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Effects of nitrogen availability on microbial activities, densities and functional diversities involved in the degradation of a Mediterranean evergreen oak litter (*Quercus ilex* L.)

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ABSTRACT

The effect of available nitrogen N ($(NH_4)_2SO_4$) amendments on various microbial variables in three different layers (OhLn, OhLv, OhLf) of a Mediterranean litter profile under an evergreen oak forest (*Quercus ilex* L.) were studied. Since Mediterranean litters are generally N limiting, the goal of the study was to understand how low (0.1 and 1%) and high (5 and 10%) N amendments impact specific biological variables such as hyphal length, community-level-catabolic-profiles (CLCPs) in ECO and FF Biolog™ plates, basal respiration, enzymatic activities (i.e. alkaline phosphatases (AlP), laccases, peroxidases and cellulases), and laccase and cellulase isoforms from three different litter layers. Results indicated that the effects of N amendments occurred over very short incubation time (3 d), and varied depending on N concentration and litter organic matter (OM) quality (i.e. depth). Thus, it appeared that the more active layer was the intermediate (OhLv) layer, which probably contained the most labile and available C pools. As a consequence, OhLv was also the layer showing globally the more intensive microbial responses following low N amendments. Indeed, in this layer, low N supplies caused several marked increases in enzymes activities (i.e. laccases, cellulases and alkaline phosphatases), hyphal length and isoenzyme patterns, suggesting a microbial reallocation of C to biomass and enzyme production. On the contrary, high N supplies resulted in adverse effects on almost all the variables, suggesting repression or cytotoxic phenomena.

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1. Introduction

At forest ecosystem level, the litter is an important layer since it is source of energy and essential elements for the metabolism of microbial communities. The renewal and the decomposition of litter are crucial to the correct functioning of the biogeochemical cycles as those of carbon, nitrogen, sulphur and phosphorus. Thus, the forest productivity is strongly related to the turn-over of the litter organic matter. The mechanisms of litter decomposition can be influenced by biotic or abiotic factors. At microbial community level, various populations of microorganisms succeed one another in the course of time degradation according to availability of organic compounds and according to changes of the environment. On this point, Toutain (1981) mentioned that among microorganisms, fungi were the first colonizers of litter layer. Due to their enzymatic systems, these microorganisms can effectively get the nutritive elements essential to their growth by degrading the litter organic matter. The degradation of organic matter (OM) varies according to the complexity of its structure. Thus, the cellulose is mainly degraded by hydrolases while lignin, a more recalcitrant biopolymer, is attacked by phenoloxidases. Increasing the availability of certain nutrients often causes a negative feedback on the synthesis of enzymes involved in the decomposition of plant polymers and the release of mineral nutrients. Indeed, such phenomena take place in order to reduce synthesis of extracellular enzymes and energy cost when nutrient levels are sufficiently high for microorganism growth. For example, phosphate fertilization causes an inhibition of phosphatase activity, which role is to release available orthophosphate ions from phosphorylated organic matter (Clarholm, 1993; Criquet et al., 2007). Nitrogen is an essential element for the living organisms and can be found in the nature either by natural processes or by anthropogenic activities. Litter decomposition provides the primary source of N for biological activity (Parton et al., 2007) in soils, and recycling of N from litter

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decomposition provides a key resource for ecosystems productivity (Waldrop et al., 2004). However, due to its short supply, N is commonly a limiting element for plant growth in many natural ecosystems (Rovira and Vallejo, 1997; Vitousek et al., 2002). Moreover, N often limits microbial growth (Carreiro et al., 2000) and therefore may decrease soil organic matter (SOM) decomposition rates (Sjöberg et al., 2004) of litter.

N availability is an important factor that controls the litter decomposition rate, in addition to the structure and the chemical composition of litter OM. Indeed, Parton et al. (2007) have mentioned, for many litters within the main terrestrial biomes, that decomposition rate and net N release from leaf litter is dominantly driven by the initial tissue N concentration. Moreover, according to Fog (1988), the oxidative decomposition of lignin would be mainly controlled by N content. Thus, chronic N addition in forest ecosystem has been reported to induce cellulase synthesis by microorganisms growing on leaf litter from low-lignin species, while phenoloxidase activity decreases in high-lignin oak litter (Knorr et al., 2005). Since lignin and cellulose are the two most abundant OM on earth, altering by N input microbial activities involved in their recycling and their mineralization may affect soil C storage, CO_2 release to atmosphere, and therefore the global carbon cycle.

In Mediterranean forest ecosystems, evergreen oak (*Quercus ilex* L.) forms characteristic climax populations that are widely distributed throughout the Western Mediterranean area (Rapp, 1969). Within these ecosystems, soils are often degraded because of the action of recurrent fires and the particular Mediterranean climate, characterized by the succession of dry and strong rainy periods. Because of the conjunction of these environmental factors and of the sclerophyllous nature (i.e. richness in cutin, waxes, lignin and tannins) of evergreen oak, litter of this species often shows low degradation rates (Nèble, 2005; Fioretto et al., 2007). In previous studies (Criquet et al., 2000, 2004), we showed that the decomposition of evergreen oak litter may be regulated by environmental factors such as moisture, temperature and seasonal patterns. These kinds of effects have been also corroborated by several studies performed by the research team of A. Fioretto (Fioretto et al., 2001; Di Nardo et al., 2004; Fioretto et al., 2007) using the same leaf material as well as other Mediterranean litters. N content of leaves is known to regulate mass loss rates of litter, especially in the early stages. However, to our knowledge, little is known about N availability, in relation with OM quality (i.e. litter decomposition stage), on microbial functional processes involved in litter degradation under Mediterranean climate. Indeed, studies about N effects on litter biology are generally performed on the whole Oh horizon. Thus, one of the original purposes of our study was to consider the influence of this factor on microbial variables of different sub-layers, corresponding to different OM qualities. In this context, we aimed to evaluate the influence of a source of nitrogen, made up of ammonium sulphate ($(NH_4)_2SO_4$), on the intensity of microbial responses of various horizons (OhLn, OhLv and OhLf) of an evergreen oak litter (*Q. ilex* L.). With this aim, different biological responses (basal respiration, enzymes activities, enzyme isoforms, community-level-catabolic-profiles (CLCPs) and microbial biomass) were monitored following increasing nitrogen fertilization under mesocosm experiments, in order to distinguish a possible inductive or repressive nitrogen effect.

2. Material and methods

2.1. Litter

Evergreen oak litter (*Q. ilex* L.) was collected in February 2005 from a 1000 m^2 dense coppice at 'La Gardiole de Rians' (Var region, France), in a Mediterranean-type bioclimate area. Litter samples were collected randomly and independently from five profiles of $5 \times 30 \times 30$ cm (depth, length, width). In each profile, three litter layers from the O horizon (Oh) were distinguished (Baize and Girard, 1995): the OhLn layer (upper layer), formed by leaves over a period of less than 1 y, non-degraded and non-compressed; the OhLv layer formed by leaves still recognizable despite decay, non-compressed; and finally the OhLf layer, formed by fragmented leaves and often compressed in lumps. All samples were air-dried at room temperature and stored at 4 °C over 70 h before their use. Dry weight was determined by weighing and drying overnight at 105 °C three replicates of a same litter sample. Total N contents of leaves were not analyzed in this study, but this kind of information has been well documented by Fioretto et al. (2007).

2.2. Nitrogen experiment

In order to evaluate the effect of nitrogen on enzymatic activities, CLCPs, respiration and hyphal length, litter layers were amended with different concentrations (0%, 0.1%, 1%, 5% and 10%) of ammonium sulphate ($(NH_4)_2SO_4$), corresponding to 0.21, 2.1, 10.6 and 21.2 g N l^{-1}, respectively. Three replicates were performed for each layer and each concentration, for a total of 45 samples. The ($(NH_4)_2SO_4$) solutions were adjusted to the pH of each litter layer in order to avoid a pH effect, which could modify the microbial metabolism. With this aim, and to avoid additional source of N, 0.1 M HCL and 0.1 M NaOH solutions were used. Samples (10 g) of each litter layer were placed 10 min in the different nitrogen solutions, allowing leaf material to be remoistened at their WHC. Remoistening was performed considering the initial water content of the leaves and their respective WHC, which differ according to the layer considered (data not shown, see Alarcón-Gutiérrez, 2007). Thus, the volume of N solutions used to attain WHC was deduced from these values and applied to leaves in plates in order to soak them until their WHC. In effect, few additional ml of N solution were used to ensure complete soaking, but leaves were never immersed in large volume to avoid litter constituent losses. Controls were carried out according to the same protocol but using bidistilled water instead of nitrogen solutions. Remoistened layers were incubated at 20 °C for 72 h.

2.3. Basal respiration

Control and nitrogen treatments (10 g of litter samples) were placed in an Oxytop apparatus (Oxytop® Model OC110, WTW Wissenschaftlich-Technische Werkstätten GmbH, Munich, Germany) to monitor O_2 consumption and CO_2 production over 72 h at 20 °C. Basal respiration was expressed in mg O_2 g^{-1} dry weight (mg O_2 g^{-1} DW). Measurements were performed on each litter sample (i.e. 45; see Section 2.2).

2.4. Enzyme assays

After the incubation time, enzyme activities were measured in extracts obtained from each sample according to Criquet et al. (1999). Unless otherwise indicated, enzyme kinetics were monitored spectrophotometrically (Kontron, model Uvikon 860). All the enzyme activity measurements were performed in triplicate for each extract.

Laccase activity in the extract was monitored using syringaldazine as substrate (Harkin and Obst, 1973; Criquet et al., 1999). For kinetic measurement, 200 µl of enzyme extract, 790 µl of 0.1 M phosphate buffer (pH 5.7) and 10 µl of a 5 mM syringaldazine-methanol solution were mixed. The oxidation rate of syringaldazine to quinone was monitored (525 nm, $\varepsilon = 65\,000$ M^{-1} cm^{-1}) at 30 °C. The results were expressed in unit, defined as µmole of quinone formed from syringaldazine min^{-1} (U) g^{-1} of dry weight (U g^{-1} DW).

For cellulase activity measurement, 100 µl of enzyme extract and 900 µl of 50 mM acetate buffer (pH 6.0) with 1% CMC

(CarboxyMethylCellulose) were mixed and incubated at 50 °C for 1 h. After incubation, the sugars released from the hydrolysis of CMC were measured using the colorimetric method of Somogyi–Nelson (Nelson, 1944; Somogyi, 1952). Cellulase activity was expressed in μmole of glucose released min^{-1} (U) g^{-1} of dry weight (U g^{-1} DW).

Peroxidase activity was measured using 2,7-diaminofluorene (DAF) as substrate (Criquet et al., 2001). For kinetics measurement, 200 μl of enzyme extract, 800 μl of 0.1 M phosphate buffer (pH 5.7), 10 μl of a 5 mM DAF solution and 10 μl of H_2O_2 1 mM were mixed and incubated at 30 °C. Kinetic of DAF oxidation was monitored at 600 nm ($\varepsilon = 10\,228\ M^{-1}\ cm^{-1}$). The results were expressed in unit, defined as μmole of oxidized substrate min^{-1} (U) g^{-1} of dry weight (U g^{-1} DW).

Alkaline phosphomonoesterase was monitored according to Tabatabai and Bremner (1969). The method was based on the measurement at 412 nm of *p*-nitrophenol (*p*-NP) released during the incubation of litter extract with *p*-nitrophenylphosphate monoester. For AlP measurement, 100 μl of enzyme extract, 900 μl of 0.1 M NaOH–Glycine buffer (pH 9) and 10 μl of 15 mM *p*-nitrophenylphosphate (*p*-NPP) were mixed and incubated 1 h at 37 °C. A *p*-NP calibration curve (0–20 mg l^{-1}) was performed under the same conditions, and results were expressed in unit defined as μmole of *p*-NP released min^{-1} (U) g^{-1} of dry litter (U g^{-1} dry litter).

2.5. Community-level-catabolic-profiles (CLCPs)

The CLCPs of litter microbial communities was assessed using Biolog™ ECO plates and Biolog™ FF plates. One gram of fresh ground litter (<0.3 mm) was suspended in 10 ml of sodium pyrophosphate (0.1%) solution and shaken 20 min. Suspensions were allowed to settle for 10 min, then decanted and diluted by 1/100 with a NaCl solution (0.8%). Each well of Biolog™ plates was inoculated with 150 μl of sample supernatant. Three independent replicates were performed for each sample. All samples were incubated at 25 °C and the optical density (O.D.) was read for each plate at 8-h intervals, over a period of 100 h. Plates were read at 590 nm and 720 nm for ECO and FF plates, respectively. Non-inoculatedwells served as controls, and the average well colour development (AWCD) was calculated for each plate. To allow comparison between the different layers and the different N amendments, an AWCD of 0.3 was chose. Moreover, the rates and lag times from AWCD curves were calculated. At the end of incubation, the numbers of negative wells were recorded in each plate. Shannon diversity index (Liu et al., 2007), neperian specific catabolic rate (μ_{max}) and lag time were calculated for each plate.

2.6. Electrophoretic analysis

To characterize cellulase and laccase isoenzymes, electrophoresis were performed according to Criquet et al. (2000) using a composite (obtained by mixing the three extracts of the same amendment modality, i.e., kind of layer *vs* N concentration) and concentrated extract. Native gels were performed according to Laemmli (1970) and Criquet et al. (2002) with some modifications. Concentrated extract (130 μl) were mixed with 20 μl of gel loading buffer (50 mM Tris–HCL pH 6.8, 0.1% bromophenol blue and 10% glycerol) and loaded on a native polyacrylamide gel (without sodium dodecyl sulphate (SDS) or β-mercaptoethanol) electrophoresis (PAGE). For the cellulase isoenzymes, a polyacrylamide separating gel (12%) layered with a 4% stacking gel and Tris–glycine electrode buffer were used. In the separating gel, the water fraction was added with 0.1% CMC. The gels were run at 200 V for 160 min at room temperature. After migration, the gels were incubated in a 50 mM sodium acetate buffer (pH 6.0) at 50 °C over 30 min. Thereafter, the gels were coloured using 0.1% Congo red solution for 30 min and discoloured with 1 M NaCl solution, until the hydrolysis bands, resulting from the activities of the isoenzymes, appeared clearly against a red back ground (Coughlan, 1988; Schwarz et al., 1987).

For the laccase isoenzymes, a polyacrylamide separating gel (10%) layered with a 4% stacking gel and Tris–glycine electrode buffer were used. The gels were run under the same conditions as for cellulases. After the migration, laccase isoenzymes were revealed by incubating the gels at 30 °C in a 0.1 M phosphate buffer (pH 5.7) with 0.1% *p*-phenylenediamine. Relative mobilities (R_f) of isoenzymes were calculated as the mobility of a particular band (i.e. the migration distance measured at the midpoint of the band) divided by the mobility of the dye-front.

2.7. Hyphal length

Total hyphal length was measured according to an adapted method from Fry (1990) and Bloem et al. (1995). Fresh samples (1.0 g) were suspended in 9.0 ml of a saline solution (NaCl 0.8%) and sonicated for 20 s at 35 W in a high intensity ultrasonic processor (Vibra Cell® Model 602). Suspensions were allowed to settle for 5 min, then decanted and diluted by 1/44 with phosphate buffer (0.1 M, pH 7.0). One milliliter of the last dilution was mixed with 1 ml of methylene blue (Sigma–Aldrich Chemical Co. Ltd.) stock solution (100 mg l^{-1}). Each final suspension (2 ml) was filtered on a Millipore® membrane (0.45 μm pore size, 25 mm diameter). The filter was thereafter mounted between a slide and a coverslip with microscopic immersion oil and examined with a Zeiss Axioscop 40 FL microscope. Thirty random microscope fields were observed for each slide at 1000× magnification. The number of intersections between the hyphae and the lines of the grid were counted, and total hyphal length was estimated (Fry, 1990).

2.8. Statistical analyzes

A multivariate analysis of variance (MANOVA) was performed using STATISTICA 6.1 (StatSoft Inc.) to determine significant effects of nitrogen concentrations on litter variable responses (basal respiration, enzyme activities, zymograms, metabolic profiles, hyphal length). A *post hoc* test (LSD, $P < 0.05$ and $P < 0.001$) was carried out to determine significant differences between means.

3. Results

3.1. Enzyme activities

Fig. 1 shows the enzyme activities measured after the different N amendments on the three litter layers evaluated. ANOVA analyzes revealed that all enzymes activities were significantly affected ($P < 00.1$) by both nitrogen concentration and litter layer.

3.1.1. Laccase activity

Fig. 1A shows that laccase activity varied significantly ($P < 0.001$) with depth. The layer the more active was the OhLv and the less active was the OhLn. Laccase activity also varied significantly with N amendments. In the OhLv layer, laccase activity increased significantly ($P < 0.05$) following low N amendments. Indeed, this activity increased by 97 and 88% following 0.1 and 1% of ($(NH_4)_2SO_4$) amendments, respectively. On the contrary, high concentrations (5–10%) decreased laccase activity by 31 and 53%, respectively. On the other hand, in the OhLn and the OhLf layers, N effect was less evident due to lower laccase activities. It can be observed that laccase activity of both OhLn and OhLf layers was significantly ($P < 0.05$) affected by high ammonium sulphate concentrations (5 and 10%). Thus, laccase activity decreased from 83 to 62% in the OhLn layer, and from 52 to 47% in the OhLf layer. The OhLn layer was the more negatively affected by N amendments,

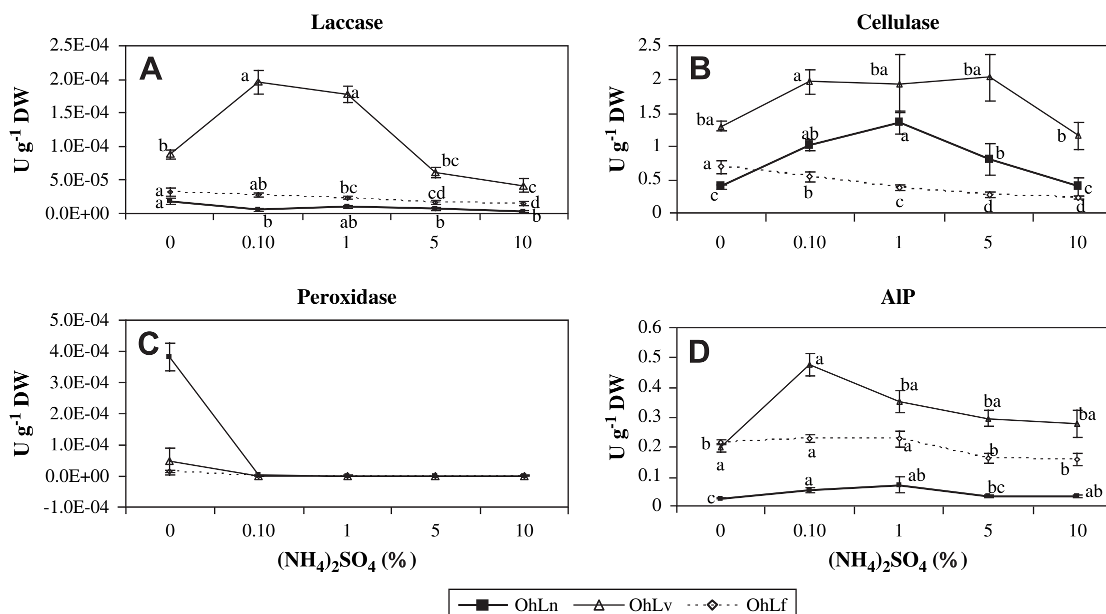

Fig. 1. Variations in enzyme activities ($U\,g^{-1}$ DW) in controls and N amended litter layers. Laccase (A); cellulases (B); peroxidases (C) and alkaline phosphatase (AlP) (D). Litter layers: OhLn (squares); OhLv (open triangles); and OhLf (solid circles). In each layer, mean values ($n = 3$) with the same letters are not significantly different ($P < 0.05$). Bars are standard error.

since amendment of 0.1% decreased laccase activity by 70% in this layer.

3.1.2. Cellulase activity

As described previously for laccases, cellulases were also the more active in the OhLv layer, and Fig. 1B shows that low nitrogen amendments increased significantly cellulase activity in both the OhLn and the OhLv layers. Thus, ammonium amendments of 0.1 and 1% increased by 150 and 232% laccase activity in the OhLn layer, and by 50 and 48% in the OhLv layer. Increasing N concentrations to 5 and 10% lead to a depletion in cellulase activity in the OhLn layer, while such a decrease occurred only for the highest concentration in the OhLv layer. Considering the deepest layer, OhLf, cellulase activities were decreased for all the N amendments applied, and a linear negative relationship between cellulase activity and N concentration was observed.

3.1.3. Phosphatase activity

In non amended controls, alkaline phosphatase activities (AlP) were low in the OhLn layer controls, while they showed high and similar values in both OhLv and OhLf layers. Following 0.1% N amendment, the highest AlP activity was observed in the OhLv layer, corresponding to an increase by 138% with regards to non amended samples. N concentrations from 0.1 to 10% decreased progressively the AlP activity in this layer. In the OhLn layer, N amendments of 0.1 and 1% increased by 95 and 150% AlP activity, respectively. However, AlP activity remained low in the OhLn layer with regards to the other layers. As described for the OhLv layer, high N amendments also showed a trend to decrease AlP activity. Considering the OhLf layer, AlP reacted only slightly to low nitrogen amendments, while high nitrogen amendments decreased by 25% AlP activity.

3.1.4. Peroxidase activity

Following N amendments, the evolutions of peroxidase activities were very different than other ones previously described (i.e. laccase, cellulase and phosphatase). Indeed, all the N concentrations applied to leaf litter layers affected significantly ($P < 0.001$) peroxidases (Fig. 1C), by inhibiting completely the activity of these enzymes. In controls without N addition, significant effect of litter depth on peroxidase activity was detected ($P < 0.001$), and the upper layer OhLn appeared far more active than the other ones.

3.1.5. Enzyme electrophoresis

Fig. 2A shows the laccase isoforms with and without N amendments in the three litter layers evaluated. The numbers of isoforms ranged between 4–6, 3–8, and 3–6 in the OhLn, OhLv and OhLf layers, respectively. Thus, the OhLv was the layer showing the highest diversity in laccase isoforms. In the OhLn layer, all the N amendments induced the synthesis of new isoforms, 0.1% N being the amendment showing the more numerous bands. In the OhLv layer, the isoform diversity was globally stable at low N amendments, but considerably decreased for high N amendments. In the OhLf layer, the diversity of laccase isoforms showed important fluctuations in all the N treatments, 0.1% being again the amendment showing the more numerous bands. Moreover, almost all N concentrations applied to the OhLf layer were characterized by the synthesis of three new isoforms with low R_f, and which were not present in control without N addition.

Fig. 2B shows the cellulase isoforms in the three litter layers with and without N amendments. The numbers of cellulase isoforms ranged between 2–5, 3–6 and 3–5 in the OhLn, OhLv and OhLf layers, respectively. As previously described for the laccases, OhLv was also the layer showing the highest diversity in cellulase isoforms. However, evolution patterns of cellulase isoforms of the different layer were different with regards to those observed for the laccases. Indeed, in the OhLn and the OhLv layers, low N amendments induced the synthesis of new isoforms, while high amendments had adverse effects. In the OhLf layer, increased N amendments resulted in a progressive decrease in the diversity of cellulase isoforms.

3.2. Respiration

Microbial litter respiration showed higher values in the OhLv, followed by the OhLn and the OhLf layers, respectively (Fig. 3). Similar patterns in the respiration responses following increased N applications were observed in the three litter layers. Indeed,

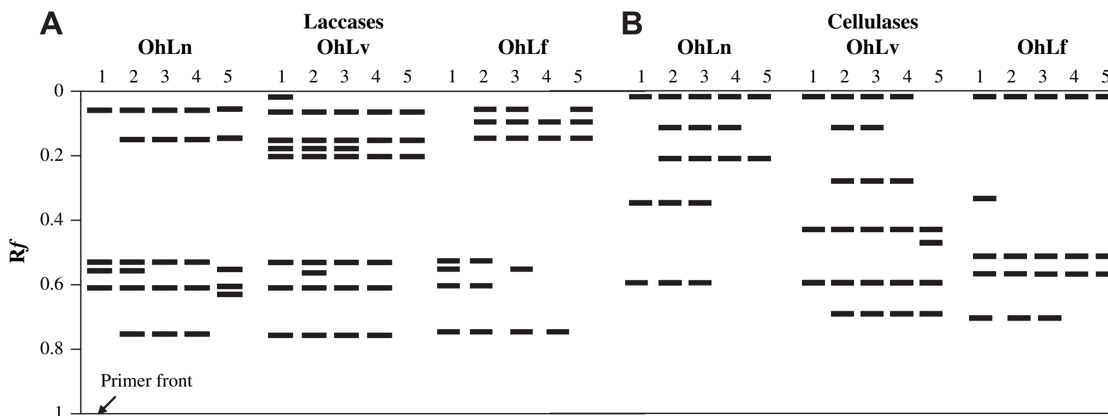

Fig. 2. Laccase (A) and cellulase (B) isoforms in control and N amended litter layers (OhLn, OhLv and OhLf). Lane 1: control without N amendment. Lanes 2 to 5: litter layers amended with 0.1, 1, 5 and 10% ($(NH_4)_2SO_4$), respectively. Negative controls were performed with boiled samples, and no isoforms were detected (data not shown). The relative mobility of proteins is shown as a "retention factor" (R_f).

microbial litter respiration was affected significantly by nitrogen amendments ($P<0.05$) and by litter depth ($P<0.001$). Low nitrogen additions did not affect, or slightly positively ($P<0.05$), microbial respiration. Nitrogen amendments of 0.1% increased by 33% respiration in the OhLn layer, by 8% in the OhLv and by 20% in the OhLf layer (Fig. 3). On the contrary, high amendments affected negatively microbial respiration in all the layers evaluated. Thus, nitrogen amendments at the concentrations of 5 and 10%, decreased microbial respiration by 35 and 50% in the OhLn layer, by 31 and 53% in the OhLv layer, and by 52 and 58% in the OhLvf layer.

3.3. Hyphal length estimation

Total hyphal length in non amended litter was higher in both OhLv and OhLf layers than in the upper layer OhLn (Fig. 4). Moreover, ANOVA results showed significant differences ($P<0.001$) in hyphal length with litter depth. The hyphal length was significantly affected ($P<0.001$) by nitrogen amendments. Following N amendments, the highest hyphal lengths were observed in the intermediate layer OhLv moistened with a 0.1% $(NH_4)_2SO_4$ solution (Fig. 4). In this layer, increasing $(NH_4)_2SO_4$ concentration above 0.1% resulted in a marked decrease in total hyphal length. In the two other layers, increasing N amendment concentration up to 1% allowed hyphal length to increase, and the use of higher concentration had adverse effects. Among these two layers, hyphal length remained always higher in the OhLf layer than in the OhLn layer.

3.3.1. Community-level-catabolic-profiles (CLCPs)

Figs. 5 and 6 show the average well colour development (AWCD 0.3) from ECO and FF plates, respectively, over 100 h of optical density (OD) detection in controls and litter layer samples amended with low (0.1%) and high (10%) ammonium sulphate concentrations. Results of ANOVA indicated that AWCD were significantly affected ($P<0.001$) by N amendments and litter depth in both ECO and FF plates (data not detailed). Similarly, the numbers of negative wells in plates were also significantly affected by N supplies (data not shown). This was also true for the litter depth effect, but only for ECO plates. Catabolic rates (μ_{max}) and lag times were calculated for all AWCD in both ECO and FF plates (Table 1). ANOVA and LSD *post hoc* results indicated that all the catabolic rates were significantly affected by nitrogen amendments ($P<0.001$). In ECO plates, μ globally decreased when increasing N concentrations, whereas in FF plates inverse patterns were observed in the OhLv and the OhLf layers. Lag times also varied with N amendments, showing marked decreases in ECO plates, and few or no fluctuations in FF plates. Shannon diversity index (H) was also calculated. Results showed that increasing N concentrations had significant negative effects on H values in all the litter layers and the Biolog™ plates (i.e. ECO & FF) considered (Table 1).

4. Discussion

4.1. Global responses of microbial variables to N addition

In our study, all the microbial variables investigated responded to N amendments, in relation with litter OM quality (i.e. depth). Thus, our results are in agreement with Caldwell (2005) who suggested that enzyme activities are the expression of the soil community to metabolic requirements and nutrient availability.

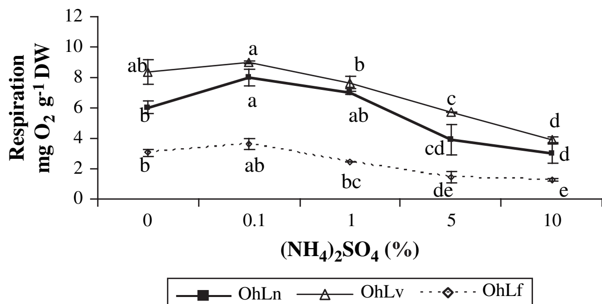

Fig. 3. Respiration values in control and N amended litter layers. OhLn (solid squares); OhLv (open triangles); and OhLf (open triangles). In each layer, mean values ($n=3$) with the same letters are not significantly different ($P<0.05$). Bars are standard error.

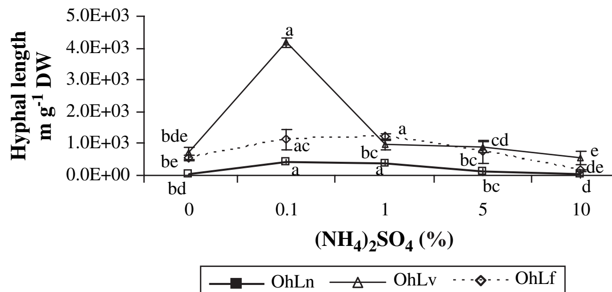

Fig. 4. Total hyphal lengths in control and N amended litter layers. OhLn (solid squares); OhLv (open triangles); and OhLf (open triangles). In each layer, mean values ($n=3$) with the same letters are not significantly different ($P<0.05$). Bars are standard error.

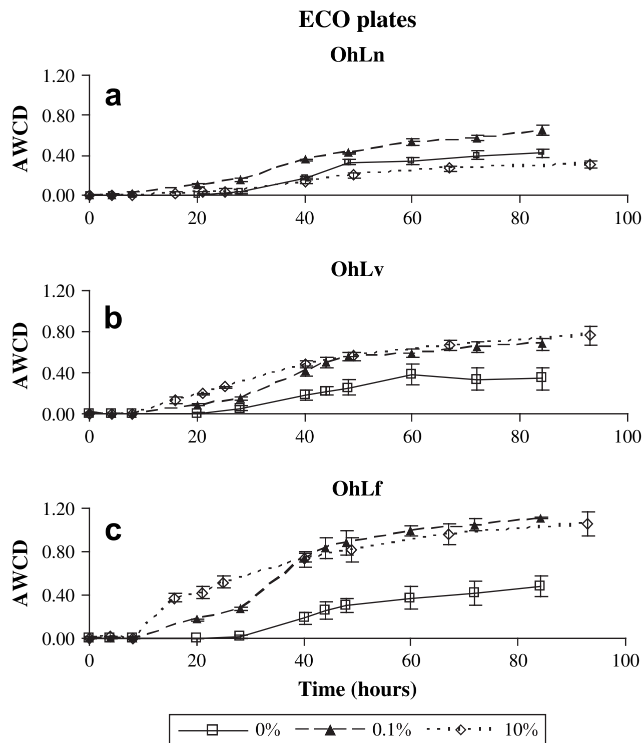

Fig. 5. Biolog™ plate results. Average Well Colour Development (AWCD) of the different litter layer samples in ECO plates. Results show the effect of three treatments (control, 0.1 and 10% (($(NH4)_2SO_4$) amendments) on the AWCD. Bars are standard error ($n = 3$).

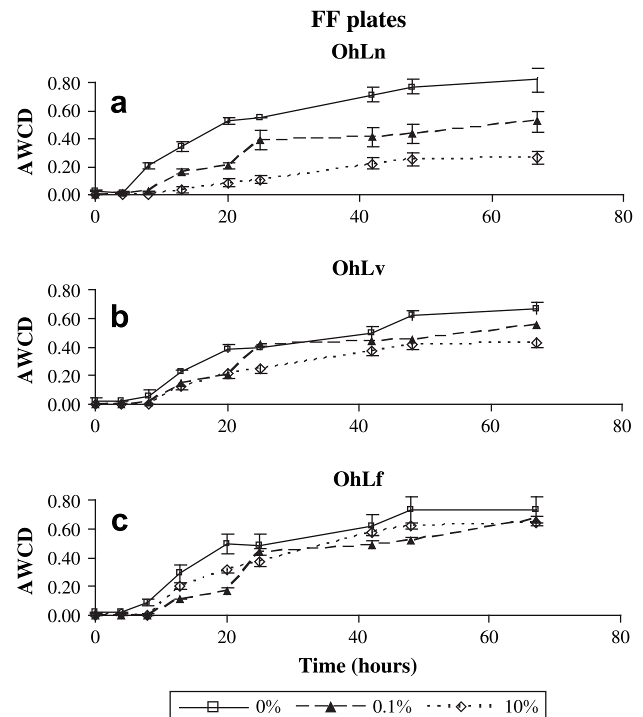

Fig. 6. Biolog™ plate results. Average Well Colour Development (AWCD 0.3) of the different litter layer samples in FF plates. Results show the effect of three treatments (control, 0.1 and 10% of (($NH4)_2SO_4$) amendments) on the AWCD. Bars are standard error ($n = 3$).

The following general trends were observed in the different layers. In all layers, high N amendments had negative effects on almost all the microbial variables measured. This may be explained by repression phenomena of enzyme synthesis, because activities and numbers of enzyme isoforms decreased after high N amendments, and/or by cytotoxic effects of high N salt concentration on microbial cells. Our results about high ammonium amendments corroborate other studies (Saiya-Cork et al., 2002; Waldrop et al., 2004; Allison and Vitousek, 2005) performed on soils. It has been suggested that a decrease of respiration after high N fertilization, reflects a microbial reallocation of C to biomass or enzyme production (Schimel and Weintraub, 2003). However, in our study on litter material, this kind of reallocation was not observed after high N fertilization. Considering low N amendments, we observed that, for most of the microbiological variables, the OhLv layer was the more active and responded more intensively to N addition than the other ones. Indeed, low N addition had generally positive effects on microbial variables of this layer. By descending order, the two other layers OhLn and OhLf showed generally lower values in microbial variables, and responded in a lesser extent to N supplies than the intermediate OhLv layer. Thus, we suggest that the differences in microbial activities and responses to N supplies between the different layers were due to the quality and the availability of C sources. Indeed, freshly fallen evergreen oak leaves of the upper layer are generally poorly degraded because of their richness in hydrophobic substances such as cutin, which make them difficult to colonization and degradation by microorganisms (Racon et al., 1988). On the other hand, OhLf layer is the deepest and the more humified layer between the three layers investigated. As a consequence, this kind of litter layer is enriched in recalcitrant compounds such as lignin, lipids or waxes (solid state ^{13}C Cross-Polarization Magic Angle Spinning Nuclear Magnetic Resonance (CPMAS NMR), Alarcón-Gutiérrez, 2007) and C availability must be scarce, explaining the lowest activity detected and the lowest microbial responses to N addition observed. Finally, OhLv was the more favourable layer for microorganism proliferation and activity, probably because of the occurrence of a more easily available C pool in this layer, which makes microbial variables of this layer very sensitive to N addition.

Table 1
N amendment effects on Shannon diversity index (*H*), catabolic rate and lag times calculated from CLCPs (ECO & FF Biology ™ plates)

Treatments	Parameters		
Layer *vs* (($NH_4)_2SO_4$)%	Lag time (h)	μ_{max} (h^{-1})	*H*
ECO plates			
OhLn 0	28	0.178 (0.037)[a]	3.96 (0.063)[a]
OhLn 0.1	8	0.077 (0.011)[b]	3.88 (0.074)[a]
OhLn 10	8	0.042 (0.007)[b]	3.58 (0.062)[b]
OhLv 0	20	0.145 (0.034)[a]	3.88 (0.035)[a]
OhLv 0.1	8	0.141 (0.021)[a]	3.84 (0.073)[b]
OhLv 10	4	0.076 (0.023)[b]	3.66 (0.054)[c]
OhLf 0	20	0.078 (0.010)[b]	3.88 (0.210)[a]
OhLf 0.1	8	0.185 (0.032)[a]	3.77 (0.038)[a]
OhLf 10	4	0.081 (0.005)[a]	3.72 (0.046)[b]
FF plates			
OhLn 0	4	0.229 (0.019)[a]	3.96 (0.063)[a]
OhLn 0.1	4	0.144 (0.010)[b]	3.88 (0.074)[a]
OhLn10	8	0.168 (0.025)[b]	3.58 (0.062)[b]
OhLv 0	4	0.178 (0.018)[b]	3.88 (0.035)[a]
OhLv 0.1	4	0.184 (0.018)[b]	3.84 (0.073)[a]
OhLv 10	4	0.240 (0.025)[a]	3.66 (0.054)[b]
OhLf 0	4	0.208 (0.015)[b]	3.88 (0.205)[b]
OhLf 0.1	8	0.204 (0.047)[b]	3.77 (0.038)[ab]
OhLf 10	4	0.365 (0.020)[a]	3.72 (0.046)[b]

Different letters indicate significant differences within each layer following N treatments ($p < 0.05$, LSD). μ: Neperian catabolic rate; *H*: Shannon diversity index.

According to the economic resources allocation theory of microbial metabolism, enzyme production should increase when simple nutrients are scarce and complex nutrients are abundant (Harder and Dijkhuizen, 1983; Koch, 1985). Other studies (Allison and Vitousek, 2005) suggested that resource limitation could constrain enzyme production. According to Allison and Vitousek (2005), low ammonium additions to N limiting soil samples, reduce soil respiration and do not stimulate enzyme activity. Conversely, in our study, low ammonium sulphate amendment caused several marked increases in enzymes activities (i.e. laccases, cellulases and alkaline phosphatases), hyphal length, diversity of CLCPs, and isoenzyme patterns, suggesting a microbial reallocation of C to biomass and enzyme production as mentioned by Schimel and Weintraub (2003). This divergence may be due to the quality of organic matter of the samples used in our work, since we used exclusively litter horizons. Moreover, the variations in microbial variables were observed mainly in the two first layer, while the deepest one (OhLn) showed no or slight modifications. This last point seems in fact to well corroborate the study of Allison and Vitousek (2005), if we consider that C pools are not readily available in both OhLf litter layer and a soil. Considering the utilisation of C sources by microbial communities in the Biolog™ plates, AWCD results showed that CLCPs diversities of both ECO and FF plates were significantly affected by both litter depth and N amendments. However, some effects induced by N supplies appeared adverse when considering ECO or FF results, because AWCD values were increased and decreased, respectively. Thus, this may reflect that N supply can promote different effects with regards to litter microbial communities, since we also observed empirically that ECO and FF plates were mainly colonized by bacteria and fungi, respectively (data not shown). Moreover, as it should be, ECO are meant for bacteria and FF to fungi.

4.2. Effects of N on enzymes involved in lignocellulose degradation

With regards to enzymes involved in lignin transformation, laccases showed higher activities and isoform diversity in the OhLv layer, and this diversity decreased when increasing litter depth. This results is coherent with the studies of Luis et al. (2004, 2005), which have indicated that the diversity of laccase sequences was higher in the organic horizon of forest soil and decreased with the depth. In previous studies, Criquet et al. (1999, 2000) also detected laccase activity in the OhLv layer, and found only three laccase isoforms. However, they used smaller gels (MiniProtean®) than in our study, resulting in lower quality of protein separation, and consequently in a lower detection of laccase isoforms. This was also true with cellulase isoforms, when comparing with results of Criquet et al. (2002). Considering the other phenoloxidase investigated, very high peroxidase activity was detected in the OhLn layer, and it seems likely that they originated much more from plants. Low N amendments affected laccase variables, since their activities were considerably increased in the OhLv layer, and some laccase isoforms were induced especially in the two other layers. Following N amendments, and even at the lowest concentrations, peroxidase activity became not detectable. With regards to cellulolytic enzymes, cellulase activities and isoforms were also induced by low N addition in the two first layers, while adverse effects were observed in the deepest one. In their review about the regulation of the synthesis of microbial extracellular enzymes involved in lignocellulose degradation, Aro et al. (2005) mentioned that N availability has a positive effect on cellulase expression, while it represses peroxidase expression. Thus, our results seem to match with these models established from pure cultures. On the other hand, the expression of laccase genes is known to be not regulated by nutrient concentration, since these enzymes are known to be often constitutive in numerous fungi. The low variability in laccase isoforms of the more active layer (OhLv) following low N amendments seems also to corroborate this *in vitro* model. However, shifts in laccase isoforms observed in the other layer may be also explained by a modification of microbial community structure following N amendments. Laccase and cellulase zymograms showed also a large range of R_f in all the litter samples considered. In particular, the numerous isoforms detected with very low mobility (i.e. low R_f) suggest that they could originate from residues of large size multienzyme complexes, such as cellulosomes (Ponpium et al., 2000). According to Bayer et al. (2004), these complexes appear to be a more common occurrence in nature, and their role is to enhance synergistic activity among different enzymes to efficiently hydrolyze plant polymers.

4.3. Conclusion

This study has shown that the effects of N amendments occurred over very short incubation time (3 d), and varied according to N concentration and litter OM quality (i.e. depth). Since these results may apply to litter in general, nutrient effects must take into account the OM quality of litters. Thus, it appeared that the more active layer was the intermediate OhLv layer, which probably contained the most labile and available C pools. As a consequence, OhLv was also the layer showing globally the more intensive microbial responses following low N amendments. On the contrary, high N supplies resulted in adverse effects, suggesting repression or cytotoxic phenomena. In further works, it will be interesting to highlight even more the observations performed during this study, and to connect the effects of N availability with those of another important limiting element, P, on microbial variables involved in litter OM transformation.

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