Annual variations of phenoloxidase activities in an evergreen oak litter: influence of certain biotic and abiotic factors

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Abstract

This study concerns ligninolysis phenomena occurring over 13 months in forest litter. Evergreen oak (Quercus ilex L.) litter was taken as a model because Quercus ilex L. is the most abundant tree species in forests of the French Mediterranean area. Several biotic and abiotic factors potentially involved in transformations of polyphenolic compounds, were measured between October 1997 and October 1998. These factors were: global fungal microflora, the fungi producing phenoloxidases (PO+), the activities of several phenoloxidases, hydrosoluble phenols, and temperature, humidity and pH of the litter. Results showed that the annual dynamics of fungi and phenoloxidase activities appear to be seasonal, i.e. that these biotic factors such as, were optimal in autumn. A multiple regression analysis showed that there was no correlation between biotic factors such as, fungal populations and phenoloxidase activity and abiotic factors such as, temperature, humidity and pH. Laccases were the preponderant phenoloxidase activities during the year, while those of Mn-peroxidases only appeared in the autumn of 1997. Other phenoloxidases, lignin-peroxidases and tyrosinases were never detected. Interactions between laccases and humic substances were also investigated. Adsorption of laccases on humic substances leads to a shift in the optimal temperature activity of these enzymes from 50 to 30°C. Activities of laccases also shifted towards more acidic values when laccases were not adsorbed on humic substances. Nevertheless, the optimal pH was the same (5.7) whether laccases were adsorbed or not to humic substances. Electrophoresis analysis showed little variations in the number of phenoloxidase isoenzymes. Indeed, laccases showed three isoenzymes during the year (Rf 0.23, 0.34 and 0.43). Only one isoform of Mn-peroxidase, with an Rf 0.21, was detected in the litter.

Keywords: Litter; Fungi; Laccase; Peroxidase; Humic substances

1. Introduction

Litter decomposition on the forest floor is an essential process in nutrient cycles and soil formation. These processes are controlled by abiotic factors, such as climate and by biotic factors, such as, chemical composition and microbial communities of litter and soil (Colotéaux et al., 1995). The continuous degradation of dead plant materials by saprophytic microorganisms, such as fungi, requires the release of various enzymes that are physically and chemically associated with insoluble organic debris and inorganic particles (Stozky and Burns, 1982). In litter, lignin is particularly important because it represents the most abundant aromatic polymer. Of particular importance are enzymes excreted by microorganisms for the initial depolymerization of lignin (Burns, 1978). The extracellular activities of the enzymes derive from exoenzymes produced by living cells or from endoenzymes released during disintegration of cells (Tabatabai and Fu, 1992). Various studies have shown the activities of numerous enzymes in soil and litter (Roberge, 1978; Sinsabaugh et al., 1991), but there is a lack of studies about the microorganisms implicated in the production of phenoloxidases in litter and the temporal distri-

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2. Materials and methods

An evergreen oak litter (*Quercus ilex* L.) was sampled monthly for one year, between October 1997 and 1998, from a dense copse at “La Gardiole de Riants” (Var, France). Samples were collected from the LV layer defined by Babel (1971): leaves still recognizable despite decay and often compressed in lumps. Five fresh samples (80 g each) were collected from the litter immediately before analysis. For the study of the microflora and electrophoretic analysis, these samples were pooled before analysis.

According to Criquet et al. (1999), phenoxidiases were extracted by shaking, at room temperature for 1 h on a reciprocal shaker, 80 g of litter in 700 ml of a 0.1 M CaCl₂ solution added with 0.05% Tween 80 and 20 g polyvinylpolypyrrolidione. The supernatant of each extract was concentrated in a cellulose-dialysis tube (Polylabo, Strasbourg, France), with a 10 kDa molecular mass cut-off, covered with polyethylene glycol, until a final volume of 1/10–1/20 of the initial volume. The activities of laccases in the concentrated extracts were measured spectrophotometrically (Kontron, model Uvikon 860) using syringaldazine (Sigma) as the substrate (Harkin and Obst, 1973). For kinetic measurements, 0.5 ml of enzyme extract, 2.5 ml of 0.1 M phosphate buffer (pH 5.7), and 10 μl of a solution of syringaldazine (5mM) were mixed in a 4 ml cuvette. The rate of oxidation of syringaldazine to quinone was measured for 5 min at 525 nm (ε₉⁰ = 65,000 M⁻¹ cm⁻¹). The results are expressed in units defined as μmol of quinone formed from syringaldazine per minute (U) and per gram of dry matter (U g⁻¹ DM).

To determine dry weight, the litter material was dried at 100°C for 24 h. The activity of Mn-peroxidases (MnP) was measured according to Pasczynski et al. (1985) using vanillylacetone as the substrate (synthesized in our laboratory). For kinetic measurements, 0.5 ml of enzyme extract, 2.17 ml of 0.1 M lactate buffer (pH 4.5), 0.2 ml of a 0.05 mM H₂O₂ solution, 0.1 ml of 0.1 mM MnSO₄ and 30 μl of a 0.4 mM vanillylacetone solution were mixed in a 4 ml cuvette. Controls did not contain H₂O₂, to subtract laccase activity on vanillylacetone. The results are expressed in unit (U) g⁻¹ DM, where U is μmol of vanillylacetone disappearing per minute. The activities of lignin peroxidases (LiP) and tyrosinases were measured according to Tien and Kirk (1984) and Dawley and Flurkley (1993), using 0.5 ml of litter extract. Litter extracts boiled for 15 min served as controls for the activities of all enzymes, respectively. No activities were detected in these boiled extracts.

The laccase fraction not adsorbed on humic substances (free laccase fraction) was estimated in the litter extract by precipitating humic substances (Mayaudon and Sarkar, 1974) with a solution of 8 g 1⁻¹ of protamine sulfate grade II (Sigma). The litter extract was then centrifuged for 20 min at 12,000 g and filtered through a 1.2 μ GF/C filter. The residual laccase activity was measured in the filtrate as described above and expressed in percentage of the total activity of laccase.

To compare laccase and peroxidase isoenzymes present in the evergreen oak litter during the year, the litter extracts were treated with protamine sulfate (2 g 1⁻¹) partly to remove humic substances, and then centrifuged for 20 min at 12,000 g. This treatment was indispensable to prevent the formation of black precipitates in the litter extracts. For electrophoresis analysis, 100 μl of each concentrated extract were
mixed with gel loading buffer (50 mM Tris–HCl pH 6.8, 0.1% bromophenol blue and 10% glycerol) and loaded on a non-denaturing (without sodium dodecyl sulfate or β-mercaptoethanol) polyacrylamide gel electrophoresis (PAGE) according to Laemmli (1970). For the analysis of phenoloxidase isoenzymes, polyacrylamide gels (7.5%) layered with 4% staking gels and Tris-glycine buffer were used. The mini-gels (Mini-Protein II, Biorad) were run at 200 V for 45 min. The electrophoresis gel were stained using p-phenylenediamine (Sigma) 0.1% (W/V) in Na-phosphate buffer (100 mM, pH 5.7) to reveal activity of the laccases. The activities of the Mn-peroxidase isoenzymes were visualized using lactate buffer (100 mM, pH 5.7) supplemented with 0.1% 2, 7-diaminofluorene (Sigma) and 3 μM MnSO₄. H₂O₂ (20 mM) was added to the lactate buffer only after 30 min to eliminate laccase activity on 2,7-diaminofluorene.

For counting fungi, the litter (2.5 g) was powdered (<0.5 mm) using a Moulinex mixer, and suspended in 250 ml of a 8.5% NaCl solution with 0.05% Tween 80. The mixture was agitated for 2 h on a reciprocal shaker (120 rpm) and sonicated in a sonic bath (Bio-Block, ultrasons 320 W, 35 kHz) for 1 min (Kilbertus et al., 1982). This treatment provided the best desorption of microorganisms from the litter. Dilutions of the mixture were plated on a medium containing 5 g malt, 15 g agar, 50 mg chloramphenicol per liter for global fungal microflora. Fungi producing phenoloxidases (PO⁺) were counted on the same medium added with 0.5 ml guaiacol (Flegel et al., 1982). Results are expressed in unit forming colony per gram of dry matter (UFC g⁻¹ DM). Water soluble phenolic compounds were measured by incubating, without agitation, the powered litter (5 g) in 75 ml of double distilled water for 24 h. The water soluble fraction was centrifuged for 10 min at 12,000 rpm, filtered through a 1.2 μm Whatman filter, and a clear supernatant was obtained following this treatment. It was then analyzed by the method of Box (1983). A calibration curve was established with catechin, and results are expressed in microgram equivalent of catechin per gram of dry matter (μg eq. catechin g⁻¹ DM).

The pH of the litter was measured (pH meter, Metrohm, Herisau, Switzerland) monthly by incubating 5 g of powdered leaves in 100 ml of double distilled water for 2 h. The temperature in the litter during the year was measured with a temperature data logger (Tinytalk II, Radiospares, Beauvais, France) and OTLM software (Orion Tiny Logger Manager, Gemini data Loggers, UK). The humidity of the litter was deducted from measure of the dry weight.

2.1. Statistical analysis

Correlation coefficients of Pearson (r) were calculated between all the variables measured during the year, except for the electrophoresis analysis. Significant correlations were retained for p ≤ 0.05 or p ≤ 0.01. The influence of humidity, pH and temperature on fungal populations and phenoloxidase activities was evaluated by multiple regression. Multiple regression...
models were analyzed by ANOVA. Levels of significance are indicated as \( p \leq 0.05 \) or \( p \leq 0.01 \). All the calculations were effected using Statview software version 1.03.

### 3. Results and discussion

#### 3.1. Relationships between phenoloxidases, fungi and abiotic factors

The activities of laccases, measured at pH 5.7 and 30°C, reached a first peak in autumn of 1997 (Fig. 1). This peak was followed by a decrease in the activity until March 1998. During the spring of 1998, the activities increased again, albeit slightly, and then sharply decreased in July 1998 because of the typical dry Mediterranean summer. Over the year, there was no significant correlation between humidity and temperature of the litter and the activity of laccases (Table 1). Once again, we observed a maximum activity of laccase in the autumn of 1998. This phenomenon may reflect a seasonal cycle of the expression of laccases in evergreen oak litter.

It was only in the autumn of 1997 that activity of Mn-peroxidases (Fig. 1) was observed in the litter. The best pH and temperature for the measurement of the activity of Mn-peroxidases were 4.5 and 30°C, respectively (Fig. 2). The same pH was found by Galliano et al. (1991) using vanillylacetone as the substrate, and a MnP purified from a culture of *Rigidosporus lignosus*. Bartha and Bordeleau (1969) extracted peroxidases from soil using a 50 mM phosphate buffer (pH 6.0) and measured their activities in the same buffer using o-dianisidine as the substrate. The peroxidase detected with this method had activities between \( 0.6 \times 10^{-2} \) and \( 2.35 \times 10^{-2} \) U g\(^{-1}\) DM, which are comparable with the activities obtained in our study with the evergreen oak litter (Fig. 1). Bollag et al. (1987) found an optimal pH value of 5.0 for a peroxidase extracted from soil. The Mn-peroxidases derived from the evergreen oak litter were totally inactive for pH values above 5.5. Consequently, distinction between the activities of peroxidases and laccases seems possible because the activities of both these enzymes are at 4.5 and 5.7, respectively.

### Table 1

Pearson correlation coefficients between microbial, enzymatic, chemical and environmental factors

<table>
<thead>
<tr>
<th></th>
<th>Hr</th>
<th>t°C</th>
<th>pH</th>
<th>HP</th>
<th>Lac</th>
<th>F. Lac</th>
<th>MnP</th>
<th>GF</th>
<th>PO*</th>
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<td>Hr</td>
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<td>-0.43</td>
<td>-0.05</td>
<td>0.08</td>
<td>0.48</td>
<td>-0.43</td>
<td>0.38</td>
<td>0.47</td>
<td>0.38</td>
</tr>
<tr>
<td>t°C</td>
<td>1</td>
<td>0.12</td>
<td>0.34</td>
<td>-0.46</td>
<td>0.82a</td>
<td>-0.31</td>
<td>-0.22</td>
<td>-0.22</td>
<td></td>
</tr>
<tr>
<td>pH</td>
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<td>-0.17</td>
<td>0.004</td>
<td>-0.34</td>
<td>-0.56</td>
<td>-0.57a</td>
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<tr>
<td>HP</td>
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<td>0.01</td>
<td>0.26</td>
<td>0.32</td>
<td>0.44</td>
<td>0.36</td>
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<td>Lac</td>
<td>1</td>
<td>-0.71b</td>
<td>-0.42</td>
<td>0.15</td>
<td>0.23</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F. Lac</td>
<td>1</td>
<td>-0.19</td>
<td>0.58c</td>
<td>0.66b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MnP</td>
<td>1</td>
<td>0.58c</td>
<td>0.66b</td>
<td></td>
<td></td>
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<td></td>
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<td>GF</td>
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<td></td>
</tr>
</tbody>
</table>

\( ^{a} \)Hr: water content of the litter; \( ^{t} \)t°C: temperature; HP: hydrosoluble phenols; Lac: laccase activity; F. Lac: free laccase activity; MnP: Mn-peroxidase activity; GF: global fungal microflora; PO*: fungi producing phenoloxidases.

\( ^{b} p < 0.01 \).

\( ^{c} p < 0.05 \).

\( ^{c} p < 0.05 \).

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Fig. 2. Effect of pH (A) and temperature (B) on activity of Mn-peroxidases of the Lv layer of the evergreen oak litter studied. Values represent the averages of five replicates; bars represent standard deviations.
spectively. Furthermore, for pH 4.5, no phenoloxidase activity was detected without adding H\textsubscript{2}O\textsubscript{2} to the enzyme extract. No activity was detected for other phenoloxidases, (i.e. lignin peroxidase and tyrosinase). Laccases appeared to be the most important phenoloxidases in the evergreen oak litter because these were present throughout the year and could be excreted in significant quantities, especially in autumn. These results agree with those of Ruggiero and Radogna (1984), who reported that a humus-phenoloxidase system extracted from soil showed only, or prevalently, laccase activity.

The global fungal microflora and fungi producing polyphenoloxidases (PO\textsuperscript{+}) were evaluated using the plate counts. Such a technique is known to be ambiguous because some colonies may arise from spores and hyphal fragments. Nevertheless, according to Witkamp (1966), this method can reflect significantly the quantitative variations of the fungal microflora in soil and litter, which is one of the purposes of this study.

The global fungal microflora and the PO\textsuperscript{+} fungi were well correlated ($r = 0.94$, Table 1) and showed a significant increase in number during the autumn of 1997 (Fig. 1). This sudden increase may be attributable to a typical and seasonal augmentation of the number of fungi, particularly basidiomycetes, in litter. Seasonal trends in fungal population dynamics are well known, and Berg et al. (1998) have recently described the dynamics and stratification of fungi in the organic layers of a Scots pine forest soil. The number of fungi PO\textsuperscript{+} only showed a new increase in the autumn of 1998. In the case of global fungal microflora, other smaller variations were observed during the year and may be the result of meteorological conditions (Berg et al., 1998), especially rainfalls, which present quite varying frequency and intensity under Mediterranean climate. The maximal expression of the activities of laccases and Mn-peroxidases occurred during the period of proliferation of the fungi PO\textsuperscript{+} (Fig. 1). Even if certain bacteria, such as Bacillus sphalericus (Claus and Filip, 1997) and Streptomyces strains (Crawford, 1978), are able to synthesize laccases, Rosenbrock et al. (1995) suggested that the laccase activity in litters was essentially attributable to lignolytic fungi.

The pH of the evergreen oak litter ranged from 5.5 to 7.0 during the year (Fig. 3). During autumn, the pH values were the lowest and favorable to the development of PO\textsuperscript{+} fungi ($r = -0.57$, Table 1). These fungi synthesized laccases and Mn-peroxidases, whose activities measured in vitro were well correlated ($r = 0.64$, Table 1). However, Figs. 3 and 4 show that the pH values in autumn were favorable for the in situ activity of laccases, but not of peroxidases since the lowest pH observed in autumn (5.5–5.7) was still too high for Mn-peroxidase activity (pH optimum 4.5). Mn-peroxidase activity was negatively correlated with the pH ($r = -0.54$, Table 1). Thus, it is difficult to hypothesize that peroxidases were really active in the evergreen oak litter during autumn, since the optimum pH for Mn-peroxidases and the pH measured in the litter extract were different.

As indicated above, significant negative coefficients were observed between pH and PO\textsuperscript{+} fungi, and between pH and Mn-peroxidase activities (Table 1). Nevertheless, multiple regression analysis (Tables 2 and 3) shows there was no relationship between biotic factors (fungal populations and phenoloxidase activity) and the abiotic ones (temperature, humidity, and pH). Thus, variations of these biological parameters over one year cannot be predicted by these classical abiotic factors.

### 3.2. Relationships between humic compounds and laccases

Among the water soluble components of the litter, water soluble phenols are chemical compounds usually integrated in humic substances, which interact with enzymes of litter. The origin of water soluble phenols...
in the evergreen oak litter is not clear, because they may be due to degradation of lignin and polyphenolic compounds by phenoloxidases or due to leaching from the upper layer of the litter, which is rich in water soluble components (Gourbière, 1983; McClaugherty, 1983). Furthermore, water soluble phenols may have an important role in the acidification observed in the Lv layer, which may also be due to the simultaneous release of various metabolic organic acids. The pH values were negatively correlated \( r = -0.69 \) (Table 1) with the concentration of water soluble phenols (Fig. 3). The values of water soluble phenols were correlated neither with the temporal dynamics of fungi nor with the activities of the phenoloxidases (Table 1). The presence of these water soluble phenols may be explained by disintegration over the year of plant cells releasing hydrolyzable and condensed tannins in evergreen oak litter (Racon et al., 1988). These tannins undergo leaching of the litter layers and, probably, water soluble phenols do not reflect the in situ catalytic activities of microorganisms on polyphenolic components of evergreen oak litter.

According to Burns (1982), high proportions of extracellular enzymes are associated with soil or litter colloids, such as humic compounds. According to Tabatabai and Fu (1992), these systems may allow great stability of laccases in soil and may affect the properties of soil or litter enzymes. However, little work has been done to find the degree of adsorption of extracellular enzymes on humic colloids over a period of a year (Burns, 1982). Laccases were selected for the present study, because these were the sole phenoloxidase observed in evergreen oak litter throughout the year.

Criquet et al (1999) has mentioned the role of humic substances on the thermic optimum for the activity of laccases in litter extracts. Maximum temperature for activity of laccases of the litter extract shifted from 30

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Table 2

<table>
<thead>
<tr>
<th>Variable name( ^a )</th>
<th>Constant</th>
<th>Moisture (coefficient ( a ))</th>
<th>Temperature (coefficient ( b ))</th>
<th>pH (coefficient ( c ))</th>
<th>( r^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laccases</td>
<td>4.073</td>
<td>0.017</td>
<td>-0.064</td>
<td>-0.423</td>
<td>0.326</td>
</tr>
<tr>
<td>Mn–peroxidases</td>
<td>9.714( ^b )</td>
<td>0.011</td>
<td>-0.046</td>
<td>-1.472( ^b )</td>
<td>0.479</td>
</tr>
<tr>
<td>Global fungi</td>
<td>27.436( ^b )</td>
<td>0.554</td>
<td>-0.063</td>
<td>-4.341( ^b )</td>
<td>0.522</td>
</tr>
<tr>
<td>Fungi PO(^+ )</td>
<td>16.676</td>
<td>0.024</td>
<td>-0.048</td>
<td>-2.618( ^b )</td>
<td>0.465</td>
</tr>
</tbody>
</table>

\( ^a \) Variable name = Constant + (\( a \) \times Moisture) + (\( b \) \times Temperature) + (\( c \) \times pH).

\( ^b \) Levels of significance of the coefficients are indicated for \( P \leq 0.05 \).

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Table 3

Analysis of variance of the multiple regression models established between activities of laccases and Mn-peroxidases, fungal numbers (global and PO\(^+ \)), moisture, temperature and pH

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F-ratio</th>
<th>Prob &gt; F</th>
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<tr>
<td>Laccase model</td>
<td>4.016</td>
<td>3</td>
<td>1.339</td>
<td>1.450</td>
<td>0.293</td>
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<tr>
<td>Error</td>
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<td>9</td>
<td>0.923</td>
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<tr>
<td>MnP model</td>
<td>4.721</td>
<td>3</td>
<td>1.574</td>
<td>2.756</td>
<td>0.104</td>
</tr>
<tr>
<td>Error</td>
<td>5.138</td>
<td>9</td>
<td>0.571</td>
<td></td>
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</tr>
<tr>
<td>Global fungi model</td>
<td>45.443</td>
<td>3</td>
<td>15.148</td>
<td>3.270</td>
<td>0.073</td>
</tr>
<tr>
<td>Error</td>
<td>41.690</td>
<td>9</td>
<td>4.632</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fungi PO(^+ ) model</td>
<td>14.091</td>
<td>3</td>
<td>4.697</td>
<td>2.608</td>
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<tr>
<td>Error</td>
<td>16.209</td>
<td>9</td>
<td>1.801</td>
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</tr>
</tbody>
</table>

\( ^a \) SS : Sum of squares; df : degrees of freedom; MS : Mean of Squares.

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Fig. 4. Effect of temperature (A) and pH (B) on activity of the laccases of the Lv layer of an evergreen oak litter studied. (–––) activity of laccases from extracts containing humic substances (Criquet et al., 1999); (–○–) activity of the “free laccase fraction” devoid of humic substances (HS). Values represent the averages of five replicates; bars represent standard deviations.
to 50°C after total precipitation of humic substances using protamine sulfate (Fig. 4). However, Ruggiero and Radogna (1984) have shown that the thermic optimum for laccases adsorbed on humic substances shifted to higher temperatures. Furthermore, Nannipieri et al. (1982) have shown that immobilization of enzymes on humic substances usually increases their thermic stability. These contradictory results may be explained by the observations of Ladd and Butler (1975), who reported certain exceptions for thermal properties of soil enzymes.

There was also a shift in laccase activity towards acidic values of the pH, when humic substances were removed from the litter extracts with protamine sulfate (Fig. 4), although the optimum pH values were the same in both extracts, with or without humic substances. A similar phenomenon has also been described for urease activity in soil (Nannipieri et al., 1978).

Fig. 5 shows that the optimal laccase activity was negatively correlated to the “free laccase fraction” \( r = -0.71 \), Table 1). Thus, it appears that the adsorption of laccases on the humic substances is a favorable factor, which allows a higher activity of these enzymes in the litter. These results could agree with the hypothesis of Gramss et al. (1999), who suggested an implication of laccases and other phenoloxidases in the biosynthesis and decomposition of humic substances in the litter. Moreover, the high correlation coefficient observed between the “free laccase fraction” and temperature \( r = 0.82 \), Table 1) may indicate that humification processes in the evergreen oak litter are probably slackened or stopped during summer dryness, which is typical of Mediterranean climate.

### 3.3. Electrophoretic analysis

The laccases detected over the year in the evergreen oak litter (Fig. 6) showed three isoenzymes with \( R_f \) 0.23, 0.34 and 0.43 (Fig. 7). These three isoenzymes were observed in the litter between October 1997 and January 1998 and in September and October 1998, when the activities of laccases generally peaked. For the rest of the year, the isoenzyme with \( R_f \) 0.23 was not detected in the litter extracts (Fig. 7). In August 1998, only one isoenzyme (\( R_f \) 0.34) was present in the litter (Fig. 7), which was probably due to the dryness of the Mediterranean summer (Daget, 1984). This dryness was probably unfavorable for the development of global fungal microflora and fungi producing polyphenoloxidases, thereby, for the expression of several laccase isoenzymes in the litter.

Only one isoenzyme of Mn-peroxidases with an \( R_f \) of 0.21 (Fig. 7) was observed in November and December 1997, in accordance with the peak activity of this enzyme detected during the same period.

### 4. Conclusion

This study has shown certain aspects of the microbial degradation of polyphenolic compounds in evergreen oak litter in the Mediterranean area. The phenoloxidases which oxidize these compounds seem
to be limited to laccases and Mn-peroxidases, since lignin-peroxidases and tyrosinases were never detected under these experimental conditions. During the autumn, we observed the maximum activities of phenoloxidases followed by an increase in the number of fungi producing polyphenoloxidases.

Laccases were the only phenoloxidases present in the litter throughout the year and were always represented by one isoenzyme (Rf 0.34) with a high activity staining. The two other isoenzymes were not detected in all the samples and their activity staining was always weaker than that observed with the permanent isoenzyme (Rf 0.34). The highest activity of laccases was observed, when they were adsorbed to humic substances. This adsorption was weak during the summer (hot, as is typical of a Mediterranean climate), since it is negatively correlated to temperature.

Since laccases are present in the litter throughout the year, polyphenolic compounds, such as lignin, are probably subjected to continuous transformations by these enzymes. Nevertheless, in the evergreen oak litter, transformation of polyphenolic compounds at an optimum rate appeared to be a seasonal phenomenon, involving the activity of phenoloxidases such as laccases or Mn-peroxidases produced by ligninolytic fungi. The presence of water soluble phenols was apparently not correlated with the development of fungi and with activities of phenoloxidases. It may be correlated with other phenomena such as the enzymatic disintegration of cells containing tannins in the evergreen oak leaf.

It will be interesting to study the evergreen oak litter at length in autumn, with samples very closely collected at very short intervals. The tannin concentration measured in the litter would allow us to study closely how these compounds are released over time. Furthermore, under these conditions, correlation between temperature, humidity, pH and other biological parameters would allow us to define the seasonal cycle, which seems so important for fungi. In other words, we should investigate the direct influence of abiotic factors on fungal enzymatic activities.

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