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Discriminating between Seasonal and Chemical Variation in Extracellular Enzyme Activities within Two Italian Beech Forests by Means of Multilevel Models

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Abstract: Enzymes play a key-role in organic matter dynamics and strong scientific attention has been given to them lately, especially to their response to climate and substrate chemical composition. Accordingly, in this study, we investigated the effects of chemical composition and seasons on extracellular enzyme activities (laccase, peroxidase, cellulase, chitinase, acid phosphomonoesterase, and dehydrogenase) by means of multilevel models within two Italian mountain beech forests. We used chemical variables as the fixed part in the model, season as random variation and layers (decomposition continuum for leaf litter and 0–5, 5–15, 15–30, and 30–40 cm for soil) as nested factors within the two forests. Our results showed that seasonal changes explained a higher amount of variance in enzyme activities compared to substrate chemistry in leaf litter, whereas chemical variation had a stronger impact on soil. Moreover, the effect of seasonality and chemistry was in general larger than the differences between forest sites, soils, and litter layers.

Keywords: seasonal trends; beech forests; soil enzymes; organic matter; multilevel models

1. Introduction

Forests cover about 30% of Earth's surface and are of vital importance for many ecosystem services [1], including the regulation of the global carbon cycle [2]. Forests act as C sinks by storing more than 650 billion tonnes of carbon of which, on average, 11% is found in plant necromass and in the organic horizon, and 45% in the mineral soil [1]. Noticeably, temperate and boreal forests are of great importance as C sinks, containing about 14% and 32% of global forest C pools, respectively [3].

Accumulation of soil organic matter, which is mainly comprised of C, depends on the balance between primary net productivity and detrital decomposition. Several biotic and abiotic factors influence the decomposition processes [4]. In detail, litter decomposition is affected mostly by soil characteristics, nature and abundance of decomposing organisms and their interactions with soil fauna, litter quality and climate [5–8].

Litter decomposition is operated mainly by soil bacteria and fungi by releasing hundreds of different extracellular enzymes into the environment, which break down complex organic compounds

into assimilable sources of carbon (C), nitrogen (N), phosphorus (P), and other nutrients. Not every species of microbe produces the same enzymes, and they show different efficiency in substrate use and nutrient demand [9]. In addition, the production of extracellular enzymes is stimulated by substrate supply or reduced nutrient availability [10]. As decomposition goes on, the quality of the organic substance changes due to loss of easily degradable components and, therefore the microbial community that colonizes it changes as well. Thus, a true succession of microbial communities is formed, which tends to be composed predominantly by *K*-strategist species in the advanced phases, decomposers of more recalcitrant components, such as lignin and cellulose [11]. In this view, measuring extracellular enzyme activities could provide functional information on specific aspects and succession of the microbial communities during decomposition [12–14], but it is also useful to assess changes of microbial soil communities in response to environmental variation [15,16]. Kaiser et al. [17] found that changes in enzyme activities during the year suggested a switch of the main substrate to decompose but also a strong relationship between microbial community composition, which responds to environmental changes, and enzyme activities over the seasons. In this view, a relationship between decomposer enzyme activities and temperature and rainfall regimes has been found [18].

Our work focused on change of activity of some enzymes involved in the decomposition process within the organic horizon of forest floor and in the mineral soil, to a depth of 40 cm. We have studied two European beech (*Fagus sylvatica* L.) forest ecosystems under different climatic conditions and on different parent material. We chose beech ecosystems because this species is one of the most important forest trees of Europe, growing in a wide range of site conditions extending from humid to semiarid climates and from alkaline to acidic soils [19]. Beech forest soils contain an extensive carbon stock [20] that is predicted to decrease sharply under climate change scenarios [21]. The two studied beech stands are located on the Italian Apennines, one in the south (Laceno) and the other in the north (Pradaccio). They have been also investigated for litter decomposition dynamics under field and laboratory conditions [22], carbon stock in forest floor and mineral soil [5], and soil fauna communities.

In this study, we investigated the effects of substrate chemical composition and season on extracellular enzyme activities by means of multilevel models. Accordingly, past research demonstrated that enzymes involved in decomposition can respond to substrate availability and can be influenced by seasonality on short timescales, yet there is a need to understand how variation in chemistry vs. temporal is expressed, and how seasonal variation may control soil enzyme activity [23]. Thus, our research is timely and novel, aiming to better understand the role of seasonality versus underlying soil nutrient pools in controlling enzyme activity in short-term ecosystem dynamics [24] The advantage of multilevel models compared to classic statistical approaches is that the hierarchical structure in the data can be specified by considering both the within- and between-group variances, leading to a partial pooling of data across all levels in the hierarchy [25]. We used chemical variables as the fixed part in the model, season as random variation and layers (decomposition continuum for leaf litter and 0–5, 5–15, 15–30, and 30–40 cm for soil) as nested factors within the two forests. As for chemical variables, we measured organic matter, cellulose, acid unhydrolysable residue (AUR, i.e., a proximate content of lignin), and nutrients in leaf litter, while for soil we measured content of organic matter and nitrogen. The extracellular enzyme activities investigated in this study were involved in the main biogeochemical cycles, namely C (laccase, peroxidase, cellulase), N (chitinase), and P (acid phosphomonoesterase), whereas dehydrogenase was chosen as an enzyme proxy of overall biological activity [26]. Given that different factors may affect litter and soil differently, our study aims to evaluate, at high-view level, the differences in controls on microbial enzyme activity between leaf litter and soil.

2. Materials and Methods

2.1. Site Description

The study was conducted in Italy on the Apennines. The northern forest (Pradaccio, N Forest) is located at 1350 m a.s.l. (44°24′ N; 10°01′ E) while the southern forest (Laceno, S Forest) lies at an elevation of 1150 m a.s.l. (40°47′ N; 15°05′ E). Comprehensive tables about forest features and soil characteristics can be found in Innangi et al. (2015) [22] and De Marco et al. (2016) [5]. Both forests have a sub-Mediterranean mountain climate, characterized by temperate and relatively dry summer. However, during the year, temperature is lower and precipitation more abundant in N forest than in S forest. N Forest has a mean temperature of 6.0 °C, averaging on 11.0 °C between April and September and 0.7 °C in the rest of the year. Precipitations have peaks during autumn and spring and a marked reduction between June and August with an overall average rainfall of 2900 mm per year. Snow cover is often very thick and lasting from late November to the beginning of May. Average temperature in S Forest is 13.7 °C between April and September, and 3.7 °C in the rest of the year with an overall mean temperature of 8.7 °C. There was a longer semiarid period (May to September) than in N Forest and an overall average rainfall of 2300 mm per year, lower than in N Forest. Snow cover does not persist long and was commonly present between December and February.

In Figure 1 temperature and precipitation trends in the two forests during the year are shown. Data, provided by Lagdei Metereological Station and Laceno Metereological Station for N and S forests, respectively, are means of 5 years of monitoring (2010–2014, including our observations).

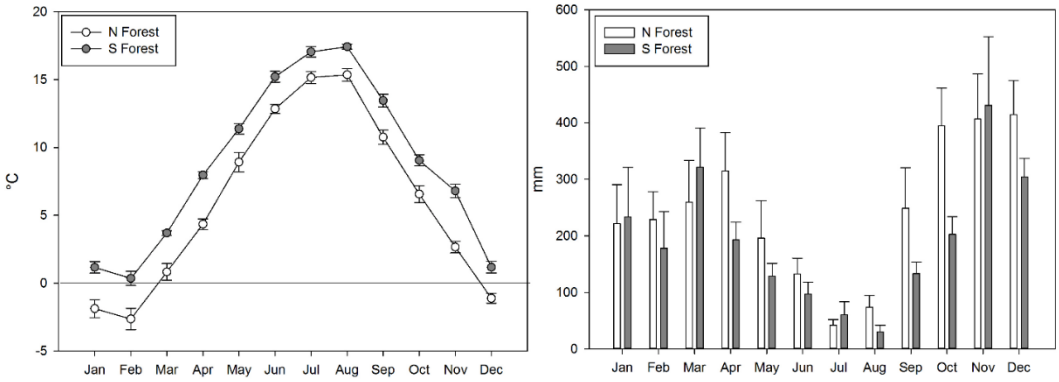

Figure 1. Temperature and precipitation regimes in the two studied forests. Data were collected for five years (2010–2014) at local meteorological stations. Values represent mean ± standard error of the mean.

Pedological surveys done in the past years in the nearby areas acknowledged the soil from Pradaccio as Lithic Haploborolls according to USDA Soil Taxonomy with a loamy-sand to sandy-clay-loam texture [27] while Laceno has been classified as Humic Haplustands according to USDA Soil Taxonomy with a loamy-sand to sandy-loam texture [28].

2.2. Litter and Soil Sampling

In each forest, 6 sampling points were randomly chosen in a 1 ha area. In each of them, litter was sampled by a 20 × 20 cm square steel frame and soil by a steel core sampler with a diameter of 5 cm and a length of 40 cm. Samplings were carried out in October 2011, May and July 2012, henceforward labelled as Autumn, Spring and Summer. Sampling points were marked in the field in order to repeat the sampling in the same spots and avoid introducing biases in the analyses. The samples, enclosed in plastic bags, were kept at 4 °C during transfer to the laboratory. Here, litter and soil were immediately processed.

Following a removal of all non-leaf litter material, leaf litter, which represented the most abundant litter component, was divided into three layers according to the degree of fragmentation/fermentation that can be defined as a decomposition continuum. The discrimination criteria were:

Li: leaf litter where leaves did not show any evident sign of fragmentation and/or chemical alteration. Such layer included mostly newly shed leaves, but also older leaves that have not yet been visibly altered. Leaves in this layer were easily separated from each other;

Lf: leaf litter with evident signs of fragmentation and/or chemical alteration but where the original structure of the leaf itself is still recognizable. Leaves in this layer can be separated from each other; and

Lhf: leaf litter with strong signs of fragmentation and/or chemical alteration, where the original shape of the leaves is hardly recognizable. Leaves in this layer are usually impossible to separate from each other and are sealed together by fungal mycelia.

Subsequently, subsamples for each layer were oven dried at 75 °C for 48 h in triplicate to assess moisture content. Other aliquots were kept at −80 °C for biological analyses and others were air dried and finely ground for chemical analyses.

Soil cores were carefully cut into four layers of 0–5, 5–15, 15–30, and 30–40 cm depth. Subsequently, soil cores were weighed and sieved with a mesh size of 2 mm. Subsamples were oven dried at 105 °C for 48 h in triplicate to assess moisture content. Other aliquots were kept at −80 °C for enzyme analyses and others were air dried and finely ground for chemical analyses.

2.3. Chemical Analyses

For chemical analyses, dry samples were ground by a Fritsch Pulverisette (type 00.502, Oberstein, Germany) equipped with an agate mortar and ball mill. Organic matter was evaluated gravimetrically as the difference between the dry weight and the ashes after incineration in a muffle furnace. Samples of leaf litter were burnt at 550 °C for 4 h into oven dried porcelain capsules [29], while those of mineral soil were burnt at 375 °C for 16 h [30].

Carbon and nitrogen were measured on finely ground dry samples [31] by a CNS elemental analyser (Vario El III, Elementar Analysensysteme GmbH, Hanau, Germany). Approximately 5 and 10 mg were weighted for litter and soil samples, respectively, using a holm oak litter as standards (C = 49.81%, N = 1.86%). As for soil samples, carbonates were removed before analysis [32].

To determine nutrient contents (Ca, P, K, Mg, Mn, and Fe), litter/soil samples were dry-mineralized in a muffle furnace at 480 °C for 16 h. Ashes were rehydrated with 1 mL 1:3 HNO_3 (70%) and 9 mL double-distilled water, filtered and then analytical determinations were performed by inductively coupled plasma atomic emission spectrometry (ICP-AES) without ultrasonic nebulization [33].

Cellulose and acid unhydrolizable residue (AUR) were evaluated according to [34] with modifications [29]. Such method allows for a semi-quantitative determination of AUR, including both native lignin and acid-insoluble lignin-like substances that are formed during decomposition, namely humic substances produced by soil microorganisms [29]. For simplicity, we will refer to this complex of lignin and lignin-like substances as lignin.

2.4. Enzyme Activities

For leaf litter, an extraction of the enzymes in appropriate buffers was performed. The extracts for cellulase and chitinase activities were prepared by suspending 1 g of leaf litter in 10 mL of 0.05 M sodium acetate buffer pH 5.5. For laccase and peroxidase, 0.5 g of fresh litter were suspended in 10 mL of sodium acetate buffer 0.05 M pH 5.0. Extracts for acid phosphomonoesterase were prepared by suspending 0.5 g of fresh litter in 10 mL of modified universal buffer (MUB) pH 6.5. Samples were homogenized using a homogenizer Polytron Heidolph Diax 600 for about 20–30 s while kept in ice to avoid heating. The homogenized samples were then centrifuged for 20 min at 22,000× *g* at 4 °C, the supernatant recovered and used as enzyme extract. For dehydrogenase, no extraction was

undertaken, but the colorimetric assay was performed directly on 50 mg homogenized litter/soil in 0.750 mL tris(hydroxymethyl) aminomethane buffer (1 M pH 7.0). Similarly, all enzymatic assays on soil samples were carried out directly on homogenized soil without separating the supernatant.

Carboxymethyl-cellulase (cellulase, EC 3.2.1.4) activity was determined according to Schinner & Von Mersi (1990) [35] with minor modifications [36] using carboxymethyl-cellulose as substrate. *N*-acetylglucosaminidase (henceforward labelled as chitinase, EC 3.2.1.14) and dehydrogenase (EC 1.1.1.x) activities were measured according to Verchot & Borelli (2005) [37] and Von Mersi & Schinner (1991) [38], respectively using 4-Nitrophenyl *N*-acetyl-β-D-glucosaminide and iodonitrotetrazolium chloride as substrates, respectively. Laccase (EC 1.10.3.2) and peroxidase (EC 1.11.1.x) were determined according to Leatham & Stahmann (1981) [39] with modifications [40], while acid (EC 3.1.3.2) phosphomonoesterase was performed according to Eivazi & Tabatabai (1977) [41] (substrates were *o*-Tolidine for laccase and peroxidase and 4-nitrophenyl phosphate bis(cyclohexylammonium) salt for acid phosphomonoesterase). All activities were measured in triplicate on each sample and data expressed as μmol/g dry weight/h. All measurements were done using a Varian Cary 1E UV/Vis spectrophotometer (Santa Clara, CA, USA).

2.5. Statistics

In order to test for variables affecting the variance in each enzyme in leaf litter and soil, we fitted multilevel models (MM), which are also known as random coefficient models and hierarchical linear models [25]. We used chemical data as fixed continuous variables, location and layer (nested within location) as fixed categorical variables and seasons as a random factor. We used grand mean centering for all continuous predictors [42] and, in order to avoid multicollinearity, we tested variance inflation factor (VIF) on each continuous variable, recursively discarding all with VIF $\geq$3 [21]. The procedure kept as chemical variables lignin, Fe, and K in leaf litter dataset and O.M. in soil. In order to ensure normality and homoscedasticity, we applied Box-Cox transformation with optimal λ to each enzyme activity. The transformation applied can be seen in Table S1. Residuals were checked for normality observing normal probability plots of studentized residuals and by fitting generalized additive models (GAMs) to evaluate the presence of non-linear patterns [43]. Multilevel model goodness of fit was expressed as conditional and marginal determination coefficients (R^2_c and R^2_m) in order to quantify unbiased measurements of variance expressed by fixed and fixed + random factors, respectively. All statistical analyses were done in R (v. 3.4.0) using packages 'lme4' and 'sjPlot'.

3. Results

3.1. Leaf Litter

Leaf litter chemical variables can be seen in Table 1. Generally, N Forest was characterized by higher values of O.M. in the leaf litter while S Forest was richer in Ca. Minor differences between forests included Mg, P, K, and Fe, which were higher in S Forest, while Mn and N had higher concentrations in N Forest. With the exception of Ca, all nutrients showed strong differences according to factor Layer, being generally at higher concentration in Lhf compared to Li. During autumn and spring there were higher values of cellulose and C:N, while all other chemical variables, including lignin, tended to increase during summer in both forests.

The trends for all enzyme activities in leaf litter can be found in Figure 2, while results from the MM in leaf litter can be seen in Table 2. Plots for the conditional models of the random effects in leaf litter, including prediction intervals, can be seen in Figure 3. Conditional determination coefficient for laccase was low (R^2_c = 0.192), while marginal determination coefficient was higher (R^2_m = 0.478), showing that there was a noticeable effect of the random parts in the model. Within the fixed parts, only lignin proved to be significant ($p < 0.050$). Laccase activity was not significantly different between forests, but Lf layer was significantly different from Li in both locations. The random part of the model, expressed by seasonal differences, clearly showed an increase in activity from autumn to summer.

Table 1. Chemical variables in leaf litter. Organic Matter (O.M.) is measured as g × g dry weight^{-1}. Cellulose and lignin are measured as mg × g O.M.$^{-1}$. All macro and micro elements are measured as mg × g dry weight^{-1}. Values are represented as mean ± standard error of the mean.

Season	Location	Layer	O.M.	Cellulose	Lignin	N	Ca	P	K	Mg	Mn	Fe	C:N
Autumn	N Forest	Li	0.95 ± 0.004	526 ± 8	276 ± 9	13.21 ± 0.45	13.15 ± 0.16	0.41 ± 0.01	0.62 ± 0.001	0.85 ± 0.01	0.07 ± 0.001	0.10 ± 0.000	39.2 ± 1.1
		Lf	0.94 ± 0.001	516 ± 12	277 ± 10	16.52 ± 0.71	13.24 ± 0.29	0.45 ± 0.007	0.63 ± 0.001	0.87 ± 0.01	0.088 ± 0.004	0.10 ± 0.000	27.96 ± 1.4
		Lhf	0.91 ± 0.007	482 ± 13	320 ± 5	22.28 ± 0.16	12.61 ± 0.12	0.47 ± 0.004	0.63 ± 0.002	0.91 ± 0.02	0.101 ± 0.003	0.11 ± 0.000	22.0 ± 0.3
	S Forest	Li	0.89 ± 0.005	537 ± 12	275 ± 11	14.06 ± 0.66	16.03 ± 0.13	0.49 ± 0.005	0.63 ± 0.001	0.96 ± 0.03	0.06 ± 0.002	0.10 ± 0.001	35.0 ± 1.2
		Lf	0.89 ± 0.008	510 ± 7	279 ± 10	13.07 ± 0.54	15.84 ± 0.12	0.49 ± 0.005	0.63 ± 0.002	0.94 ± 0.02	0.059 ± 0.002	0.10 ± 0.000	37.1 ± 1.4
		Lhf	0.77 ± 0.017	466 ± 10	313 ± 12	17.69 ± 0.73	15.63 ± 0.32	0.56 ± 0.008	0.64 ± 0.004	1.04 ± 0.004	0.081 ± 0.003	0.12 ± 0.000	22.4 ± 0.45
Spring	N Forest	Li	0.93 ± 0.008	498 ± 11	306 ± 8	18.53 ± 0.32	13.02 ± 0.15	0.44 ± 0.005	0.62 ± 0.001	0.81 ± 0.006	0.074 ± 0.001	0.10 ± 0.000	28.1 ± 0.36
		Lf	0.92 ± 0.010	505 ± 13	312 ± 11	21.35 ± 0.62	12.53 ± 0.23	0.45 ± 0.003	0.62 ± 0.001	0.85 ± 0.01	0.09 ± 0.004	0.10 ± 0.000	23.9 ± 0.8
		Lhf	0.87 ± 0.021	492 ± 13	300 ± 7	19.75 ± 0.94	12.32 ± 0.23	0.46 ± 0.007	0.63 ± 0.003	0.95 ± 0.04	0.098 ± 0.005	0.11 ± 0.000	22.3 ± 0.4
	S Forest	Li	0.89 ± 0.007	503 ± 6	292 ± 2	12.83 ± 0.65	15.79 ± 0.32	0.47 ± 0.006	0.62 ± 0.001	0.86 ± 0.01	0.05 ± 0.003	0.10 ± 0.000	39.4 ± 2.5
		Lf	0.85 ± 0.012	479 ± 18	313 ± 9	15.37 ± 0.67	16.27 ± 0.29	0.51 ± 0.007	0.62 ± 0.001	0.93 ± 0.03	0.06 ± 0.002	0.11 ± 0.000	31.3 ± 1.8
		Lhf	0.75 ± 0.016	450 ± 13	338 ± 4	17.52 ± 0.61	16.38 ± 0.39	0.54 ± 0.013	0.63 ± 0.002	1.00 ± 0.02	0.067 ± 0.003	0.12 ± 0.000	24 ± 0.7
Summer	N Forest	Li	0.954 ± 0.002	426 ± 23	353 ± 20	28.87 ± 1.69	13.87 ± 0.23	0.57 ± 0.011	0.64 ± 0.027	0.92 ± 0.042	0.122 ± 0.007	0.13 ± 0.030	17.6 ± 1.13
		Lf	0.940 ± 0.003	409 ± 20	383 ± 32	37.32 ± 2.43	13.67 ± 0.18	0.60 ± 0.023	0.64 ± 0.022	0.99 ± 0.04	0.150 ± 0.019	0.26 ± 0.070	13.37 ± 0.69
		Lhf	0.906 ± 0.015	398 ± 15	429 ± 38	43.59 ± 2.67	12.55 ± 0.64	0.68 ± 0.017	0.71 ± 0.027	1.14 ± 0.06	0.0163 ± 0.015	0.50 ± 0.150	10.72 ± 0.46
	S Forest	Li	0.932 ± 0.002	426 ± 8	341 ± 25	28.14 ± 1.48	16.33 ± 0.44	0.67 ± 0.014	0.67 ± 0.005	0.95 ± 0.016	0.086 ± 0.006	0.18 ± 0.03	17.7 ± 0.9
		Lf	0.916 ± 0.003	379 ± 11	338 ± 13	31.98 ± 1.12	17.74 ± 0.68	0.75 ± 0.030	0.69 ± 0.015	1.06 ± 0.027	0.111 ± 0.006	0.24 ± 0.04	15.1 ± 0.49
		Lhf	0.830 ± 0.020	387 ± 11	387 ± 25	36.69 ± 0.89	17.11 ± 0.35	0.78 ± 0.014	0.704 ± 0.030	1.28 ± 0.05	0.128 ± 0.003	0.62 ± 0.15	12 ± 0.19

Fixed parts of the model had a weaker effect for peroxidase (R^2_c = 0.158), with no chemical variable being significant. There was no significant difference between the two forests, and little to no effect of decomposition layer. Like laccase, random parts improved the model (R^2_m = 0.283) with similar activities in spring and summer and lower values in autumn.

Chitinase showed a low effect of the fixed part of the model (R^2_c = 0.160). No chemical variable proved to be significant. There was a significant difference between N Forest and S Forest ($p < 0.050$), with the latter expressing lower activity than N Forest. No particular effect of layer was detected. Marginal determination coefficient was sensibly higher (R^2_m = 0.673) with a strong seasonal effect (ICC_{season} = 0.611). Yet, compared to laccase and peroxidase, the seasonal trend was opposite, with highest activity in autumn and lowest in summer.

As for dehydrogenase, conditional determination coefficient was low (R^2_c = 0.160) and no chemical variable was significant. The activity was not different between forests, but in N Forest there was a strong difference between layers compared to S Forest. There was a trend to increase activity from autumn to summer, but this effect was relatively low (ICC_{season} = 0.284).

The activity of cellulase also showed a weak effect of the fixed parts of the model (R^2_c = 0.149). Accordingly, no chemical variable proved to be significant in the MM. Yet, differences between locations were sharp ($p < 0.001$), with N Forest showing higher activity than S Forest. Little effect of layer could be detected, and only in N Forest. The random part of the model increased the overall explained variance (R^2_m = 0.566), with a clear pattern of increasing activity from summer to autumn.

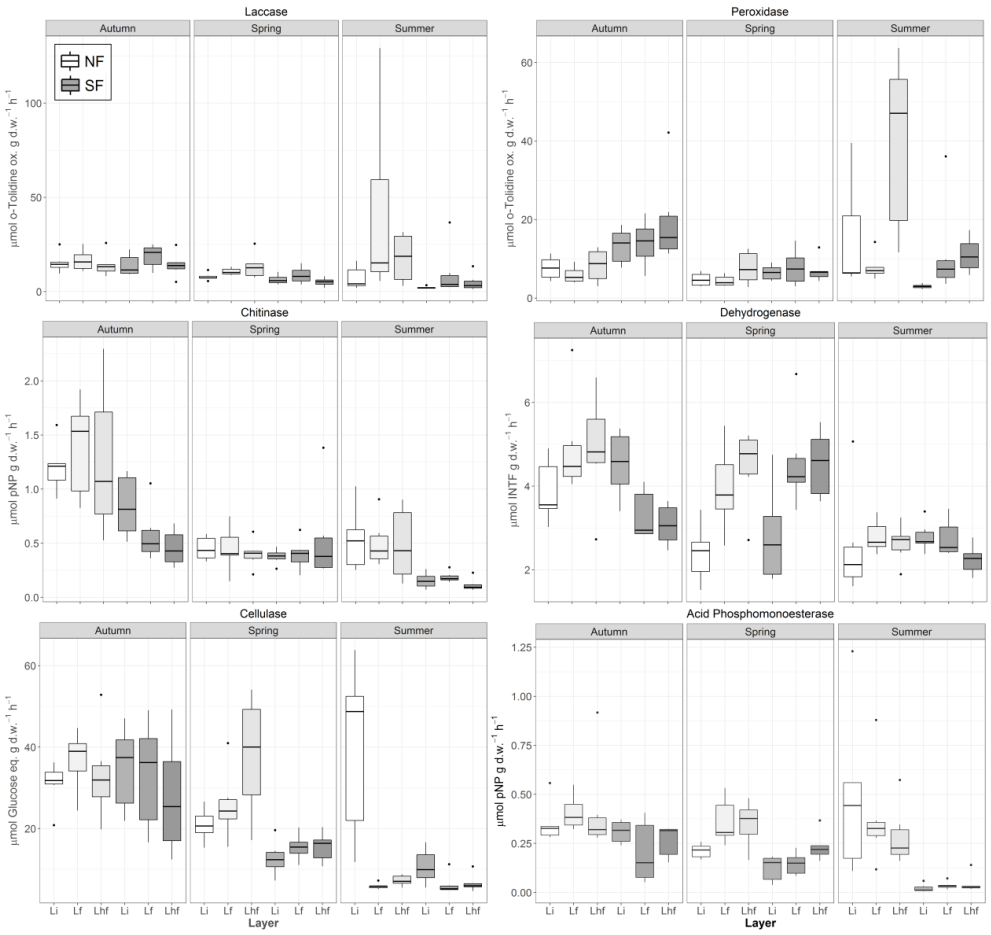

Figure 2. Boxplot representation of extracellular enzyme activities in leaf litter subdivided by season.

Table 2. Results from the MM analysis of Leaf Litter. Dependent variables are reported in columns, while predictors (both fixed and random parts) are reported in rows. Dependent variables have been transformed with Box-Cox transformation as reported in Materials and Methods. Reference levels for the fixed categorical variables have been chosen as N Forest for Location and Li for Layer, respectively. Statistical significance is reported as * ($p < 0.050$), ** ($p < 0.010$), *** ($p < 0.001$).

	Laccase	**Peroxidase**	**Chitinase**	**Dehydrogenase**	**Cellulase**	**Acid Phosph.**
	Estimate (CI)	*Estimate (CI)*	*Estimate (CI)*	*Estimate (CI)*	*Estimate (CI)*	*Estimate (CI)*
			Fixed Parts			
(Intercept)	0.8 (0.76 − 0.84) ***	0.47 (0.40 − 0.54) ***	0.95 (0.89 − 1.02) ***	0.82 (0.78 − 0.86) ***	1.93 (1.72 − 2.14) ***	0.63 (0.49 − 0.76) ***
Lignin	−0.27 (−0.50 − −0.04) *	−0.07 (−0.60 − 0.46)	0.16 (−0.07 − 0.39)	0.15 (−0.07 − 0.37)	−0.83 (−1.72 − 0.06)	0.86 (0.21 − 1.51) *
Fe	0.03 (−0.03 − 0.10)	0.00 (−0.15 − 0.15)	−0.06 (−0.13 − 0.00)	0.03 (−0.03 − 0.09)	−0.17 (−0.42 − 0.07)	−0.24 (−0.42 − −0.06) *
K	0.13 (−0.18 − 0.43)	−0.47 (−1.17 − 0.22)	0.08 (−0.22 − 0.38)	−0.08 (−0.38 − 0.21)	0.50 (−0.66 − 1.66)	0.35 (−0.50 − 1.20)
Location **S Forest**	0.02 (−0.01 − 0.06)	0.03 (−0.04 − 0.10)	−0.05 (−0.08 − −0.02) *	−0.02 (−0.05 − 0.01)	−0.20 (−0.32 − −0.09) ***	−0.21 (−0.29 − −0.12) ***
Location **N Forest:** Layer **Lf**	−0.04 (−0.07 − −0.01) *	0.03 (−0.03 − 0.10)	0.00 (−0.03 − 0.03)	−0.05 (−0.08 − −0.02) **	−0.14 (−0.25 − −0.02) *	0.04 (−0.04 − 0.13)
Location **S Forest:** Layer **Lf**	−0.03 (−0.06 − −0.00) *	−0.07 (−0.14 − 0.00)	−0.01 (−0.03 − 0.02)	−0.01 (−0.04 − 0.02)	−0.03 (−0.15 − 0.08)	−0.01 (−0.10 − 0.07)
Location **N Forest:** Layer **Lhf**	−0.03 (−0.06 − 0.01)	−0.06 (−0.13 − 0.02)	−0.01 (−0.04 − 0.02)	−0.06 (−0.09 − −0.03) ***	−0.07 (−0.19 − 0.06)	0.00 (−0.08 − 0.09)
Location **S Forest:** Layer **Lhf**	0.00 (−0.03 − 0.03)	−0.09 (−0.16 − −0.02) *	−0.02 (−0.05 − 0.01)	−0.01 (−0.04 − 0.03)	−0.01 (−0.13 − 0.11)	0.05 (−0.04 − 0.14)
			Random Parts			
σ^2	0.002	0.011	0.002	0.002	0.031	0.016
$\tau_{00,\ Season}$	0.001	0.002	0.003	0.001	0.029	0.011
ICC_{Season}	0.354	0.148	0.611	0.284	0.490	0.406
R^2_c/R^2_m	0.192/0.478	0.158/0.283	0.160/0.673	0.126/0.374	0.149/0.566	0.349/0.613

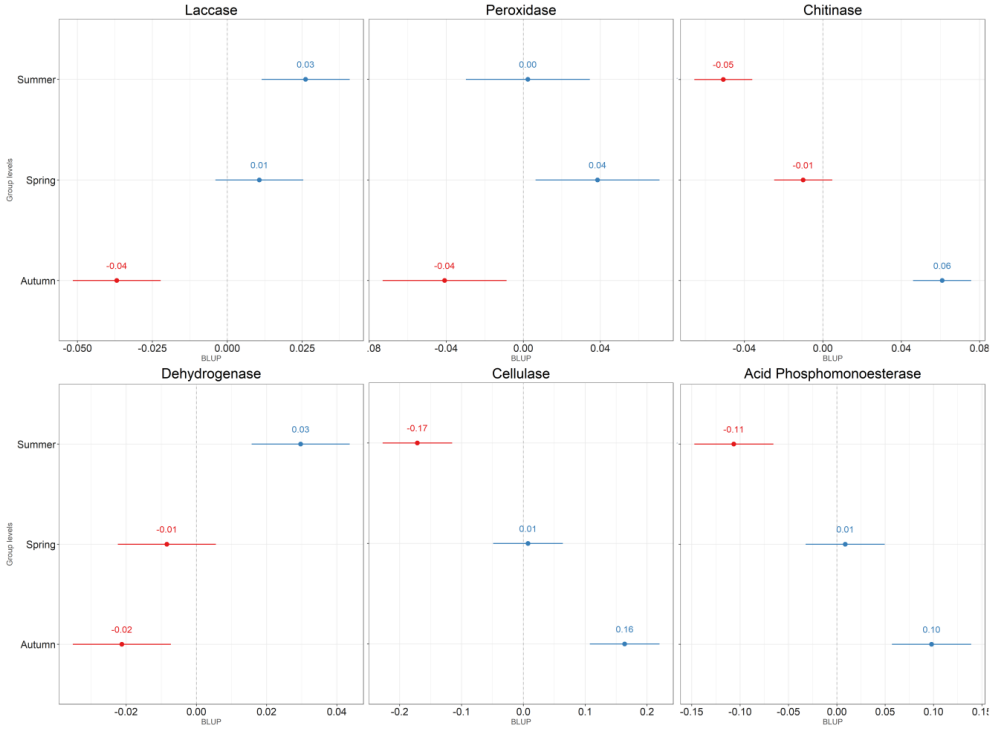

Figure 3. Plots for the conditional models of the random effects in leaf litter, including prediction intervals. Colors indicate a negative value for the conditional model (red) or a positive value (blue). BLUP stands for Best Linear Unbiased Predictor.

Finally, acid phosphomonoesterase showed the highest effect of the fixed parts of the model ($R^2_c = 0.349$). Both lignin and Fe were significant ($p < 0.050$), although with opposite signs. Like cellulase, there was a strong difference between locations ($p < 0.001$) but no relevant layer effect. The seasonal trend was similar to chitinase, although weaker ($ICC_{season} = 0.406$).

3.2. Soil

The chemical variables for soil can be seen in Table 3. The proportion of organic matter, with the exception of topsoil (0–5 cm), was generally higher in S Forest compared to N Forest. S Forest showed a slower decrease of O.M. with depth, whereas in N Forest there was a sharp difference between the topsoil (0–5 cm) and the subsoil (5–40 cm). No particular seasonal effect could be detected in either site. Nitrogen was also different between forests, although there were differences driven by seasons. Topsoil (0–5 cm) of N Forest had higher N content, whereas S Forest showed greater concentrations in the deeper layers of the soil. In contrast to O.M., a general tendency to increase in N was detected from autumn to summer, especially in N Forest. C:N ratio was different between sites, being generally narrower in S Forest. There was an overall similarity of C:N between layers, whereas a sharp seasonal effect was noticed. Although autumn and spring values were largely overlapping, C:N ratio was noticeably lower in summer in both sites.

Results from the MM analysis of the soil can be seen in Table 4, while the trends for enzymes in soil can be seen in Figure 4. Plots for the conditional models of the random effects in soil, including prediction intervals, can be seen in Figure 5. Laccase showed little effect of the random part of the model given that marginal and conditional determination coefficients were similar ($R^2_c/R^2_m = 0.488/544$). Compared to litter, a large amount of variance was derived from the fixed part of the model. In detail, O.M. was significant ($p < 0.050$), whereas there were no differences between forests but a significant effect of layer. Although seasonal effect was not particularly strong, summer was markedly different from both spring and autumn (Figure 5).

Table 3. Chemical variables in studied soil. Organic Matter (O.M.) is measured as g × g dry weight^{-1}. Total nitrogen (N) is measured as mg × g dry weight^{-1}. Values are represented as mean ± standard error of the mean.

Season	**Autumn**							
Location	**N Forest**				**S Forest**			
Layer	**0–5 cm**	**5–15 cm**	**15–30 cm**	**30–40 cm**	**0–5 cm**	**5–15 cm**	**15–30 cm**	**30–40 cm**
O.M.	0.31 ± 0.06	0.11 ± 0.01	0.10 ± 0.01	0.08 ± 0.01	0.27 ± 0.02	0.20 ± 0.02	0.16 ± 0.01	0.15 ± 0.01
N	6.64 ± 1.01	2.29 ± 0.14	1.42 ± 0.14	1.30 ± 0.10	8.58 ± 0.88	6.39 ± 0.57	3.97 ± 0.52	3.88 ± 0.15
C:N	17.05 ± 0.69	19.37 ± 1.86	21.21 ± 0.44	21.94 ± 0.32	13.11 ± 0.36	12.27 ± 0.16	12.82 ± 0.27	13.2 ± 0.27
Season	**Spring**							
Location	**N Forest**				**S Forest**			
Layer	**0–5 cm**	**5–15 cm**	**15–30 cm**	**30–40 cm**	**0–5 cm**	**5–15 cm**	**15–30 cm**	**30–40 cm**
O.M.	0.42 ± 0.08	0.10 ± 0.02	0.09 ± 0.01	0.08 ± 0.01	0.32 ± 0.03	0.21 ± 0.01	0.16 ± 0.01	0.18 ± 0.00
N	10.14 ± 2.06	2.44 ± 0.51	1.53 ± 0.02	1.35 ± 0.12	7.81 ± 0.86	5.25 ± 0.45	3.8 ± 0.29	4.14 ± 0.35
C:N	14.01 ± 0.68	20.82 ± 2.57	21.99 ± 0.42	20.20 ± 0.32	13.20 ± 0.42	13.24 ± 0.42	12.94 ± 0.19	13.15 ± 0.37
Season	**Summer**							
Location	**N Forest**				**S Forest**			
Layer	**0–5 cm**	**5–15 cm**	**15–30 cm**	**30–40 cm**	**0–5 cm**	**5–15 cm**	**15–30 cm**	**30–40 cm**
O.M.	0.42 ± 0.1	0.12 ± 0.02	0.10 ± 0.01	0.08 ± 0.01	0.25 ± 0.02	0.20 ± 0.01	0.20 ± 0.01	0.21 ± 0.01
N	21.03 ± 4.21	5.19 ± 0.95	4.95 ± 0.69	4.77 ± 1.11	7.81 ± 0.86	5.25 ± 0.45	3.8 ± 0.29	4.14 ± 0.35
C:N	10.68 ± 0.24	9.68 ± 0.73	6.32 ± 1.39	6.93 ± 1.63	6.99 ± 0.28	6.39 ± 0.11	6.6 ± 0.21	6.51 ± 0.14

Table 4. Results from the MM analysis of studied soil. Dependent variables are reported in columns, while predictors (both fixed and random parts) are reported in rows. Dependent variables have been transformed with Box-Cox transformation as reported in Materials and Methods. Reference levels for the fixed categorical variables have been chosen as N Forest for Location and 0–5 cm for Layer, respectively. Statistical significance is reported as * ($p < 0.050$), ** ($p < 0.010$), *** ($p < 0.001$).

	Laccase	**Peroxidase**	**Chitinase**	**Dehydrogenase**	**Cellulase**	**Acid Phosph.**
	Estimate (CI)	*Estimate (CI)*	*Estimate (CI)*	*Estimate (CI)*	*Estimate (CI)*	*Estimate (CI)*
			Fixed Parts			
(Intercept)	0.69 (0.50 − 0.88) ***	0.16 (0.09 − 0.23) ***	0.95 (0.92 − 0.98) ***	0.81 (0.74 − 0.88) ***	2.37 (1.93 − 2.81) ***	0.62 (0.56 − 0.69) ***
OM	−0.74 (−1.36 − −0.12) *	−0.19 (−0.41 − 0.03)	−0.14 (−0.19 − −0.09) **	0.68 (0.43 − 0.94) ***	−1.07 (−1.63 − −0.51) *	0.44 (0.28 − 0.61) ***
Location **S Forest**	0.01 (−0.17 − 0.18)	0.18 (0.11 − 0.24) ***	0.00 (−0.01 − 0.02)	0.09 (0.01 − 0.16) *	−0.43 (−0.59 − −0.27) **	−0.04 (−0.08 − 0.01)
Location **N Forest:** Layer **5—15 cm**	−0.07 (−0.30 − 0.17)	−0.02 (−0.11 − 0.06)	0.00 (−0.02 − 0.02)	−0.01 (−0.11 − 0.09)	0.16 (−0.05 − 0.37)	−0.08 (−0.15 − −0.02) *
Location **S Forest:** Layer **5–15 cm**	0.23 (0.06 − 0.40) *	0.01 (−0.05 − 0.08)	0.01 (−0.00 − 0.03)	−0.1 (−0.17 − −0.03) **	0.15 (−0.01 − 0.30)	0.02 (−0.03 − 0.06)
Location **N Forest:** Layer **15–30 cm**	0.23 (−0.02 − 0.47)	0.13 (0.04 − 0.22) **	0.00 (−0.02 − 0.02)	−0.05 (−0.15 − 0.05)	0.23 (0.01 − 0.44)	−0.06 (−0.12 − 0.01)
Location **S Forest:** Layer **15–30 cm**	0.45 (0.27 − 0.63) ***	0.07 (0.01 − 0.14) *	0.03 (0.02 − 0.05) **	−0.17 (−0.25 − −0.10) ***	0.3 (0.14 − 0.46) *	0.03 (−0.01 − 0.08)
Location **N Forest:** Layer **30–40 cm**	0.41 (0.16 − 0.66) **	0.21 (0.12 − 0.30) ***	0.01 (−0.01 − 0.03)	−0.18 (−0.29 − −0.08) ***	0.43 (0.21 − 0.66) *	−0.1 (−0.17 − −0.04) **
Location **S Forest:** Layer **30–40 cm**	0.46	0.06	0.03	−0.28	0.38	0.03
	(0.28 − 0.64) ***	(−0.00 − 0.13)	(0.02 − 0.05) **	(−0.35 − −0.20) ***	(0.21 − 0.54) **	(−0.02 − 0.08)
			Random Parts			
σ^2	0.058	0.007	0.000	0.010	0.046	0.004
$\tau_{00,Season}$	0.007	0.001	0.001	0.000	0.133	0.002
ICC_{Season}	0.109	0.135	0.561	0.000	0.742	0.280
R^2_c/R^2_m	0.488/0.544	0.569/0.627	0.359/0.718	0.678/0.678	0.397/0.844	0.515/0.651

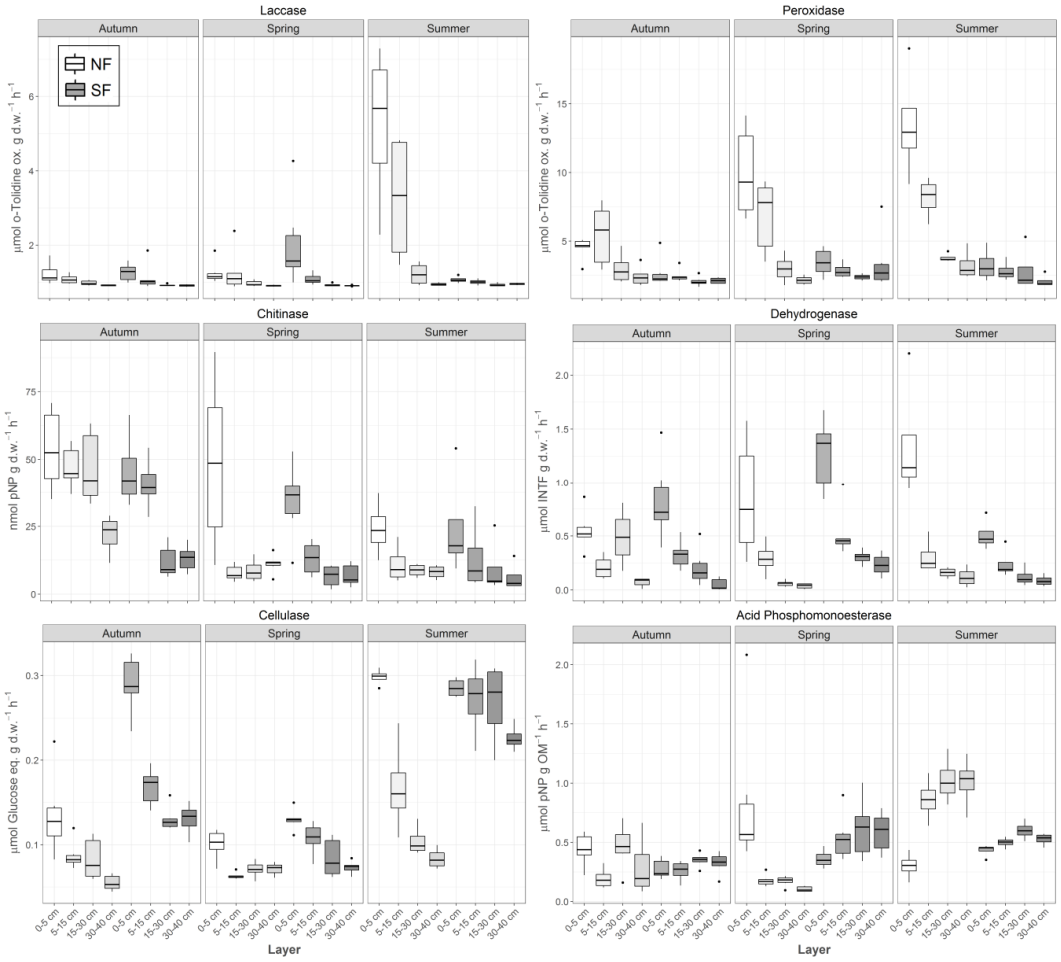

Figure 4. Boxplot representation of extracellular enzyme activities in studied soil subdivided by season.

Fixed parts of the model in peroxidase explained higher amount of variance compared to laccase (R^2_c = 0.569), but O.M. was not significant. In contrast to laccase, locations had highly significant differences for peroxidase ($p < 0.001$), with higher activity in N Forest. Differences between layers were sharper in N Forest compared to S Forest. Although the random part moderately contributed to variance (R^2_m = 0.627), a weak trend of decreasing activity in autumn was detected when compared to spring and summer.

In contrast with previous enzymes, chitinase had a large difference in conditional and marginal determination coefficients. The proportion of O.M. was highly significant ($p < 0.010$), although fixed parts of the model explained not much variance (R^2_c = 0.359). There was no difference between forests and little differences between layers, except for the deeper part of the soil in S Forest. The random part of the model expressed a large amount of variance (R^2_m = 0.718) with a clear seasonal effect (ICC_{season} = 0.561) with sharp differences in autumn compared to spring and summer.

Dehydrogenase had identical determination coefficients ($R^2_{c/m}$ = 0.678), meaning that there was no random effect at all. The proportion of O.M. was highly significant ($p < 0.001$). There was a weak, although significant, difference between forests ($p < 0.050$), with higher activity in S Forest, where also significant differences between layers were found to be more conspicuous than N Forest. As already mentioned, there was no detectable seasonal effect for this enzyme.

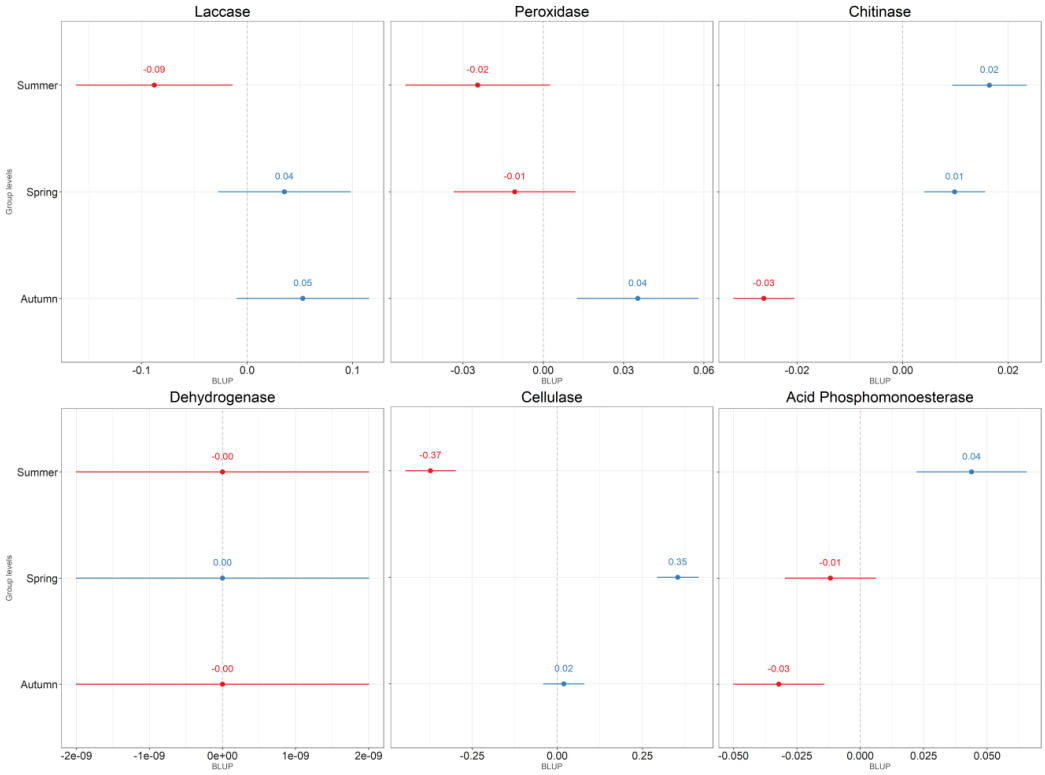

Figure 5. Plots for the conditional models of the random effects in studied soil, including prediction intervals. Colors indicate a negative value for the conditional model (red) or a positive value (blue). BLUP stands for Best Linear Unbiased Predictor.

Like chitinase, a large difference in conditional and marginal determination coefficients was found in cellulase. Fixed parts of the model explained half of the variance (R^2_c = 0.397) when compared to random effects (R^2_m = 0.844). Again, O.M. proved to be significant ($p < 0.050$) and there was a large difference between forests ($p < 0.010$), with greater activity in S Forest. The deeper layers of the soil, especially in S Forest, showed significant differences. The seasonal effect was broad (ICC_{season} = 0.742) with a complex pattern. Although autumn was relatively more similar to summer, the latter season and spring showed sharp differences.

Finally, acid phosphomonoesterase, like laccase and peroxidase, exhibited small differences between fixed and random parts of the model. The proportion of O.M. was highly significant ($p < 0.001$), but no difference was detected between locations. Layers had little effect, being the activity significantly different only in the deepest layer of N Forest. A weak trend of increasing activity from autumn to summer was detected, although seasonal effect was small (ICC_{season} = 0.280).

4. Discussion

4.1. Leaf Litter

The trend of organic matter along the decomposition continuum was consistent with the previously observed trend of litter decomposition in the two forests [22] and with the forest floor features in autumn and spring [5]. At the same time, a change in quality was observed as well. Accordingly, there was a decrease of cellulose and an increase of lignin and N (Table 1). Similar trends in the content of cellulose and lignin, mostly for the litter or organic layers, are known from other studies [29,44–47]. The cellulose-rich secondary wall must be at least partially degraded before fungi can attack the lignin. This may explain the increase of ligninolytic enzyme activity with litter decay stage and the significant effect of lignin in MM for laccase (Figure 2 and Table 2). Rihani et al.

(1995) [48] found that, in beech litter, lignin decay rates increased significantly after more than 20% of the cellulose content had disappeared. The small decline of cellulose concentration and the increase of lignin along the decomposition continuum at both sites indicated that even in the most decayed litter, (Lhf) lignin degradation was still at the beginning.

Within the decomposition continuum, the increase of nitrogen suggests that this element was limiting for microbial growth in both forests. Similar trends for N have been already seen during litter decay [4], and similar dynamics in beech litter are known as well [49–51]. The higher N content in summer could be linked to a combined effect of soil fauna activity and higher presence of labile nitrogen compounds, which are subsequently leached away during autumn precipitation [52]. Accordingly, in prairie ecosystems, warming was linked to an increase in labile N, whereas recalcitrant N remained stable [53]. As a consequence of decomposer activities, the C:N ratio decreased along the decomposition continuum, especially in summer, reaching humus-like values in Lhf [54]. This suggested the achievement of a certain stabilization of the organic matter in this leaf litter layer. Our finding is consistent with Michel and Matzner (2002) [55], who hypothesized that low C:N ratios in later stages of decomposition stabilized soil organic matter and could be responsible for increased accumulation of C in forest floors. The sharp decrease of C:N ratio in summer compared to the other seasons could be linked to the subsequent N leaching during autumn. This statement is supported by soluble N data in autumn for the two studied forests, where a decrease from autumn to spring was witnessed [5].

Generally, enzyme activities vary with the qualitative composition of the substrate and are influenced by environmental conditions that act directly on the released enzymes and indirectly by affecting the organisms that produce them [56]. The statistical model applied to leaf litter coming from both forests, showed significant seasonal effects on the activity of all considered enzymes, although effects varied in magnitude between enzymes (Table 2). Activities, such as chitinase, cellulase and, to a lesser degree, acid phosphomonoesterase, were higher in autumn, likely due to higher moisture associated to mild temperature, compared to summer. Other studies showed strong seasonal effects on enzyme activities in Mediterranean ecosystems, with the lowest activity during summer's drought and the winter coldest periods [29,57–59]. Nevertheless, laccase and dehydrogenase showed an opposite trend although, compared to the previous ones, they showed a smaller seasonal effect. Laccase variability was largely due to variation in chemical composition, mostly lignin. The activity of peroxidase, on the contrary, was influenced neither by season nor chemistry, at least for the variables we have measured. Although only laccase showed a weak seasonal trend and there was no difference between locations, another study that used isoelectric focusing on high-stability pH gradients with high resolving power showed strong differences in both laccase and peroxidase isoenzymes, with higher activities of both enzymes in summer and greater diversity of isoenzymes in N Forest [60]. Finally, as for the comparison of the two forests, our model indicated a significant difference only for cellulase, chitinase, and acid phosphomonoesterase.

4.2. Soil

The concentration of organic matter in the soil was shown to be much higher in N Forest in the uppermost layer, but larger in S Forest in the deeper layers. The explanation could be derived to the higher annual litter input of S Forest, due to the milder climate that promotes a longer vegetative season [5]. This allows trees to grow larger leaves and in greater quantity. Thus, in spite of a higher rate of decomposition observed in southern site [22], a greater accumulation of organic matter was evident (Table 3). Quantitative and qualitative changes along soil depth are consistent with current literature [5,28,61–63]. The increased nitrogen content during summer that we have recorded was congruent with reported higher N mineralization and availability in labile-compounds during warmer seasons in temperate ecosystems [64–66].

Along soil depth, enzyme activity varied in relation to the decreasing content of organic matter (Figure 3). The statistical model applied to soil put forward some differences compared to leaf litter: (1) chitinase and, to a lesser degree, acid phosphomonoesterase variation was largely influenced by

seasonality but with an opposite trend to leaf litter, being larger in autumn and smaller in summer; (2) laccase and peroxidase were barely influenced by season, but also had opposite trends compared to leaf litter; and (3) the content of organic matter was largely responsible for the variability of enzyme activities, with the exception of peroxidase, as already shown in other beech mountain ecosystems [61].

These findings were congruent with what was observed by Baldrian et al. (2013) [18], given that, along seasons, temperature, humidity and chemical quality have greater changes in litter than soil. The seasonal trend of most enzymes in soil is consistent with previous research in beech forests in Northern Europe, where the activity of chitinase and cellulase was higher during summer [66].

4.3. Conclusions

Admittedly, we cannot make insights beyond a certain resolution, as many factors are not included the modeling analysis and cannot account for the complexity of forest ecosystems. Nevertheless, our work showed that litter is relatively more controlled by seasonality while soil is relatively more controlled by soil properties. Our work contributed to understanding aboveground-belowground interactions and differences in enzyme activities in a short-time perspective, with particular focus on enzymes involved in nutrient cycles that is pivotal to further understand the factors that regulate, for instance, the transfer of plant C to soil and its importance for the microbial community [23]. In conclusion, the main results of our study can be summarized as: (i) seasonal changes, expressed as random variation in our MM, had a stronger effect than chemical variables in leaf litter, whereas chemical variation (organic matter) had a stronger impact on enzyme activities soil; (ii) lignin was an important variable in explaining the variance of laccase and acid phosphomonoesterase in leaf litter, while O.M. was the driving variable for all soil enzymes with the exception of peroxidase; and (iii) the effect of seasonality and chemistry was in general larger than the differences due to both forest origin and layers.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/9/4/219/s1.

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