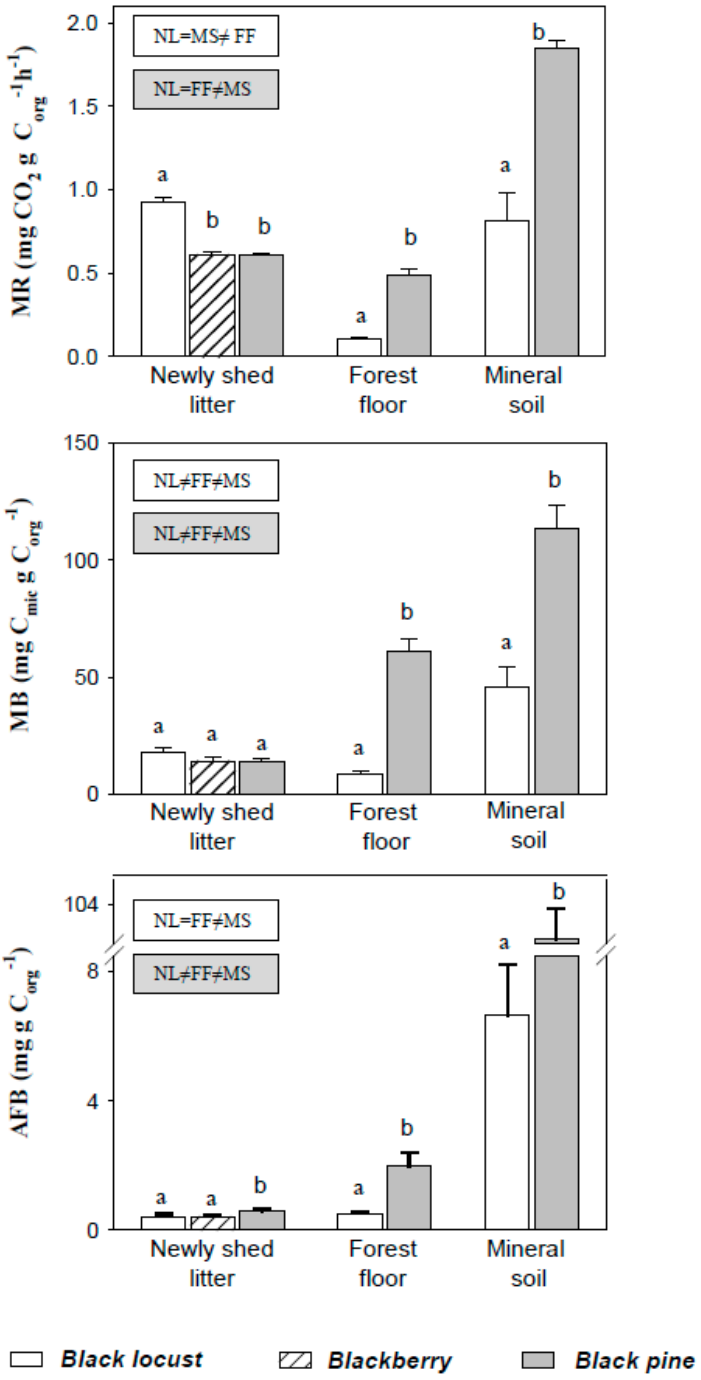


Figure 1. Mineralization rates (MR), microbial biomass (MB) and active fungal biomass (AFB) for Newly-shed leaf litter, Forest-floor litter, and Mineral soil collected in two 40-year-old paired forest stands at Mount Vesuvius. Data are reported as the mean (±SE) from 10 samples for newly-shed litter and 20 samples for forest-floor litter and mineral soil of 0–5 cm depth. Different lowercase letters indicate statistically significant differences ($p < 0.05$) between types of newly-shed litter and between stands for forest floor and mineral soil.

Table 1. Main characteristics of newly-shed litters, forest-floor litter and mineral soil of paired stands of black locust (*Robinia pseudoacacia)* and black pine (*Pinus nigra*) at Mount Vesuvius.

	pH-H_2O	Ash/CEC	P	Mn	N	C-to-N	N-to-P	Soluble C	Soluble N	Soluble N	Soluble C-to-N
			mg g^{-1}	μg g^{-1}	mg g^{-1}			mg g^{-1}	mg g^{-1}	% of total N	
Newly-shed		Ash (mg g^{-1})									
Black locust	-	§ 7.0 ± 0.3 ^{a}	1.7 ± 0.6 ^{a}	§ 42.1 ± 7.9 ^{a}	§ 24.6 ± 0.4 ^{a}	§ 19.5 ± 0.9 ^{a}	14.5 ± 0.8 ^{a}	122.3 ± 4.4 ^{a}	10.6 ± 0.9 ^{a}	43.1 ± 1.5 ^{a}	11.7 ± 0.9 ^{a}
Blackberry	-	§§ 2.0 ± 0.3 ^{b}	0.8 ± 0.07 ^{a}	§§ 65.2 ± 1.9 ^{b}	§§ 16.9 ± 0.3 ^{b}	§§ 27.4 ± 1.3 ^{b}	21.2 ± 0.5 ^{b}	107.3 ± 3.5 ^{a}	7.1 ± 0.3 ^{b}	28.8 ± 1.1 ^{b}	15.2 ± 0.5 ^{ab}
Black pine	-	§ 2.8 ± 0.9 ^{b}	2.56 ± 0.8 ^{b}	§ 29.0 ± 2.0 ^{a}	§ 23.0 ± 1.8 ^{a}	§ 22.1 ± 1.0 ^{a}	9.0 ± 1.8 ^{c}	57.7 ± 5.8 ^{b}	3.4 ± 0.4 ^{c}	14.0 ± 1.4 ^{c}	17.0 ± 1.3 ^{b}
Forest floor											
Black locust	6.03 ± 0.13 ^{a}	4.5 ± 0.6 ^{a}	0.51 ± 0.11 ^{a}	57.4 ± 3.0 ^{a}	23.01 ± 0.61 ^{a}	16.20 ± 0.98 ^{a}	45.1 ± 0.8 ^{a}	81.54 ± 2.31 ^{a}	9.05 ± 1.03 ^{a}	39.46 ± 1.56 ^{a}	8.90 ± 1.33 ^{a}
Black pine	4.75 ± 0.17 ^{b}	6.9 ± 0.8 ^{b}	1.11 ± 0.18 ^{b}	62.1 ± 13.1 ^{a}	13.40 ± 1.52 ^{b}	31.57 ± 3.15 ^{b}	12.1 ± 1.1 ^{b}	16.40 ± 0.82 ^{b}	2.49 ± 0.62 ^{b}	18.67 ± 1.63 ^{b}	5.96 ± 1.25 ^{a}
Mineral soil		CEC (cmolc Kg^{-1})									
Black locust	§ 5.59 ± 0.10 ^{a}	§§ 12.19	0.43 ± 0.06 ^{a}	§ 18.6 ± 0.9 ^{a}	§ 5.20 ± 0.58 ^{a}	6.48 ± 0.79 ^{a}	12.1 ± 0.8 ^{a}	9.11 ± 0.90 ^{a}	3.22 ± 0.13 ^{a}	61.92 ± 1.24 ^{a}	2.81 ± 1.11 ^{a}
Black pine	§ 4.17 ± 0.18 ^{b}	§§ 4.92	0.28 ± 0.04 ^{b}	§ 12.1 ± 1.4 ^{b}	§ 3.10 ± 0.18 ^{b}	8.86 ± 1.19 ^{a}	11.1 ± 0.6 ^{a}	5.77 ± 1.21 ^{a}	1.12 ± 0.10 ^{b}	36.13 ± 0.98 ^{b}	5.24 ± 0.89 ^{b}

Values are means ± standard error (N = 10 for newly-shed litter; N = 20 for forest floor, and mineral soil 0–5 cm depth). Different lowercase letters indicate statistically significant differences between litter species (One-way ANOVA, $p < 0.05$) or between forest stands (*t*-test, $p < 0.05$). § Source: De Marco et al., [12]; §§ Source: De Marco et al., [13].

Table 2. Mass of C fractions (^{13}C-NMR data), Methoxyl C-to-Phenol C ratio, Aromaticity (Phenol C + Aryl-C -to- O-Alkyl C) and Hydrophobicity (Aryl-C + Phenol C + Alkyl-C –to- Carboxyl-C + O-Alkyl C) indices in newly-shed litter and forest-floor litter from two paired forest stands at Mount Vesuvius.

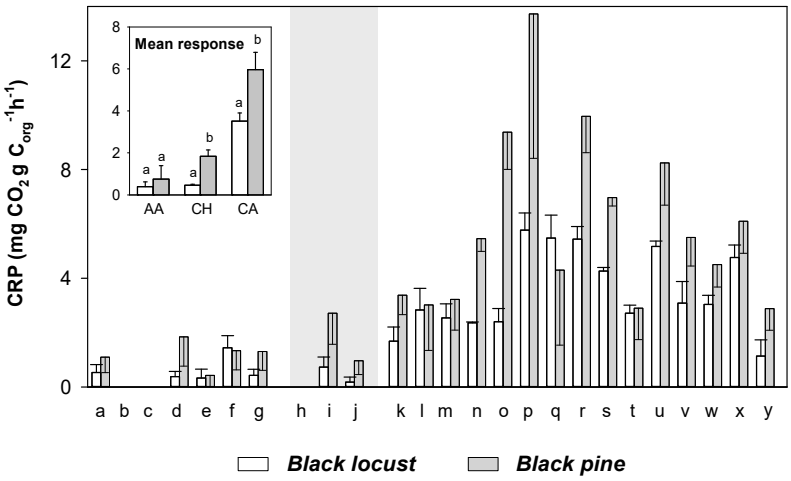
	Newly-Shed Litter			Forest-Floor Litter	
	Black Locust	Blackberry	Black Pine	Black Locust Stand	Black Pine Stand
Total C (mg g^{-1})	480.5 ± 10.0 ^{a,A}	463.4 ± 10.3 ^{a,B}	509.1 ± 15.0 ^{b,A}	425.9 ± 11.2 ^{a,B}	471.0 ± 18.1 ^{b,A}
Carboxyl C (200–160 ppm)	29.3 ± 0.1 ^{a,A}	26.9 ± 0.1 ^{a,A}	18.8 ± 0.1 ^{b,A}	39.2 ± 1.3 ^{a,B}	24.5 ± 1.2 ^{b,B}
Phenol C (160–145 ppm)	34.1 ± 0.6 ^{a,A}	27.3 ± 0.1 ^{b,B}	25.5 ± 0.2 ^{b,A}	32.6 ± 1.1 ^{a,A}	29.2 ± 1.4 ^{a,B}
Aryl C (145–110 ppm)	48.5 ± 0.8 ^{a,A}	53.8 ± 0.3 ^{b,A}	44.8 ± 0.3 ^{a,A}	44.1 ± 1.4 ^{a,B}	39.6 ± 1.9 ^{b,B}
O – Alkyl C (110–60 ppm)	258.5 ± 4.2 ^{a,A}	243.3 ± 1.2 ^{a,A}	312.6 ± 1.8 ^{b,A}	199.1 ± 6.4 ^{a,B}	263.4 ± 9.9 ^{b,B}
Methoxyl C (60–45 ppm)	29.8 ± 0.5 ^{a,A}	30.1 ± 0.1 ^{a,A}	25.5 ± 0.2 ^{b,A}	33.8 ± 1.1 ^{a,B}	34.8 ± 1.6 ^{a,B}
Alkyl C (45–0 ppm)	80.2 ± 1.3 ^{a,A}	82.0 ± 0.4 ^{a,A}	82.0 ± 0.6 ^{a,A}	76.9 ± 2.5 ^{a,A}	86.6 ± 4.1 ^{b,A}
Methoxyl C to Phenol C	0.87 ± 0.008 ^{a,A}	1.11 ± 0.001 ^{b,A}	1.00 ± 0.0001 ^{c,A}	1.03 ± 0.007 ^{a,B}	1.19 ± 0.0001 ^{b,B}
Aromaticity index	0.32 ± 0.002 ^{a,A}	0.33 ± 0.001 ^{a,A}	0.22 ± 0.002 ^{b,A}	0.38 ± 0.003 ^{a,B}	0.26 ± 0.007 ^{b,B}
Hydrophobicity index	0.56 ± 0.0001 ^{a,A}	0.61 ± 0.005 ^{b,A}	0.46 ± 0.006 ^{c,A}	0.65 ± 0.004 ^{a,B}	0.55 ± 0.003 ^{b,B}

Mass of C fractions are mg g^{-1} dry litter (mean ± SE; N = 10 for newly-shed litter; N = 20 for forest-floor litter). Different lowercase letters indicate a significant difference among the three species of newly-shed litter and between stands for forest-floor litter. Different uppercase letters indicate a significant difference between newly-shed litter and forest-floor litter. In the black locust stand, each of the two types of newly-shed litter was separately compared to forest-floor litter, composed of indistinguishable residues of black locust and blackberry (Two-way ANOVA, $p < 0.05$). Source of data for newly-shed litter of black locust and black pine: De Marco et al., [12].

3.3. Catabolic Response Profile of Mineral Soil

All the added substrates, with the exceptions of two amino acids (L-asparagine and L-glutamic acid) and D-glucosamine, induced an increase in respiration rate in both black locust and black pine soils. However, there was a higher ($p < 0.05$) response to many substrates in the black pine soil than in the black locust soil (Figure 2).

In both stands, the patterns of the catabolic response profile were similar in that carboxylic acids induced the highest respiration response (Figure 2). We may see that the mean response for carbohydrates and carboxylic acids was significantly higher ($p = 0.001$ and $p = 0.005$, respectively) in the pine soil than in black locust soil (Figure 2 insert).



Amino acids	AA
L-arginine	a
L-asparagine	b
L-glutamic acid	c
L-glutamine	d
L-histidine	e
L-lysine	f
L-serine	g

Carbohydrates	CH
D-glucosamine	h
D-glucose	i
D-mannose	j

Carboxylic acids	CA
L-ascorbic acid	k
citric acid	l
fumaric acid	m
gluconic acid	n
α-ketobutyric acid	o
α-ketoglutaric acid	p
α-ketovaleric acid	q
L-malic acid	r
malonic acid	s
pantothenic acid	t
quinic acid	u
succinic acid	v
tartaric acid	w
uric acid	x
urocanic acid	y

Figure 2. Catabolic Response Profile (CPR) of mineral soil from a black locust and a black pine stand. CRP was measured in soil samples (0–5 cm depth) amended with 25 low-molecular simple organic substrates in the paired stands (see legend). The mean response for type of substrate (AA: amino acids; CH: carbohydrates; CA: carboxylic acids) is shown in the inner panel. Different lowercase letters indicate statistically significant differences (*t*-test, $p < 0.05$) between stands.

3.4. Organic Components of Litter Water Extracts

Based on ^{1}H NMR spectra, the most abundant components of water extracts for newly-shed, and forest-floor litter were Carbohydrates, followed by Alkyls (Table 3). Newly-shed litter from blackberry was poorest in Carbohydrates and Alkyls, and the richest one in Carboxylic acids/Aldehydes as well as Aromatic compounds (Table 3). Forest-floor litter of the black locust stand, compared with that of black pine, had a water-soluble fraction richer in Carboxylic acids/Aldehydes and O-Alkyl compounds, and poorer in Aromatic compounds and Alkyls (Table 3). The water-soluble fraction of the black locust soil was richer in Aromatic compounds and O-Alkyls and poorer in Alkyls compared to black pine soil.

Table 3. Water extract components of newly-shed and forest-floor litter and mineral soil from two paired forest stands at Mount Vesuvius.·

Water Extract Components (%)	Newly-Shed Litter			Forest-Floor Litter		Mineral Soil	
	Black Locust	Blackberry	Black Pine	Black Locust Stand	Black Pine Stand	Black Locust Stand	Black Pine Stand
Carboxylic ac./Aldehydes (12–8 ppm)	8.11	15.39	4.71	12.44	4.54	5.35	3.27
Aromatic compounds (8–6 ppm)	5.41	12.31	5.88	9.61	14.50	8.23	5.23
Carbohydrates (6–4 ppm)	59.46	49.23	58.83	51.16	44.56	53.50	45.75
O-Alkyls (4–3 ppm)	5.41	6.15	7.06	7.80	4.73	12.35	6.54
Alkyls (3–0 ppm)	21.61	16.92	23.53	18.99	31.67	20.58	39.22

Data are percent of total area for ^{1}H-NMR spectra (chemical shift ranges in ppm are reported in parenthesis).

3.5. Effects of Litter Water Extracts on Mineralization Rates, Microbial Biomass, and Active Fungal Biomass of the Mineral Soil

The effects of the amendment of soil samples with litter water extracts were expressed as percent of the control. There was no significant difference among soil samples amended with litter water extracts at different concentrations. Thus, we report the mean value for the whole range of concentrations for each treatment (types of newly-shed litters, and forest-floor litter).

Extracts of newly-shed litter from black locust and blackberry caused a reduction of MR and MB in both pine and black locust soils (Figure 3). The amount of AFB was affected by the amendment only in black pine soil (Figure 3). Extracts of newly-shed litter from black pine had no effect either in black pine or in black locust soil (Figure 3).

Water extracts of black locust forest floor reduced MR in soil samples from the pine stand as well as in those from the black locust stand (Figure 4). However, in pine soil, MR was reduced more than in black locust soil ($p < 0.05$).

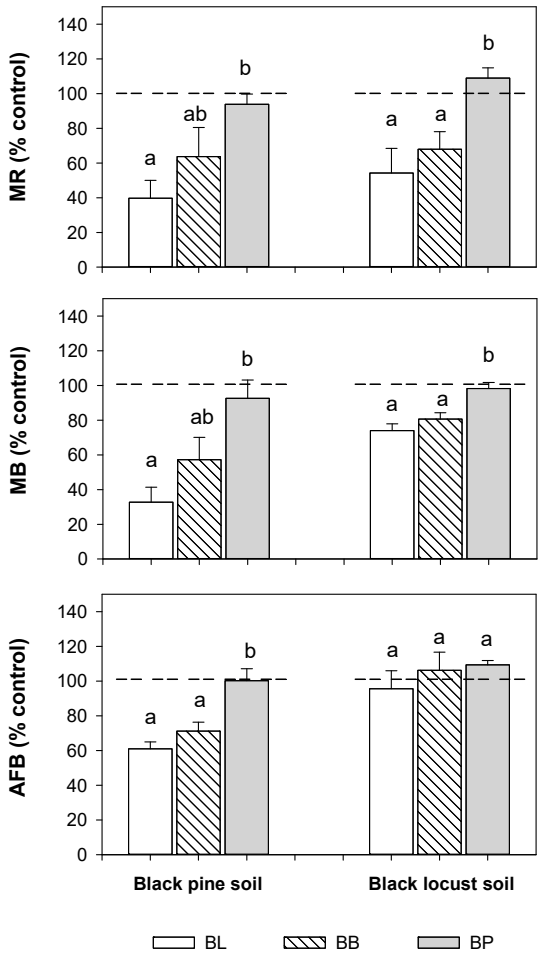


Figure 3. Changes in mineralization rate (MR), microbial biomass (MB), and active fungal mycelium (AFB) after amendment of black pine and black locust mineral soil with water extracts of newly-shed black locust, black pine and blackberry leaf litter. Changes are presented as percent of MR, MB, and AFB in non-amended (control: dashed line) soil (0–5 cm depth) and reported as the mean (±SE) from 20 samples. Different lowercase letters indicate statistically significant differences between soil samples amended with water extracts from different litters. In the boxes within panels statistically significant differences between black pine and black locust soils (two-way ANOVA, $p < 0.05$) are shown.

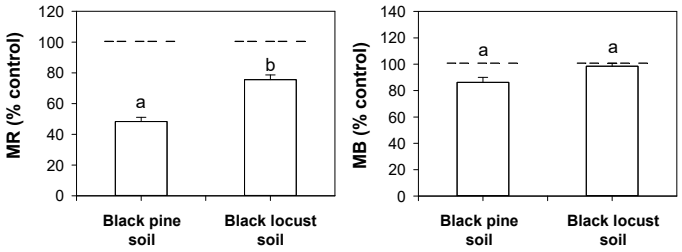


Figure 4. Changes in mineralization rate (MR) and microbial biomass (MB) after amendment of black pine and black locust mineral soil with water extracts of black locust forest-floor litter. Changes are presented as percent of MR and MB in non-amended (control: dashed line) soil (0–5 cm depth) and reported as the mean (±SE) from 20 samples. Different lowercase letters indicate statistically significant differences between the two soils (*t*-test, $p < 0.05$).

3.6. Changes in the Catabolic Response Profile of Mineral Soil Amended with Litter Water Extracts

The amendment with water extracts of black locust litter induced changes in CRP patterns of both soils (Figure 5). Extracts of newly-shed litter significantly suppressed the response of black pine soil to the amendment with carboxylic acids ($p = 0.03$) and increased the response to amino acids in both soils (Figure 5; $p < 0.01$). Forest-floor extracts significantly increased ($p = 0.008$) the response to amino acids in black pine soil (Figure 5).

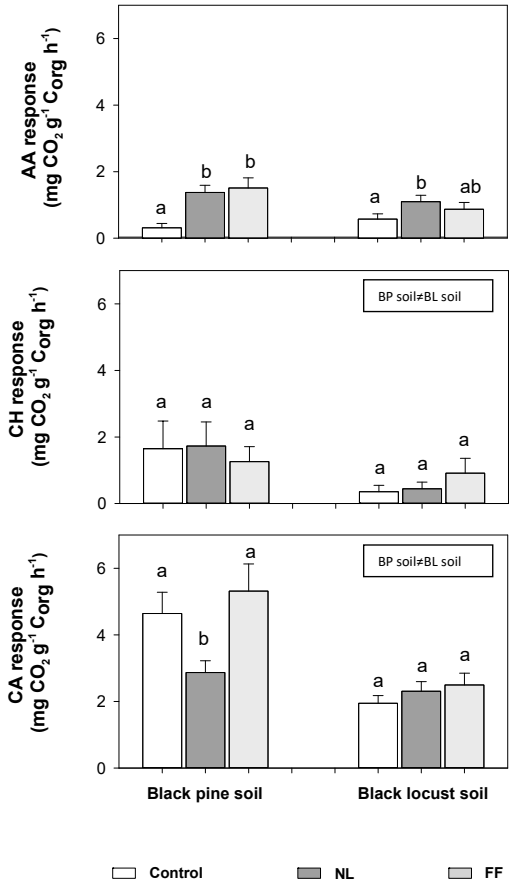


Figure 5. Mean catabolic response for type of substrate (AA: amino acids; CH: carbohydrates; CA: carboxylic acids) using black pine and black locust mineral soil amended with water extracts from newly-shed litter (NL) and forest-floor litter (FF) from a black locust stand. Different lowercase letters indicate statistically significant differences within control and amended samples. In the boxes within panels statistically significant differences between black pine and black locust soil (two-way ANOVA, $p < 0.05$) are shown.

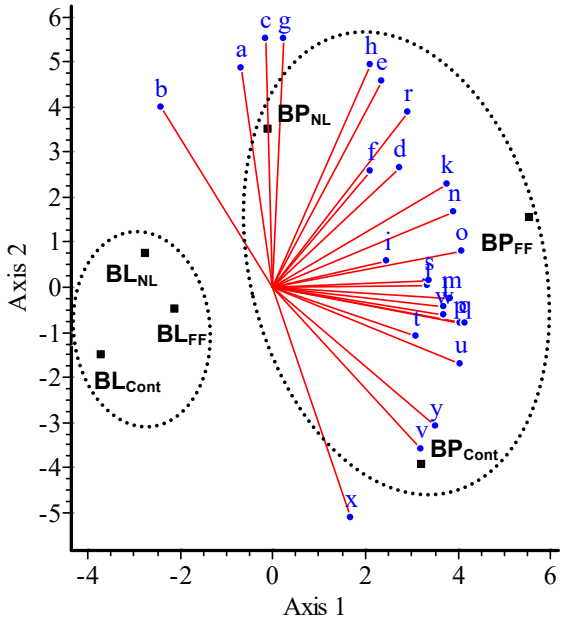


Figure 6. PCA applied to catabolic response profiles of black locust (BL) and black pine (BP) mineral soil amended with NL (subscript) and FF (subscript) litter extracts of black locust. Control (non-amended soil) are indicated as Cont (subscript). Legend for substrates in Figure 2.

PCA performed on CRP of black locust and black pine soils (Figure 6) showed a clear separation between the two soils, viz. that of black pine and that of black locust (Axis 1) as well as between control soils and soils amended with litter extracts (Axis 2). Three substrates, i.e., ketoglutaric acid (p), succinic acid (v), and malonic acid (s) explained 41%, 13%, and 6%, respectively, (altogether 60%) of the total variance.

4. Discussion

Consistent with our hypothesis, the present study demonstrates that water extracts of black locust and blackberry litter have a negative effect on the amount, activity, and community-level catabolic diversity of soil microorganisms. Hence our results give evidence for a role of litter inhibitory effects on soil organic matter mineralization that could contribute to explaining the pattern of C sequestration in soil.

4.1. Some Main Factors Influencing Carbon Sequestration in Forest Soils and Their Roles in The Present Study

Tree cover may influence carbon accumulation in the soil in different ways, e.g., through the amount of litter fall, through the quality of litter fall in terms of decomposability; through litter content of nutrients, such as N, P, and Mn, which regulate microbial activity; through the occurrence among litter components of substances, which may inhibit microbial activity; through the interactive effects of these factors.

As the amount of litter fall in the black locust stand is lower than in that with black pine [13], we may exclude this factor as a determinant of the higher C pool in the black locust soil. Moreover, our previous results [12] also show that the stable, remaining fraction (100-limit value) is smaller in black locust litter than in black pine litter (33 versus 47%, respectively). The higher potential mineralization rate of the newly-shed litter of black locust reflects its higher quality compared to those of blackberry and black pine. Blackberry NL decomposition is likely P and N limited.

In addition, the quality of forest-floor litter, i.e., of litter at later stages of decomposition, was different in the two stands. The black locust forest floor had higher pH, was richer in N as well as in soluble C and N, and had a lower C-to-N ratio, properties being considered indices of a higher quality and, thus, theoretically more decomposable litter. Nonetheless, the black locust forest

floor has a lower mineralization rate compared to the pine stand. Concentrations of Mn, a factor known to have a positive effect on late-stage decomposition [41], did not differ between forest floors. One reason for the difference between stands could be the higher N content of the black locust forest floor. Forest-floor litter is in the late stage of litter decomposition, and many authors have reported a strong and negative influence of N on lignin degradation. High N concentrations increase holocellulose degradation in the early stage but slow down the degradation of lignified tissue in the late and near-humus stages [41]. Moreover, the lower P concentration and the higher N-to-P ratio in black locust forest floor compared to black pine forest floor suggests that decomposition in the black locust stand is P-limited [42]. On the other hand, a lower resistance to degradation of black pine forest floor compared to that of black locust is consistent with its significantly lower aromaticity and hydrophobicity indices.

A factor influencing carbon sequestration in the soil of the two stands is that black locust forms arbuscular mycorrhiza (AM) while black pine forms ectomycorrhiza (EM). A recent study [43] has demonstrated that AM inhibits the development of fungal and bacterial groups in the soil at the later stage of decomposition while enhancing the rates of decomposition in the earlier stage. It has been shown that ectomycorrhizal forests are characterized by higher C content in the organic layers (as in the black pine stand), whereas arbuscular mycorrhizal forests are characterized by higher C content in the mineral layer (as in the black locust stand) [44]. There is much evidence that AM increase soils' micro aggregates formation, and this can physically protect organic carbon from decay by soil microbes [45].

A set of mechanisms for C accumulation in the soil relates to the MEMS (Microbial Efficiency-Matrix Stabilization) model of Cotrufo et al. [17]. According to the MEMS framework, black locust litter has higher decomposability and, therefore, can make more microbial biomass per unit of litter C, which then is stabilized in the mineral soil. The C-to-N ratio in the mineral soil of the black locust stand is lower than in the black pine stand (6.5 versus 8.9) and consistent with a larger microbial contribution to the higher C accumulation in the mineral soil, but the difference is not significant. The capacity for stabilization of microbial C is high in fine-textured soils where micro aggregates and clay-surfaces are abundant, and in soil with high allophane content [17]. The coarse-textured Lepti-Vitric Andosol at our site is the same in both stands and, thus, also the capacity for stabilization of microbial C should be the same. However, soil aggregate formation in the black locust stand is eventually promoted by AM. A confusing factor is that litter input in the black locust stand is made of similar amounts of a high-quality litter (black locust) and a less decomposable low-quality litter (blackberry). Thus, it is very difficult to conceive the role of MEMS framework as a driving factor in the accumulation of soil organic matter in the two stands.

Lastly, we may speculate about the role of belowground biomass and litter. Uselman et al. [46] found that root exudation of black locust has little potential to contribute to C sequestration in the soil because (1) it does not exceed 0.9–1.2% of the net C fixed by photosynthesis, and (2) approximately 60% of the exudates are rapidly decomposed with a turnover time of less than 1 day. On the other hand, belowground litter is generally more recalcitrant to decomposition than leaf litter [41], thus, maintaining C sequestered in the debris, although N resorption before tissue senescence in black locust [22] likely leads to more decomposable, nitrogen-poor belowground residues.

A C allocation pattern similar to that in our black locust and black pine stands has been reported in a recent study [47]. Both black locust and black pine are able to mitigate soil degradation in extreme site conditions increasing C sequestration, but the C stock in litter layers, as a fraction of total C (aboveground stock plus litter layers stock), is greater in the afforestation with black pine (21% vs. 18%), whereas C stock in the mineral soil, as a fraction of total aboveground and soil C stocks, is greater in the afforestation with black locust (23% vs. 19%). The mechanisms underlying such C allocation pattern are not clear

4.2. Are There Additional Factors for Carbon Sequestration-Allelopathy?

The bulk of the data indicates that in the two stands the litter's quality only in part explains the different organic matter decomposition rates in late to very late stage. Despite the better environmental conditions for soil microbes (CEC, fractions of soluble C and N), we find that the soil of the black locust stand has a lower mineralization rate, a lower microbial biomass, and a lower respiratory response to an array of simple organic substrates normally found in the rhizosphere, as shown by the Catabolic Response Profile.

The microbial communities of the two soils differ in activity and size. We may see that AFB is one order of magnitude higher in the mineral soil of black pine compared to that of black locust. As the two stands were planted on the same soil, we conclude that the difference in soil microbial abundance and activity are due to the different plant covers. That is a confirmation of earlier findings that distinct soil microbial communities have been found under different tree species planted in a common soil in several common garden experiments [4,48].

Our results suggest that inhibitory substances present in both black locust and blackberry litter may be responsible for the differences between the two paired stands in the abundance and activity of the microbial communities. Consequently, this may be a constraining factor for the lower decomposition rate of SOM and, thus, for the higher C sequestration rate in the black locust soil. The occurrence of inhibitory substances in black locust litter, such as 4–hydroxyacetophenon [20], is consistent with the lower microbial fraction of organic C and the lower mineralization rate in the black locust stand.

The results of the inhibition assays performed on soils of black pine (not conditioned by the presence of black locust) and black locust (likely hosting a microbial community naturally selected for the resistance to allelopathic substances occurring in the litter) show that extracts from newly-shed litter of both black locust and blackberry inhibit mineralization rate and reduce the amount of microbial biomass of both soils, but the effect is lower in that of black locust. No effect of the amendment was observed on the active fungal biomass of the black locust soil. This suggests that the smaller fungal community of the black locust soil is made up of strains resistant to the inhibitory substances present in black locust litter. Similarily, it has been reported that fungal strains isolated from the same site as the needle litter of Scots pine were not inhibited by litter extracts, whereas some strains isolated at other sites were strongly inhibited [26].

It is worth emphasizing that the inhibition tests evidenced no concentration dependence of the amount of allelopathic substances. This is consistent with the findings of Adamczyk et al. [49] showing that tannins can accelerate as well as decelerate the enzymes responsible for soil organic matter decomposition. Indeed, at low concentrations tannins increase the coiled structures of the enzymes boosting their catalytic activity; high concentrations of tannins act in the opposite way, thereby diminishing the catalytic activity. Thus, the activity of beta-glucosidase, a key enzyme in C cycling, shows weak changes over a wide range of tannin concentrations [49].

Forest-floor litter in the black locust stand is a mixture of black locust and blackberry residues indistinguishable from each other. Thus, the observed inhibitory effect may be due to both litter species. It is worth emphasizing that litter inhibitory effect decreases from newly-shed to forest-floor litter, likely because some of the inhibitory compounds have been degraded. The persistence of allelochemicals in soil environment is an important factor [50]. It has been found that the allelopathic potential of black locust against understorey species is modulated by soil as the phytotoxic compounds are absorbed into the soil organic matter and clays and/or are modified by soil microorganisms [51].

Extracts of newly-shed black pine litter had no effect, neither in the black locust soil nor in that of black pine. Based on the lack of inhibitory effects of the newly-shed black pine litter, no test was performed for inhibitory effects of black pine forest-floor litter that, however, had higher mineralization rate than the black locust forest floor.

The amendment of black pine and black locust soil with litter extracts from black locust, both newly-shed and forest-floor litters, altered the CRP pattern, i.e., the range and relative expression

of microbial activities involved in the decomposition process of organic matter components. Changes induced by the amendment with extracts from newly-shed black locust litter mainly included an increase in both soils in the response to amino acids, likely due to the priming effect [52]. Indeed, the increased mineralization of amino acids likely reflects the increased microbial N-demand to synthesize enzymes required to decompose the additional C sources supplied to the soil with the litter extracts.

The amendment of black pine soil with litter extracts induced a decrease in the response to carboxylic acids. Carboxylic acids are involved in many soil processes, and their decomposition is controlled by differences in microbial community structure [53,54]. The decrease of the response to carboxylic acids suggests that the inhibition of the microbial populations by litter extracts has an overall crucial role in the degradation of SOM and carbon accumulation in the soil. Three of the carboxylic acids may explain up to 60% of the total variance and contribute the highest proportion of the total respiration response.

As indicated by the PCA runs, extracts of black locust litter affect the catabolic response profile of black pine soil much more markedly than that of black locust soil. This suggests that an increase in the concentration of the inhibitory compounds which usually are present in the black locust soil produces no, or only a small effect, whereas the introduction of inhibitory compounds in the black pine soil induces evident changes in the activity of the microbial community.

We have tested the inhibitory effect of black locust and blackberry litters on soil microbial biomass and activity and the results are consistent with our hypothesis that water extracts of newly-shed and forest-floor litter reduce organic matter mineralization, which may explain the higher C sequestration in the mineral soil of the black locust stand despite the lower litter fall and the smaller litter residue. The inhibition of soil microbial community by allelopathic substances present in litter seems to be a valid candidate mechanism to explain the differences in C sequestration in the two stands. But other possible mechanisms as those discussed above have to be tested to evaluate their importance.

5. Conclusions

Our results suggest that an inhibitory effect of litter on decomposition is a potential driver of carbon accumulation in soil and a mechanism by which plant cover influences carbon sequestration. The inhibitory effect of black locust and blackberry litter on soil microbial activity and, hence, on the mineralization of soil organic matter, may help to explain why the input to the soil of the black locust stand of a litter with a potentially high decomposition rate results in a higher C pool in the mineral soil, despite better environmental conditions for soil microbes compared to the paired black pine stand. Litter of black locust and blackberry, as well as black locust soil, are richer in soluble components than litter and soil of the black pine stand. The water-soluble fraction of these litters is able to inhibit the microbial community and, thus, through the decrease of decomposition, to cause the storage of a higher amount of organic matter in the soil. Still other possible mechanisms as those discussed above have to be tested to fully elucidate their role in C sequestration in the black locust stand. We emphasize the multifaceted influences of plant cover on C accumulation in soil that is mainly modulated by microbial activity. Plant cover influences the activity of decomposer microorganisms through the nutrient content, organic components, and inhibitory substances of litter, as well as through the types of mycorrhiza and their interaction with other components of soil microbial community, and their effect on soil aggregate formation.

The dinitrogen-fixing tree black locust, native to North America, has been used for afforestation worldwide (despite the impact on floristic diversity of this invasive tree) due to its ability to grow in extreme site conditions, as on new volcanic substrate or on degraded soil. According to a study performed in the Yellow River Delta region, C sequestration in the soil of black locust forests, 20 years after afforestation, accounted for 32.56% of the carbon in the ecosystem (154.61 t ha^{-1}) and was increasingly more important with increasing stand age [55]. The results of our research suggest further investigations in the potential of C sequestration in the soil under black locust to better understand the

mechanisms behind and to improve afforestation policies for mitigation of climatic changes. The role of allelopathy in C sequestration in the soil under black locust still needs to be investigated in field experiments, focusing on the persistence of allelopathic substances in different types of soil, as well as on their leaching through the soil profile; moreover the impact of different climatic conditions and understorey vegetation has to be explored.

Author Contributions: A.D.M., B.B. and A.V.D.S. conceived and designed the experiments, and wrote the paper. A.D.M. and A.V.D.S. analyzed the data; F.E. performed field and lab measurements; A.Z. performed ^{1}HNMR measurements. All authors have read and approved the final manuscript.

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