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Title

Slow decomposition of leaf litter from mature *Fagus sylvatica* trees promotes offspring nitrogen acquisition by interacting with ectomycorrhizal fungi

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ABSTRACT

1. Leaf litter chemistry and ectomycorrhizal (ECM) fungi are key drivers of the belowground nitrogen (N) cycling within forest ecosystems. Their combined effects on litter decomposition and N competition between microbial decomposers and plants are still uncertain.

2. We conducted a greenhouse microcosm experiment with low or high ECM-colonized beech (*Fagus sylvatica*) saplings, growing with litter collected from old or young beech trees growing on the same loamy soil. After 6 months of growth, we investigated litter decomposition rates, microbial respiration and the N pools within leaf litter, soil (different pools), microbial and plant shoot biomass.

3. We found that the mass loss of the litter from young trees was always higher than the litter from the mature trees. The microbial biomass N per unit soil carbon was low when the litter, especially from mature trees, was added, suggesting that the litter provided recalcitrant

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compounds and limited soil microbial activity. In contrary to the '*Gagdil effect*' hypothesis, the high ECM-colonized roots increased the litter decomposition rate and N immobilization in poorly decomposable litter in comparison to the litter incubated with the low ECM-colonized roots. Finally, the high ECM-colonized plants that received the poorly-decomposable leaf litter exhibited the highest shoot N amount and biomass and were associated with the lowest microbial biomass N.

4. Two-way ANOVAs revealed that litter and ECM fungi occurrence together impacted final particulate organic N, microbial biomass N, the amount of shoot N and shoot biomass. Four N pools are key drivers of microbial biomass N and shoot N: particulate organic N, total N, soil ammonium concentration and litter N concentration.

5. *Synthesis*. Our results support the hypothesis that poorly decomposable leaf litter produced by mature beech trees and ECM fungi together decrease microbial N immobilization but increase tree N acquisition. Increasing N retention within the recalcitrant N forms in soil was identified as a key mechanism by which beech alters soil N cycling with potential positive feedbacks on its acquisition by the plant. Our result emphasizes the importance of considering within-species litter-trait variability in litter decomposition.

Keywords: leaf litter decomposability, ectomycorrhizal fungi, *Fagus sylvatica*, belowground N cycling, microbial biomass N, microcosm experiment

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INTRODUCTION

Our understanding of the nitrogen (N) cycle within terrestrial ecosystems has gained considerable awareness recently (Chapman et al., 2006, Schimel and Bennett, 2004, van der Heijden et al., 2008, Nasholm et al., 2009, Kuzyakov and Xu, 2013). A notable advance concerns the ability of trees to influence soil N cycling and compete actively for N with microbial decomposers, suggesting that plants are not a priori the least competitive organisms (Kuzyakov and Xu, 2013, Blagodatskaya et al., 2014) and can influence soil N through both aboveground and belowground pathways (Kuzyakov, 2002, Smith and Read, 2008, Kieloaho et al., 2016).

Among the aboveground pathways, litter traits (also called '*litter quality*') represent key drivers by which trees influence soil N cycling (Scott and Binkley, 1997, Aerts, 1997, Cornwell et al., 2008, Makkonen et al., 2012). Usually, leaf litter with low N concentrations and high C:N and lignin:N ratios correlates with slow rates of decomposition, low soil net N mineralization and net nitrification and high microbial N immobilization (Satti et al., 2003, Cornwell et al., 2008). Likewise, condensed tannins in litter have a great ability to precipitate proteins and directly affect belowground N cycling (Schweitzer et al., 2004, Northup et al., 1995, Madritch and Lindroth, 2015, Kraus et al., 2003). The amount of N released during litter decomposition that becomes available for plants is thus controlled by litter traits and the nutrient requirements of microbial decomposers (Manzoni et al., 2010, Stump and Binkley, 1993, Mooshammer et al., 2012).

Belowground pathways include ectomycorrhizal (ECM) fungi-root associations that strongly regulate soil N pools (Hodge et al., 2000, Courty et al., 2010, Phillips et al., 2013, Tibbett and Sanders, 2002). Through high mycelium prospecting and absorbing abilities, ECM fungi facilitate tree nutrient acquisition (Wu, 2011, Smith and Read, 2008, Becquer et al., 2014).

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Furthermore, the fungi have a strong ability to uptake N directly from organic sources (Phillips et al., 2013, Finlay et al., 1992, Wallenda and Read, 1999, Persson et al., 2003, Plassard et al., 2000) and possibly contribute to the breakdown of organic matter through the production of extracellular enzymes (Courty et al., 2010, Cullings and Courty, 2009, Courty et al., 2006).

Leaf litter and ECM fungi-root associations do not affect belowground N cycling independently but interact one with the other (Wurzburger and Hendrick, 2009,Gadgil and Gadgil, 1971). However, the magnitude and direction with which litter-ECM interactions affect organic matter and nutrient recycling are still uncertain. For example, ECM fungi can have negative effects on leaf litter decomposition, a phenomenon known as the 'Gadgil effect' (Gadgil and Gadgil, 1975). However, they can also have no effect (Mayor and Henkel, 2006) or positive effects (Brzostek et al., 2015, Zhu and Ehrenfeld, 1996). Importantly, the benefits of these interactions for plant N acquisition are unclear, even if a growing body of evidence suggests that N stored in recalcitrant compounds may limit microbial N immobilization but can be used by mycorrhizal plants (Wurzburger and Hendrick, 2009, Madritch and Lindroth, 2015, Wu, 2011, 2015, Schimel et al., 1998, Nasholm et al., 1998, Kieloaho et al., 2016). In this way, Hattenschwiler et al. (2011) formulated the '*decomposer starvation*' hypothesis, which assumes that poorly decomposable litter in tropical forests may limit the supply of easily available energy and nutrients to microbial decomposers while increasing plant nutrition via mycorrhizal associations. Bypassing competition for N would thus confer a clear competitive advantage for trees over microbial decomposers, causing higher tree productivity (Wu, 2011). These mechanisms are expected to induce large positive plant N acquisition feedbacks (Wurzburger and Hendrick, 2009), especially in nutrient-limited ecosystems where trees have high N use efficiency (Vitousek, 1982) and forage organic rather than mineral N (Madritch and Lindroth, 2015).

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In previous field studies, we found a higher proportion of total N tied up within recalcitrant compounds (lignin) in leaf litter produced by old beech (*Fagus sylvatica*) trees in comparison to leaf litter from young trees (Trap et al., 2013). These age-related shifts in litter N traits were strongly related with litter decomposition and soil N transformations (Trap et al., 2011a, Trap et al., 2013). We thus hypothesized that leaf litter produced by mature beech trees makes litter N poorly available for microbial decomposers but increases plant N acquisition from ECM-colonized beech roots. In this study, we aimed at testing this hypothesis by conducting a greenhouse experiment in microcosms with low and high ECM-colonized beech (*Fagus sylvatica*) saplings growing with litter from mature (poorly decomposable) or young (highly decomposable) beech trees.

MATERIALS AND METHODS

Soil and microbiota materials

The soil material was collected in the uppermost 20 cm from a grassland (Upper Normandy, France) that was not influenced by the presence of beech. After being sieved to 5 mm, the soil was mixed with sand at a ratio of 5:1 and autoclaved twice (1 h at 110°C) at a 1 week interval. The autoclaved material contained 46.5% sand, 27.2% silt and 26.3% clay, 7.2% organic matter, 21.3 % water, and had a pH of 6.5. In order to inoculate in the soil with a microbial community composed of a mixture of slow- and fast-growing microorganisms, fresh loamy soil taken from the same grassland was shaken in demineralized water (1:20 soil:water) for 1 hour using an orbital mechanical shaker and filtered through a 175 µm plastic mesh to remove larger particles. The same extraction procedure was applied to fresh composite loamy soil taken from a young immature beech stand and from an old mature beech stand (50:50) from the Eawy forest (France, Upper Normandy, 01°18’ E; 49°44’ N;

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7200 ha). We thus obtained two distinct microbiota filtrates, one from the grassland and one from the beech forest, dominated by bacterial- and fungal-based energy channels, respectively.

Leaf litter collection and preparation

The chemistry and decomposability of beech leaf litter vary significantly according to the age of tree producing the litter (Trap et al., 2013) but did not vary significantly within age-class changes (Supporting Information 1). We thus decided to collected beech leaf litter in the same two pure beech stands used in Trap et al. (2011a, 2011b) from the Eawy forest (France, Upper Normandy, 01°18' E; 49°44' N; 7200 ha), and each sample was representative of the litter decomposability of their respective age. We selected a young non-reproductive stand (20-yr-old, 8.0 ha, dysmull humus form, soil pH_{water} = 4.48, soil P = 0.13 g kg^{-1}, soil CEC = 6.19 $cmol^{+}$ kg^{-1}, soil C:N = 16.6) and an old reproductive stand (90-yr-old, 16.4 ha, eumoder humus form, soil pH_{water} = 3.83, soil P = 0.22 g kg^{-1}, soil CEC = 5.56 $cmol^{+}$ kg^{-1}, soil C:N = 15.4). Both stands were managed as an even-aged forest from natural regeneration by the French Forestry Service. Freshly fallen leaves were collected using a tight net one meter above the forest floor in order to avoid any colonization by soil biota, air-dried over three months, oven dried at 60°C for 48 hours and cut into pieces 3 cm in length. The leaf litter collected from the young and the old stands are Highly and Poorly Decomposable, respectively (Trap et al., 2011a), and are thus referred to as HD-litter and PD-litter. At the beginning of the experiment, the HD- and PD-litters have C:N ratios of 40.1 and 50.6 and the initial N concentrations were 1.2% and 0.9%, respectively. The initial percentage of lignin was significantly higher in the PD-litter (35.0%) than that in the HD-litter (28.5%).

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Low *versus* high ECM-colonized plants

Plants consisted of two-years old beech saplings collected in March from an old purebeech forest (Eawy forest, 18.7 ha, LUVISOLS, Eumoder humus forms, soil pHwater = 3.91, soil P = 0.15 g kg^{-1}, soil CEC = 5.75 $cmol^{+}$ kg^{-1}, soil C:N = 14.8) and from a commercial nursery located in the Eawy forest centre (Essarts nursery, France, Upper Normandy, LUVISOLS, soil pH_{water} = 7.30, soil P = 0.61 g kg^{-1}). Beech saplings were collected using a spade. Soil was gently removed from the roots by hand and the roots were then washed with water. We used five plants from both the old forest and the nursery to measure the initial plant biomass values and ensure that no significant phenotypic differences related to individual origin occurred at the beginning of the experiment (mean height 58 cm, mean total biomass 11.9 dry g, mean shoot biomass 7.2 dry g, mean root biomass 4.6 dry g, mean shoot-to-root ratio 1.6). We measured the ergosterol concentration in fine roots of each origin to establish plant ECM status. Ergosterol is a fungus-specific component of membranes which is a good indicator of living ECM-fungal biomass (Ekblad et al., 1998). Briefly, 0.5 g of fresh washed roots (three analytic replicates) were treated with methanol (ratio 1:10 w:v) and 4 g acid-washed glass beads, vortexed for 10 s, and shaken for 1 h at 320 rev min-1. The solution was centrifuged at 110000 rev min^{-1} at 5°C for 10 mn and filtered on 0.20 µm filter (Gong and Witter, 2001). The water concentration in fresh fine roots was measured by weight difference after drying roots at 65°C for 48 h. The ergosterol was measured on the filtrate by High-Performance Liquid Chromatography at 282 nm (SPD-10A SHIMADZU LC-6A, Japan) and expressed as µg-ergosterol per g of dry fine root. We found a 12-times higher ergosterol content in the fine roots of plants from the old forest than in those from the nursery ($P < 0.001$, n=5) (Supporting Information 2). Plants from the old natural stand were highly colonized by ECM fungi in

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contrast to those from the nursery. Low and high ECM-colonized plants are refereed as LC- and HC-plants, respectively.

Microcosm establishment and experimental design

The experiment was conducted in microcosms consisting of PVC pots (inner diameter 22.5 cm, height 20.5 cm) that were sealed at the bottom with a 270 µm plastic mesh and filled with 5 kg of soil material. An aliquot of 500 ml of each microbiota filtrate was sprayed at the soil surface of each microcosm. Ten grams of dry leaf litter were placed at the soil surface in litter treatments; this is equivalent to the amount of litter annually produced in the forest stands where litter was sampled (Trap et al., 2011a). To avoid any loss of litter material, a plastic net with large mesh (2 cm) was placed on the top of the microcosms.

The experiment comprised nine treatments with five replicates each. Treatment 1 consisted of a control with soil material alone. Treatments 2 and 3 had no plants and comprised highly or poorly decomposable leaf litter from young trees (HD-litter) and from old trees (PD-litter), respectively. Treatments 4 and 5 consisted of soil without litter and planted with high (HC-plants) and low (LC-plants) ECM-colonized beech saplings, respectively. The four other treatments result in a full cross-combination of the two types of leaf litter and saplings (HD + HC, PD + HC, HD + LC, PD + LC). The experiment was conducted over 6 months inside a greenhouse. The experiment started in March 2008. The positions of the microcosms in the greenhouse were randomized every 4 weeks. The soil moisture was controlled by irrigating two times per week.

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Plant harvesting at the end of the experiment

Plants were harvested 6 months after transplantation. The roots were separated from the soil by hand, then sorted and washed with distilled water. The presence of ECM fungi was checked by macro- and microscopic observations of beech roots. Shoot and root biomass values were determined after oven drying plant material at 60°C until constant weight and the relative biomass gain was computed for each individual as the difference between the final and the initial values. Biomass allocation during the 6-month experiment was calculated for both shoot and roots as a proportion of the total biomass gain. Aliquots of initial and final shoots and roots were ground with a laboratory mill (Culatti) to a mesh fraction smaller than 1 mm. Total N was then measured by gas chromatography with a CHN pyrolysis micro-analyser (137 Flash 2000 Series, CHNS/O Analysers Thermo Scientific, IRCOF, France) and expressed as percentage (concentration) and as mg-N per plant (total N amount). We also subtracted the initial N from the final shoot N amount in order to calculate the shoot N gain during the 6 months of growth.

Analytical procedures in litter and soil at the end of the experiment

Leaf litter remaining at the surface of the microcosms was collected, air-dried for 1 week at room temperature, oven dried at 60°C for 48 h and weighed. The total N content in the remaining leaf litter was determined as described above. Soil was removed from the microcosms, sieved at 2 mm and stored at 4°C. An aliquot of 20 g of fresh soil was shaken in 100 ml K_2SO_4 (0.2 M) for 1 h at 100 rev min^{-1}. The obtained extracts were then filtered through Schleicher & Schuell 0790 ½ filter papers (pre-leached with 0.2 M K_2SO_4 in order to avoid any mineral N contamination) and a subsample of the extract was frozen for mineral N

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analysis. Concentrations of NH_4^+-N and NO_3^--N were determined colorimetrically with an AutoAnalyser (AA3, BRAN+LUEBBE, Norderstedt, Germany). In order to determine the total dissolved N (TDN) in solution, an aliquot of each K_2SO_4 extract was mineralized by a persulfate digestion following the procedure of Öhlinger (1996) and mineral N was analysed subsequently with the same AutoAnalyser. Aliquots of each soil sample were air-dried until a constant weight was reached, and the total C and N were measured by gas chromatography with a CHN pyrolysis micro-analyser (Flash 2000 Series, CHNS/O Analysers Thermo Scientific, France). The dissolved organic N (DON) was calculated as the difference between the TDN and total mineral N (ammonium and nitrate). We defined the "particulate organic N" (PON) as the difference between total N and TDN. Soil aliquots were dried at 105°C for 24 h to obtain the gravimetric water content.

An aerobic incubation was conducted to determine the potential net N mineralization (i.e., ammonification and nitrification) and microbial respiration. The incubation was conducted in triplicate for each sample using 40 g of soil in 500 ml airtight glass beakers incubated for 28 days in the dark at 28°C. The soil moisture was adjusted to 85% of the maximum water holding capacity. A 20 ml flask filled with NaOH (0.2 M) was placed in each microcosm. Microbial respiration was assessed by measurements of the CO_2 released using the conductimetry method (Heemsbergen et al., 2004). Microbial respiration was defined as the mg-C released per initial (at the beginning of the aerobic incubation) g-C in the soil and was calculated by comparing the initial and final NaOH conductivity (millisiemens cm^{-1}) after a 7-d incubation period during the 28 days. Microcosms without soil were used as a negative control to measure the NaOH conductivity of the glass beaker atmosphere at the beginning and the end of every week. Potential soil N transformations were assessed by subtracting the final to initial ammonium/nitrate contents (extracted and analysed as describe above) divided by the incubation time and dry soil carbon content, expressed as µg-N per initial g-C per day

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(Hart et al., 1994). Potential net N mineralization corresponded to the sum of ammonification and nitrification.

Microbial biomass N (N_{mic}) in the soil was determined by the chloroform fumigation-direct extraction method (Brookes et al., 1985). After 24 h of fumigation with $CHCl_3$, the soil was shaken in 0.2 M K_2SO_4 solution (soil:solution ratio of 1:10) for 1 hour at 100 rev min^{-1}. Extracts were filtered through pre-leached Schleicher & Schuell 0790 ½ filter papers. The persulfate digestion of K_2SO_4 extracts was performed. The concentrations of NH_4^+-N and NO_3^--N were determined as described previously. We expressed the microbial N per g of dry soil (N_{mic}), per unit soil (total) C (N_{mic}:C_t, expressed in µg-N mg-C^{-1}) and also in proportion to the total soil N in the soil (N_{mic}:N_t, expressed in %).

Statistical analysis

Means and standard deviations were calculated by treatments for all variables (n = 5 replicates). In order to test the interaction significance between 'litter' and 'plant' treatments, we performed a two-way ANOVA for all variables with 'litter' and 'plant' as factors, including the controls, namely three treatments per factor (9 treatments in total): "absent", "high" and "low" decomposability (for litter) and ECM-colonization (for plants). The two-way ANOVAs were followed by Tukey's HSD post hoc tests to localize the significant differences among treatments and display letters of pair-wise comparisons. When the interaction was significant, Tukey's HSD post hoc results from the interaction were displayed. When the interaction was not significant, Tukey's HSD post hoc results for the main effects were displayed. We did not display letters when there was a non-significant main effect. ANOVA residuals were inspected fornormality using Wilk-Shapiro tests.

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To detect the N pools and flows likely to explain beech sapling shoot N amount and soil microbial biomass N, we used Partial Least Squares regression (PLS-R) models (Tenenhaus, 1998, Wold et al., 2001). The model strength was assessed by the proportion of variance in the dependent variable that is explained by the model (R^2) and the proportion of variance in the dependent variable that can be predicted by the model (Q^2). A PLS-component is considered significant when Q^2 exceeds a critical value of 0.097 (Tenenhaus, 1998). Cross-validation was used to test the predictive significance. The relative influence of each independent explanatory variable (soil and litter N pools) may be estimated using the Variable Importance of Projection (VIP), which is the sum of the variable's influence over all model dimensions divided by the total variation explained by the model. Independent variables with VIP > 1 are the most relevant and significant for explaining the dependent variable. Two PLS-R models were performed with the microbial biomass N (model 1) or shoot N amount (model 2) as the dependent (response) variables. The first model included the ten explanatory variables listed in table 1, excluding microbial biomass N, but including plant N. The second PLS-R model included the ten explanatory variables listed in Table 1. We also tested the relationships between microbial biomass N, plant N amount and biomass using linear regression analyses and the relationship between leaf litter mass loss and soil basal respiration. The statistical significance of the regression models was tested using the function 'lm' from the package 'stats'. The 95% confidence intervals of regressions were calculated using the function "predict" from the package 'stats'. PLS-R analyses were performed using TANAGRA 1.4.40 (Rakotomalala, 2005). All other tests were computed with the R software (R, 2008) and statistical significance was set at $P < 0.05$.

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RESULTS

Leaf litter decomposition

Litter mass loss was not significantly impacted by the interaction between 'litter' and 'plant' factors (Table 1). It was always higher for HD (highly decomposable) than for PD (poorly decomposable) litter, irrespective of the presence and type of plants (Fig. 1). The presence of high ECM-colonized (HC) plants tended to increase PD-litter mass loss, while the presence of low ECM-colonized (LC) plants decreased litter mass loss. We found a significant relationship between leaf litter mass loss and soil basal respiration in the absence of plants ($R^2 = 0.56$, $P < 0.05$, slope 0.94) and in presence of HC-plants ($R^2 = 0.43$, $P<0.05$, slope 0.54) (Fig. 1). Interestingly, we did not find relationship between these two variables in the presence of LC-plants ($R^2 = 0.01$, slope -0.02).

Soil and litter N pools

The total N content in the soil was significantly affected by the interaction between 'litter' and 'plant' factors ($P < 0.05$) (Table 1). We observed higher soil total N values when litter was present while the plant alone had no observable effect in comparison to the controls. The highest total N mean (4.1%) was observed in soil with HC-plants covered by PD-litter. We found a similar pattern for particulate organic N (PON). In contrast, the soil dissolved organic N (DON) content in the soil was not significantly affected by the litter and plant interaction. DON values did not change according to litter treatments, but strongly decreased in the presence of plants, especially with LC-plants. Likewise, the presence of plants induced lower final soil ammonium and nitrate contents in comparison to the controls (-94%) (Table 2), but this effect was dependent on the litter treatments (significant interaction) (Table 1).

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Interestingly, the presence of both litter and HC-plants did not significantly change the nitrate content in the soil in comparison to the controls. This finding was not observed for LC-plants where both soil ammonium and nitrate contents decreased significantly in comparison to the litter treatments.

The interaction between 'litter' and 'plant' treatments significantly affected the soil microbial biomass N (N_{mic}) (Table 1), even when N_{mic} is expressed per unit soil C (N_{mic}:C_t) or in proportion to the total soil N (N_{mic}:N_t). The presence of litter or LC-plants increased N_{mic} (Table 2). HC-plants have positive effects on N_{mic} only when HD-litter was present, while LC-plants increased N_{mic} only in absence of litter. In contrast to N_{mic}, N_{mic}:C_t did not increase with the addition of litter. Instead, we observed lower N_{mic}:C_t values when PD-litter was added to the microcosms in comparison to the controls. This negative effect was more pronounced when HC-plants were present. The presence of LC-plants without litter increased N_{mic}:C_t. We found similar trends for N_{mic}:N_t with the lowest values of the N_{mic}:N_t means observed in presence of litter, especially with HC-plants and PD-litter (7.5% of total N).

We did not find significant interaction effect between litter and plant on the potential net ammonium production and nitrification (Table 1). The potential net ammonium production was significantly higher in the presence of plants in comparison to the control. In contrast, the presence of PD-litter significantly decreased ammonification in comparison to the control (without litter) and HD-litter treatments. Nitrification was strongly negatively affected by litter input while LC-plants increased it (Tables 1 and 2). Concerning the litter N content, HD-litter always showed lower final N content in comparison to PD-litter, irrespective of plant and ECM occurrence (Table 2).

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Plant shoot N

Final shoot N amount and concentration were both affected by the 'litter' and 'plant' interaction (Table 1). HC-plants growing with PD-litter exhibited the highest means of shoot N amount (Fig. 2A) and concentration (Fig. 2B). We also observed high shoot N concentration sfor LC-plants growing without leaf litter but for this treatment, total shoot N amount was low (~90 mg-N). All other treatments exhibited similar, rather low, shoot N amounts and shoot N concentrations.

Plant biomass

Plant biomass was affected by both litter and plant treatments (Table 1). Higher means were found when litter was added in comparison to the controls (Table 3). For HC-plants, only PD-litter significantly increased plant biomass. Shoot and root biomass values were also impacted by the litter and plant interaction (Table 1). The maximal shoot biomass mean was found for the following treatment: HC-plants growing with PD-litter (20.9 g). In contrast, the highest root biomass mean was found for LC-plants growing with HD-litter (29.7 g), but it was not significantly different from LC-plants growing with PD-litter (28.0 g). The shoot biomass allocation of HC-plants was significantly higher than for LC-plants (Fig. 3). Shoot biomass allocation was higher when PD-litter was present, but only for HC-plants.

N sources for decomposers and plants

The first PLS-R model explained 50% of the variability in microbial biomass N ($R^2 = 0.50$, *P*-value < 0.001) and showed a cross-validated goodness of prediction (Q^2) of 0.4 (Fig. 4a).

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Four N pools were good predictors of the microbial biomass N and exhibited significant VIP values: PON, total N, soil ammonium concentration and litter N concentration at the end of the experiment. PON was the best predictor (VIP = 1.31). Except for the soil ammonium concentration, all three other N pools were negatively related to the microbial biomass N. The second PLS-R model explained 48% of the shoot N variability ($R^2 = 0.48$, *P*-value < 0.001) and also showed a cross-validated goodness of prediction (Q^2) of approximately 0.4 (Fig. 4b). The final litter N concentration was the best predictor (VIP = 1.27) and was positively related with the amount of shoot N. The total N and PON at the end of the experiment were also two significant predictors (VIP > 1) positively related to the amount of shoot N. The final concentration of ammonium in the soil (VIP = 1.19), potential net ammonification (VIP = 1.14) and microbial biomass N at the end of the experiment (VIP = 1.11) were negatively related with the amount of shoot N (Fig. 4b). The linear regression analyses between the microbial biomass N (explanatory variable) and total N gain in shoot ($R^2 = 0.37$, $P < 0.001$) or shoot biomass ($R^2 = 0.32$, $P < 0.001$), both as response variables, revealed significant negative relationships (Fig. 5).

DISCUSSION

Leaf litter decomposition and N immobilization

Litter recalcitrance of *Fagus sylvatica* has been found to vary greatly with tree age (Trap et al., 2013) but the impact of this within-species age-related variability on litter decomposition and plant N acquisition remains unclear. Since the main feature of the variability of beech litter recalcitrance with tree age is a decrease in litter decomposability while trees reach sexual maturity, we question the apparently counterintuitive consequence of this behaviour: the nutrient availability for the next beech generation is thus reduced precisely when this

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cohort is produced. The litter produced by young trees has higher initial total N, a lower C:N ratio, and lower lignin concentrations than litter produced by old trees (Trap et al., 2013). Additionally, a higher proportion of total N is associated with lignin-like compounds in poorly decomposable litter (Trap et al., 2013). The analysis of litter mass loss confirmed that old trees produced more slowly decomposable litters than young trees when litters are incubated in the same environment. Because litter from mature trees accumulated more N than litter from young trees, litter quality strongly governed decomposition and litter N mineralization (Zeller and Dambrine, 2011).

In contrast to the '*Gadgil effect*' (Gadgil and Gadgil, 1975, Gadgil and Gadgil, 1971), the presence of high ECM-colonized plants did not alter beech leaf litter decomposition rates. Likewise, Staaf (1988) showed that the exclusion of ECM-roots by trenching the soil in a 95-100-year-old beech forest in Sweden did not have an effect on decomposition. Other experiments did not support the '*Gadgil effect*' (Mayor and Henkel,2006, McGuire et al., 2010, Dighton et al., 1987). No consensus can be made yet concerning the effect of interactions between free decomposers and ECM fungi on litter decomposition (Fernandez and Kennedy, 2015). Here, we showed that different beech litter decomposability did not change the effects of ECM-fungi on the initial phase of litter decomposition. We also found that the presence of low ECM-colonized plants significantly decreased the litter decomposition rate. In parallel, we observed a high final root biomass for low ECM-colonized plants. The input of energy-rich labile carbon by roots (Jones et al., 2009) is known to support a large rhizosphere microbial community (Grayston et al., 1997, Bonkowski et al., 2009). Labile carbon resources alter the microbial community composition (Benizri et al., 2002, Puglisi et al., 2013) and favours fast-growing bacteria (Li et al., 2016, Blagodatskaya et al., 2009), mainly *Proteobacteria* (Fierer et al., 2007, Goldfarb et al., 2011). Fast-growing microorganisms are able to produce high amounts of various constitutive enzymes while the

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production of enzymes by slow-growing microorganisms is induced and is thus probably more specific to substrate composition (Fierer et al., 2007). Consequently, microbial communities growing on labile resources usually exhibit lower functional abilities (Fanin et al., 2016, Strickland et al., 2009, Fanin and Bertrand, 2016) which can have important consequences on N cycling (Schimel et al., 2005). The reduced litter decomposition rate in the presence of low ECM-colonized roots may thus result from the narrow catabolic capacities of the microbial decomposers following the selection of fast-growing microorganisms by high rhizo-deposition rates. Our results are thus in agreement with the '*functional breadth*' hypothesis (Keiser et al., 2011).

We found a significant relationship between litter decomposition and soil respiration in absence of plants or in the presence of high ECM-colonized plants. This was not the case in the presence of low ECM-colonized roots. This observation suggests that different sources of carbon were used by the microbial decomposers according to the treatment. In the absence of plants or in presence of high ECM-colonized saplings, litter may act as the main source of carbon, while in presence of a low ECM-colonized sapling, roots exudates may supply the carbon. The analysis of litter carbon loss during decomposition (Supporting Information 3.B) supported this '*decoupling carbon source*' hypothesis: high ECM-colonized plants increased litter carbon loss in comparison to the control while the presence of low ECM-colonized plants decreased litter carbon loss. The saprophytic activity of ECM fungi is still debated (Baldrian, 2009, Cullings and Courty, 2009) but our results suggest that ECM fungi probably used carbon and mined N from poorly decomposable litters for their own growth, while free microbial decomposers did not do so in the presence of low ECM-colonized plants (Buée et al., 2007, Read and Perez-Moreno, 2003). Litter N immobilization was higher when high ECM-colonized plants were present, but only for poorly decomposable litter. ECM fungi can

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produce nutrient-acquiring enzymes such as proteases (Baldrian, 2009) that could probably explain a high amount of the N directly immobilized from poorly-decomposable litter.

Soil N retention

The input of poorly-decomposable litter increased the N proportion within particulate organic compounds (PON) (defined here as the difference between total N and total dissolved N), in accordance with the previous findings of Guo et al. (2013). As discussed by Vitousek (1982), litter with a high C:N ratio should increase N retention and reduce soil N availability. Litter from mature trees has a higher C:N ratio and lignin concentration than litter from young trees (Trap et al., 2013) which may favour N binding with tannins (Kraus et al., 2003, Wurzburger and Hendrick, 2009, Verkaik et al., 2006), making N highly recalcitrant. Because PON is probably less mobile than dissolved organic N and mineral N forms (Neff et al., 2003), increasing the size of PON may increase soil N retention. Also, we found that low ECM-colonized plants did not accumulate as much N within PON as did high ECM-colonized plants, signifying that ECM fungi was crucial to the increase in the PON pool size. Knowing that mineral N can be easily lost by leaching or by denitrification (Hayatsu et al., 2008), soil N retention was also favorred by lower net N mineralization (Supporting Information 4) in the presence of litter. The high levels of tannins in litter may decrease N mineralization by binding microbial enzymes or enzyme substrates (Kraus et al., 2003, Fierer et al., 2001), while nitrification may have been inhibited by monoterpenes (Smolander et al., 2006) or polyphenols (Northup et al., 1995) leaching from the litter. Interestingly, we found lower nitrification in the presence of high ECM-colonized plants. Nitrification may have been inhibited either by competition for ammonium between microbial decomposers and ECM

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fungi (De Boer and Kowalchuk, 2001) or by inhibiting compounds released by ECM fungi (Subbarao et al., 2007).

Microbial biomass N

Microbial biomass N greatly increased when litter was added. Likewise, the presence of low ECM-colonized roots increased the microbial biomass N in comparison to the control when litter was absent (+0.57 g-N microcosm^{-1}). Knowing that soil decomposers are strongly limited by energy-rich labile carbon (Demoling et al., 2007), litter and rhizo-deposition may thus provide energy for microbial decomposers, allowing them to immobilize more N (Dannenmann et al., 2009, Murphy et al., 2015, Farrell et al., 2014).

However, the soil microbial biomass N per unit C (or N) decreased when litter was added in comparison to the control, but increased in presence of low ECM-colonized roots. These observations suggest that litter provided poorly available C compounds, limiting microbial activity per unit C in contrast to beech root exudates. This antagonism supported our '*carbon source-decoupling*' hypothesis discussed earlier between litter and rhizo-deposition acting as carbon sources for microorganisms. We also observed that the presence of high ECM-colonized plants decreased microbial biomass N per unit N in comparison to the control. ECM fungi may compete with microbial decomposers for N, or limit the carbon supply for microbial decomposers, supporting the '*decomposerstarvation hypothesis*' (Hattenschwiler et al., 2011).

The PLS regression analysis showed that PON, total N and litter N were the best predictors of the microbial biomass N. Higher PON and litter N concentrations were associated with low microbial N immobilization per unit soil N, while a higher final ammonium concentration and potential ammonification exhibited the opposite trend. Kuzyakov and Xu (2013) found

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that microorganisms exhibited very high affinity (low K_m values) and capacity (high V_{max} values) for ammonium, while plants have higher affinity and capacity for nitrate. We thus supposed that microbial decomposers immobilize a high amount of ammonium during the experiment, but only when carbon was not limiting. When they become carbon-starved, they may use amino acids for energy. The excess of N is thus released as ammonium in the soil solution according to microbial stoichiometric constraints (Mooshammer et al., 2014, Mooshammer et al., 2012, Kuzyakov and Xu, 2013). This release may occur when LC-plants or litter without ECM fungi were added in the microcosms.

Plant N acquisition and growth

We observed that the presence of both litter with a high proportion of recalcitrant N and roots highly colonized by ECM fungi was required to maximize plant N acquisition. Our observations are in accordance with the literature concerning the crucial role of ECM fungi in plant N nutrition (Bending and Read, 1996) and agree with the findings of Madritch and Lindroth (2015), which showed that ^{15}N recovery from frass or litter of defoliated trees increased with increasing concentrations of tannins in the leaf litter produced by *Populus tremuloides* genotypes. Microbial biomass N was significantly negatively correlated with shoot N amount (Fig. 5), suggesting that the microbial community and the beech sapling strongly competed for N during the experiment. Gessler et al. (1998) showed that beech preferentially take up ammonium rather than nitrate to supply their N requirements. This may explain why the PLS-regression identified the final ammonium concentration as an important predictor of shoot N amount.

The soil mineral N concentration did not change in the presence of high ECM-colonized plants and litter together in comparison to litter treatments, which could indicate direct N

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uptake from the litter by ECM fungi (Ehrenfeld et al., 2005). This assumption is supported by the PLS regressions that showed that the final N amount in shoot was largely explained by the size of the final litter N concentrations. The ability of ECM fungi to take up N directly from litter may thus constitute an important mechanism involved in the competition of microbial decomposers and ECM fungi for N. Finally, PON and litter N are key N pools that explain the final amount of shoot N.

CONCLUSIONS

The experiment provided evidence that poorly decomposable litter produced by mature beech trees and ECM fungi together decrease microbial N immobilization but increase tree N acquisition. Litter-ECM fungi interactions maximized the N nutrition of beech saplings probably by increasing recalcitrant N pools. These interactions inhibit mineral N production and decrease access of labile C for microbial decomposers, resulting in lower microbial N demand and higher soil N retention. Growing with poorly decomposable litter from mature trees and ECM fungi may thus confer a competitive advantage to beech saplings for N over microbial decomposers. Our study did not test whether such leaf litter-ECM interactions improved (or limited) N recovery for other plant species. It would thus be interesting to conduct plant competition experiments using plant species, ECM fungi and litter quality as factors. Such studies may help to provide an understanding of how these ecological mechanisms operate in temperate forests and impact the natural regeneration of these ecosystems.

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Data Accessibility

All data is included in the paper and supplementary information.

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Figure 1. (A) Leaf litter mass loss (% of initial) at the end of the experiment according to treatments. The vertical bars represent the standard deviation. The microcosms were without plant ("- plant", square), planted with high ("HC", triangle) or low ("LC", circle) ECM-infected beech saplings. The soil was covered by highly-decomposable ("HD", white symbols) or by poorly-decomposable litter ("PD", black symbols). The interaction from the two way-ANOVA was not significant, thus letters "x and y" and "α, β and γ" indicate main effect significance among "litter" and "plant" treatments, respectively, according to Tukey HSD test ($P < 0.05$, n=5). (B) Relationships (Pearson correlations) between leaf litter mass loss and soil basal respiration rate (i) in absence of plant (R^2: 0.56), (ii) in presence of HC-plants (R^2: 0.46) and (iii) in presence of LC-plants (no correlation). *$P < 0.05$.

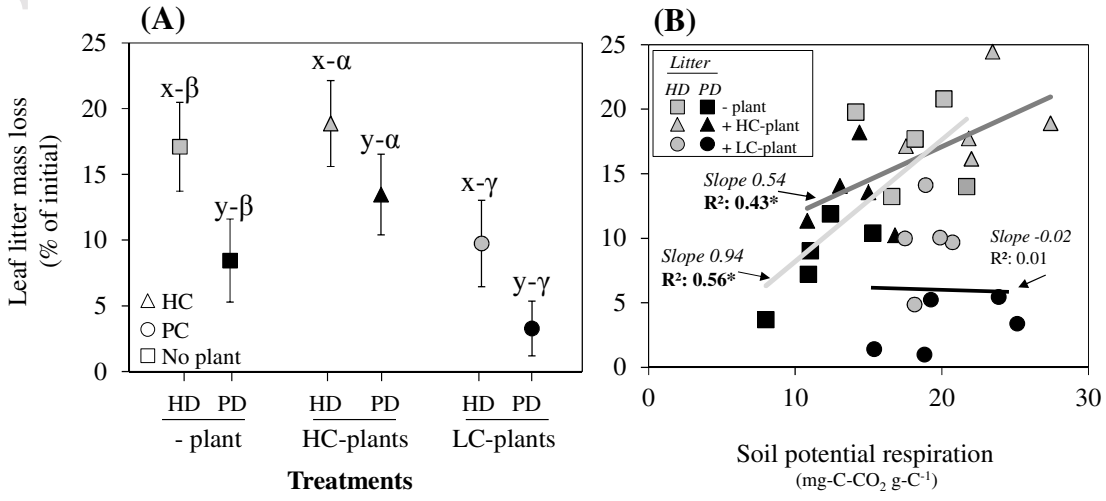

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Figure 2. (A) N amount and (B) N concentration in plant shoot at the end of the experiment according to treatments. Vertical bars represent standard deviation. The microcosms were planted with high ("HC", triangle) or low ("LC", circle) ECM-infected beech plants. The soil was uncovered ("alone", white symbols), covered by highly-decomposable ("HD", white symbols) or poorly-decomposable litter ("PD", black symbols). The dark dotted line corresponds to initial values. Letters (a, b and c) indicate significant differences among treatments according to two-way ANOVA and post hoc Tukey HSD test ($P < 0.05$, n=5).

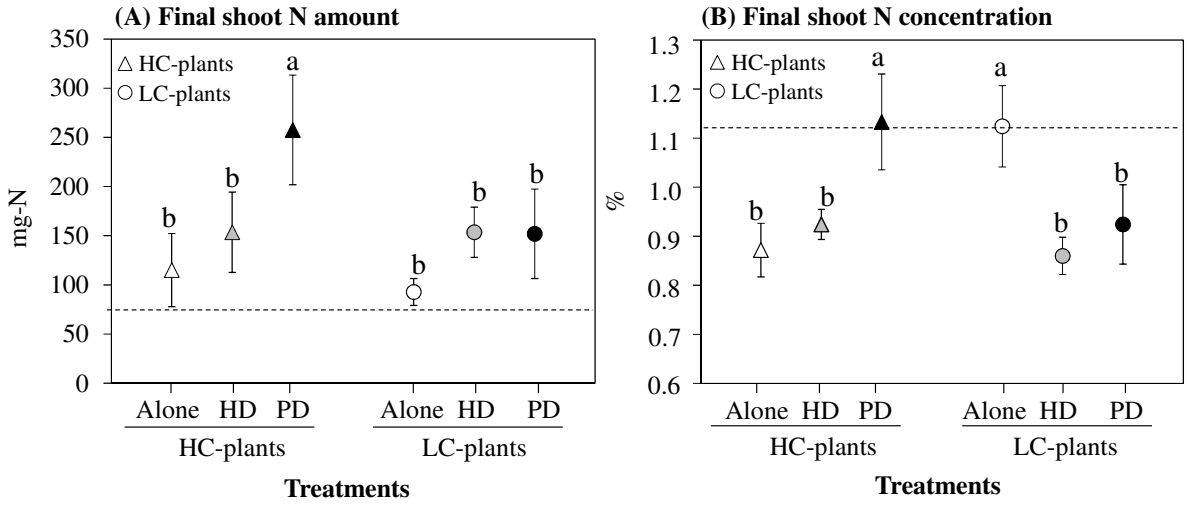

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Figure 3. The allocation of biomass in shoot at the end of the experiment (expressed in proportion of total biomass gain) according to treatments. Vertical bars represent standard deviation. Microcosms were planted with high (HC) or low (LC) ECM-infected beech plants and covered by highly (white bars) or poorly (black bars) decomposable litters. Letters (a, b and c) indicate significant differences among treatments according to two-way ANOVA and post hoc Tukey HSD test ($P < 0.05$, n=5). Interaction significance was P-value = 0.04.

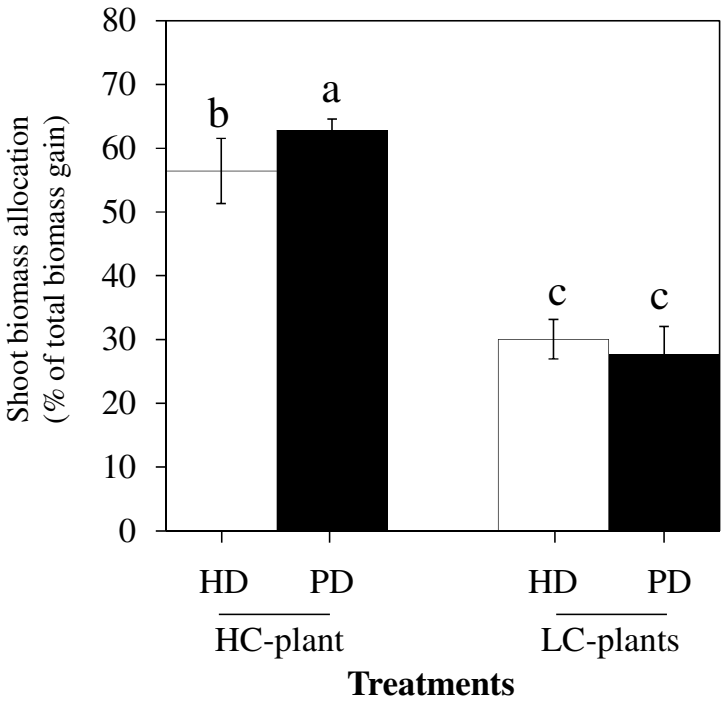

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Figure 4. Scatter plots and Variable Importance of Projection (VIP) from the PLS-R models of predicted *versus* measured values of (A) microbial biomass N and (B) shoot N amount. Microbial biomass N is expressed in proportion of total N in soil and shoot N corresponds to total N amount in shoot measured at the end of the experiment. The explanatory variables are the different N pools measured at the end of the experiment (see table 1). Q^2 = cross-validated R^2. Signs (+ or -) in front of each explanatory variables indicates trend of standardized regression parameters. The dotted lines correspond to 95% confidence interval. "*n*" corresponds to total number of observations. ***$P < 0.05$.

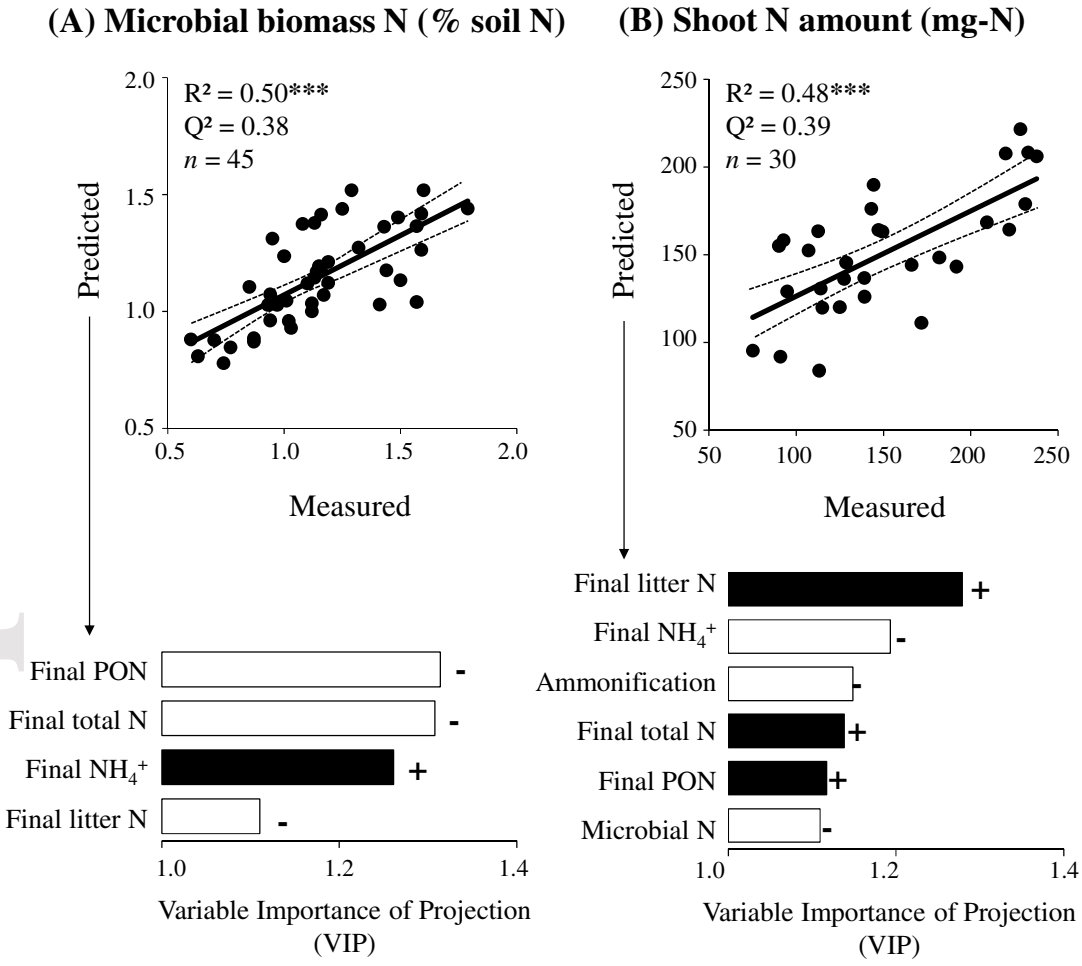

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Figure 5. Linear regression between (A) the amount of shoot N (mg-N shoot^{-1}), (B) shoot biomass (response variables) and soil microbial biomass N (explanatory variable). Microbial biomass N is expressed in proportion of the total N in soil and shoot N corresponds to total N amount in shoot measured at the end of the experiment. "*n*" corresponds to total number of observations. ***$P < 0.001$.

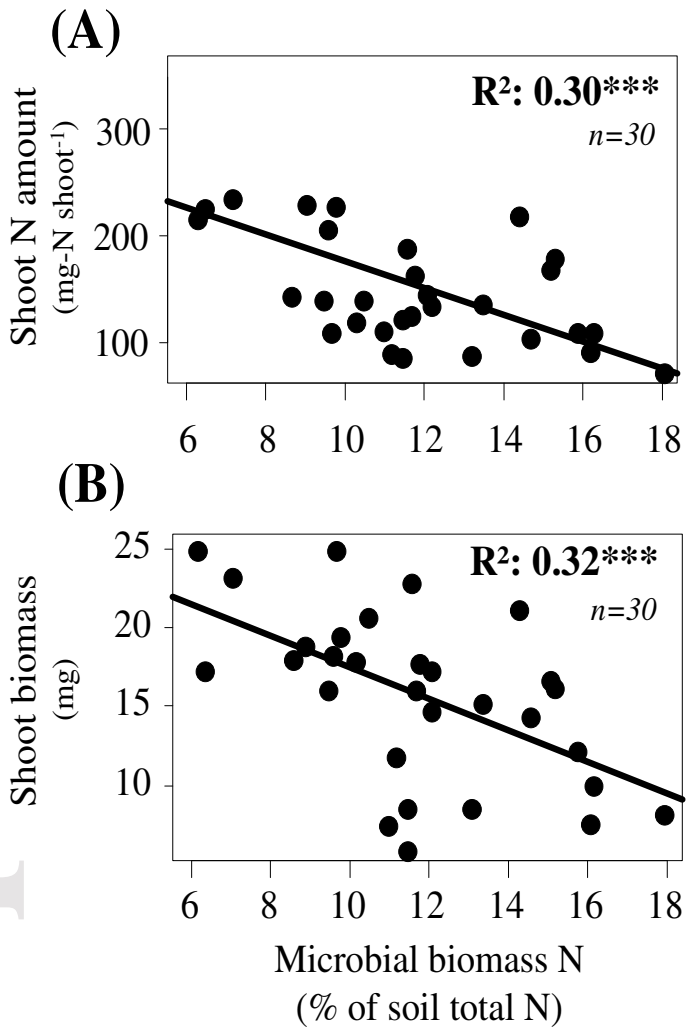

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Table 1. Two-way ANOVA results for all variables with "litter" (absent, highly and poorly decomposable litter) and "plant" (no plant, low and high ECM-colonized roots) as factors.

Variables	Units	$n^{\$}$	Two-way ANOVA		
			Factors		
			Litter	*Plant*	*Interaction*
Soil-based variables					
Total N	mg g^{-1}	9	***	*	***
Particulate organic N	mg g^{-1}	9	***	N.S	***
Dissolved organic N	µg g^{-1}	9	N.S	***	N.S
Ammonium	µg g^{-1}	9	**	***	**
Nitrate	µg g^{-1}	9	N.S.	***	**
Microbial biomass N (N_{mic})	µg g^{-1}	9	N.S	N.S	***
N_{mic} per unit C	µg mg-C^{-1}	9	N.S	**	***
N_{mic} per unit N	% of total N	9	***	N.S	*
Ammonification	µg g-C^{-1} d^{-1}	9	***	***	N.S
Nitrification	µg g-C^{-1} d^{-1}	9	***	***	N.S
Respiration	mg-C-CO_2 g-C^{-1}	9	***	***	***
Litter-based variables					
Final litter N content	% of initial	6	***	N.S	N.S
Litter mass loss	% of initial	6	***	***	N.S
Litter-C loss	% of initial	6	***	***	N.S.
Plant-based variables					
Total biomass	g	6	***	*	*
Shoot biomass	g	6	***	*	*
Root biomass	g	6	***	***	*
Final shoot N amount	mg-N	6	***	**	*
Final shoot N concentration	%	6	N.S.	N.S.	**

$^{\$}$ "n" corresponds to the total number of treatments

N.S. non significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

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Table 2. N pools and flows in the litter and the soil at the end of the experiment according to treatments (mean values).

Variables	Units	Treatments															
		Without plant (- plant)						High ECM-colonized plant (HC)						Low ECM-colonized plant (LC)			
		Litter occurrence[$]						Litter occurrence						Litter occurrence			
		Absent		*HD*		*PD*		*Absent*		*HD*		*PD*		*Absent*		*HD*		*PD*	
Total N	mg g^{-1}	2.0	**d**	3.4	**abc**	4.0	**ab**	2.6	**cd**	3.1	**bc**	4.1	**a**	2.7	**cd**	2.8	**cd**	2.9	**cd**
Particulate organic N	mg g^{-1}	1.7	**d**	3.1	**abc**	3.7	**ab**	2.4	**cd**	2.8	**bc**	3.9	**a**	2.5	**cd**	2.6	**cd**	2.7	**cd**
Dissolved organic N	µg g^{-1}	224.2	**α**	230.0	**α**	256.8	**α**	177.7	**β**	193.2	**β**	193.9	**β**	156.5	**γ**	140.6	**γ**	165.2	**γ**
Ammonium	µg g^{-1}	3.1	**a**	2.6	**ab**	2.6	**ab**	0.2	**d**	2.2	**abc**	2.5	**ab**	0.3	**d**	1.0	**cd**	1.1	**bcd**
Nitrate	µg g^{-1}	5.9	**ab**	6.5	**a**	6.7	**a**	2.8	**a**	5.7	**ab**	6.1	**a**	3.3	**bc**	2.0	**c**	2.4	**c**
Microbial biomass N (N_{mic})	µg g^{-1}	279.5	**b**	348.3	**ab**	354.1	**ab**	249.1	**b**	397.7	**a**	304.5	**ab**	394.4	**a**	296.0	**ab**	309.7	**ab**
N_{mic} per unit C	µg $mg\text{-}C^{-1}$	8.3	**c**	8.8	**abc**	7.7	**bc**	8.6	**abc**	11.8	**ab**	6.7	**c**	12.8	**a**	8.7	**abc**	9.7	**abc**
N_{mic} per unit N	% of total N	13.9	**ab**	10.3	**bcd**	9.1	**cd**	10.2	**abc**	13.0	**abc**	7.5	**d**	14.8	**a**	10.4	**abcd**	10.8	**abcd**
Ammonification	µg $g\text{-}C^{-1}$ d^{-1}	111.6	**x-α**	107.9	**x-α**	91.9	**y-α**	175.2	**x-β**	139.8	**x-β**	100.1	**y-β**	169.1	**x-β**	151.6	**x-β**	135.0	**y-β**
Nitrification	µg $g\text{-}C^{-1}$ d^{-1}	15.3	**x-α**	7.5	**y-α**	8.5	**y-α**	10.3	**x-α**	8.8	**y-α**	7.7	**y-α**	17.7	**x-β**	11.6	**y-β**	12.0	**y-β**
Final litter N content	% of initial	/		108.7	**x**	119.2	**y**	/		109.7	**x**	123.1	**y**	/		108.2	**x**	118.3	**y**

When the interaction from the two way-ANOVA was significant (see Table 1), letters "a, b, c and d" indicate significant differences among treatments (vertical reading) but when the interaction from the two way-ANOVA was not significant, letters "x and y" and "α, β and γ" indicate main effect significance among "litter" and "plant" treatments, respectively, according to Tukey HSD test ($P < 0.05$, n=5).

[$]Litter was poorly- (PD) or highly-decomposable (HD) (see text for details)

$*P < 0.05$; $**P < 0.01$; $***P < 0.001$

Table 3. Plant biomass at the end of the experiment according to treatments.

Plant biomass	Units	Treatments								
		High ECM-colonized plant (HC)						Low ECM-colonized plant (LC)		
		Litter occurrence[$]						Litter occurrence		
		Absent		*HD*		*PD*		*Absent*	*HD*	*PD*
Total biomass	g	21.5	**bc**	29.0	**abc**	33.7	**ab**	17.1 **c**	47.6 **a**	44.2 **a**
Shoot biomass	g	13.1	**bc**	16.9	**ab**	20.9	**a**	7.8 **c**	17.9 **ab**	16.2 **ab**
Root biomass	g	8.5	**b**	12.1	**b**	12.8	**b**	9.2 **b**	29.7 **a**	28.0 **a**

Letters "a, b, c and d" indicate significant differences among treatments (vertical reading) according to two way-ANOVA and Tukey HSD test.

[$]Litter was poorly (PD) or highly decomposable (HD) (see text for details)

$*P < 0.05$; $**P < 0.01$; $***P < 0.001$

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