Effects of environmental factors and leaf chemistry on leaf litter colonization by fungi in a Mediterranean shrubland

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KEYWORDS

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Ergosterol;
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Soil moisture;
Total phenolic
compounds

Summary

Estimation of litter colonization by fungi, using ergosterol, an indicator of fungal biomass, is a reliable way to describe the process of leaf litter decomposition. This litter colonization by fungi is regulated both by exogenous or environmental factors, and endogenous factors, i.e. litter chemistry. In this work, we have examined the effects of some of these factors on litter fungal colonization in a Mediterranean ecosystem, by determining ergosterol content of *Quercus coccifera* leaf litter.

Environmental factors have been studied through the fertility of the soil, by comparing plots amended with two rates of compost and plots without amendment. Results indicated that (i) compost had a significant effect on soil fertility but did not increase ergosterol content of leaf litter and (ii) soil humidity improved leaf litter colonization by fungi.

Endogenous factors have been studied through measurements of total phenolic and ergosterol concentrations of seven shrub species leaf litter. We have shown (i) a negative significant correlation between total phenolic compounds and ergosterol concentrations of leaf litter and (ii) a positive significant correlation between total phenolic compound concentrations in green leaves and in leaf litter. We conclude that, in this Mediterranean shrub ecosystem, leaf litter colonization by fungi is controlled by soil moisture and plant leaf litter quality.

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Introduction

Litter is an important source of dead organic matter in terrestrial ecosystems, with inputs of 50×10^9 tons of litter per year (Isidorov and Jdanova, 2002). Litter decomposition contributes directly to nutrient availability both for plant growth and ecosystem productivity (Koukoura et al., 2003). The studies that have taken microorganisms into account agree in giving the fungi as the main contributors to leaf litter decomposition (Toutain, 1987; Isidorov and Jdanova, 2002).

These eukaryotes are able to hydrolyse and assimilate refractory compounds such as lignin (Criquet et al., 1999) or tannins (Iacazio et al., 2000), although bacteria are not thought to degrade the leaf material before it has become partially broken down by microarthropods and partially decomposed by fungi (Périsol et al., 1993; Dilly et al., 2001).

Litter fungal colonization is regulated both by exogenous or environmental factors and endogenous factors. Environmental factors include climate and soil nutrient availability (Cortez et al., 1996), endogenous factors are leaf litter nutrient content (e.g. C, N, P) and secondary metabolites (e.g. phenolic compounds) content (Melin, 1930; Koukoura et al., 2003).

With regard to environmental factors, Mediterranean shrublands are characterized by low water availability and low soil organic matter content, the latter parameter becoming worse with recurrent fires (Borghetti et al., 2004). One of the methods employed for improving nutrient budgets in these low productive ecosystems is to spread composted sewage sludges as organic amendments. Compost may effectively reactivate the biogeochemical cycles since it brings nutrients to microorganisms, and enhances the water retention into the soil (Borken et al., 2002).

With regard to endogenous factors, the vegetation of these shrublands is dominated by evergreen sclerophyllous species which produce high amounts of leaf secondary metabolites, including phenolic compounds (Gershenzon, 1984). Plants produce these compounds in response to different stress factors, such as interspecific competition (Ferrat et al., 2001), animal overconsumption of leaves (Van Hoven, 1984) and atmospheric pollution (Pasqualini et al., 2003). Plant secondary metabolite content has been suggested to be a major inhibiting factor of the activity of microorganisms (Anderson, 1973; Hättenschwiler and Vitousek, 2000; Isidorov and Jdanova, 2002), particularly in nutrient-poor soils (Northup et al., 1998). Consequently, soil organic

matter is easily humified instead of mineralized (Shindo and Kuwastuka, 1976).

In view of the characteristic features of Mediterranean ecosystems, we assumed that the influence of factors controlling leaf litter colonization by fungi and then decomposition, could be of major importance in the matter cycle in these ecosystems.

The aim of this study is to provide comprehensive data on leaf litter colonization by fungi in a Mediterranean shrubland by determining the effects of (i) an organic amendment by biosolids and (ii) phenolic content on litter colonization by fungi. As fungi associated with decomposed leaves are the main actors of leaf litter breakdown (Toutain, 1981), these eukaryotes offer a reliable way to describe the process (Baldy et al., 1995; Gessner et al., 1999). Consequently, the impact of factors controlling litter breakdown could be studied by monitoring changes in fungal biomass dynamics (Gessner and Chauvet, 1994; Isidorov and Jdanova, 2002) and relating them to factors controlling the process.

Materials and methods

Study site and experimental design

The experiment was carried out on 6000 m² in the plateau of Arbois (Southern Province, France; 5°18'6"E–43°29'10"N in WSG-84 Geodetic system), at an altitude of 240 m above sea level under Mediterranean climatic conditions (Fig. 1). The soil was a silty-clayey chalky rendzina, with a high percentage of stones (77%) and low average depth (24 cm). The last fire occurred in June 1995 and the site was colonized by a Mediterranean sclerophyllous vegetation, with a 70% total cover, *Quercus coccifera* L. and *Brachypodium retusum* Pers. being the two dominant species. This natural vegetation belongs to the holm oak (*Q. ilex* L.) succession series, and we observed *Cistus albidus* L., *C. salvifolius* L., *Rosmarinus officinalis* L., *Ulex parviflorus* L., and some groves of *Pinus halepensis* Miller, *Q. ilex* L., and *Q. pubescens* Willd.

Experimental setup and field procedures

Effects of environmental factors, added organic matter and its effects on soil properties and on litter colonization by fungi, were determined using litter from kermes oak (*Q. coccifera* L.), as it is the dominant species of the Mediterranean garrigue

Figure 1. Mean air temperature and precipitation from January 2002 to April 2003 (Météo France).

ecosystem, accounting generally for 60–70% of the total litter (Cañellas and San Miguel, 1998).

Compost was surface applied in January 2002 with a complete randomized block of twelve 500 m² plots as experimental design. Four plots did not receive any compost (D0 = control), four plots received 50 Mg ha⁻¹ (D50) and four plots received 100 Mg ha⁻¹ (D100). The compost was produced by Biotechna (Ensuès, South Province, France) and is certified as being in conformity with the NF U 44-095 (2002) norm on composts made with materials of sewage treatment origin. This compost was made with greenwastes ($\frac{1}{3}$ volume), pine bark ($\frac{1}{3}$ volume) and local municipal sewage sludge ($\frac{1}{3}$ volume). The mixture was composted for 30 days at 75 °C to kill pathogenic microorganisms and decompose phytotoxic substances, and then sieved (<20 mm mesh) to remove large bark pieces and stored in swathes. The swathes were turned (mixed) several times within the next 6 months to promote organic matter humification. The final compost met the French legal standards for pathogenic microorganisms, organic trace elements and heavy metals. Compost characteristics are shown in Table 1. With this experimental design, the soil surface organic layer was entirely collected down to mineral soil, every 2–3 months from April 2002 to April 2003. Organic samples were then 2 mm mesh sieved and separated into two fractions: a coarse fraction >2 mm and a fine fraction <2 mm. Chemical analysis were only performed on the fine fraction. Each analysed sample was a mix of three samples randomly collected on each plot. Entire *Q. coccifera* senescent leaves were separated from the coarse fraction to determine fungal biomass.

Effect of the endogenous factor “total phenolic compounds” on leaf litter colonization by fungi was

determined in green and litter leaves of seven Mediterranean species collected in May 2003 on the same site, but outside the experimental plots. The species were: the semi-deciduous malacophyllous shrubs *C. albidus* L. and *C. salvifolius* L., the evergreen sclerophyllous oaks and shrub *Q. coccifera* L., *Q. ilex* L. and *R. officinalis* L., the deciduous oak *Q. pubescens* Willd., and *P. halepensis* Mill. Mature green leaves were collected randomly from several individuals and leaf litter under the same individuals.

For both sampling (endogenous and exogenous factors), leaf litter was sampled in the most superficial layer where the decomposition is the most efficient (Toutain, 1987). In the laboratory and before we measured the ergosterol and/or the phenol content, green leaves or leaf litter was frozen, later lyophilized (Lyovac GT2®) and finally crushed.

Fungal biomass

Fungal colonization of the litter of the species studied was estimated using ergosterol concentration. Ergosterol is a fungal indicator which offers an efficient measure of living fungal biomass (Gessner et al., 1991; Davis and Lamar, 1992; Djajakirana et al., 1996; Gessner and Schmitt, 1996). Analyses were performed with 50 mg of lyophilized leaf litter. Ergosterol was extracted from leaf litter by 30 min refluxing in alcoholic base (Gessner et al., 1991) and purified by solid-phase extraction (Gessner and Schmitt, 1996). Final purification and quantification of ergosterol was achieved by high-performance liquid chromatography (HPLC) on a HP series 1050 chromatograph. The system was run with HPLC-grade methanol at a flow rate of

Table 1. Soil (0–24 cm: maximal depth; $N = 12$) before amendment and compost ($N = 3$) physical–chemical characteristics

Parameter	Soil		Compost	
	Mean (SD)	Authorized French limit values before sewage sludge amendment	Mean (SD)	Authorized French limit values (08/01/1998)
pH _{H2O}	7.34 (0.008)		7.7 (0.05)	
Moisture (% FM)			4.8 (0.29)	
CEC (cmol ⁺ kg ^{−1})	23.12 (0.31)			
Total CaCO ₃ (%DM)	4.17 (0.13)			
OM (% DM)	7.58 (0.12)		46.8 (2.74)	
Total N (% DM)	0.36 (0.005)		2.03 (0.03)	
C/N	12.42 (0.09)		13.4 (0.78)	
Total P (% DM)	0.037 (0.001)		3.24 (0.03)	
Available P (ppm)	23.3 (0.35)		2514.8 (7.82)	
Copper (mg kg ^{−1} DM)	19.8 (0.14)	100	144.1 (0.84)	1000
Zinc (mg kg ^{−1} DM)	78.2 (0.24)	300	265.0 (5.49)	3000
Cadmium (mg kg ^{−1} DM)	0.31 (0.002)	2	0.8 (0.0)	15
Chrome (mg kg ^{−1} DM)	67.3 (0.33)	150	27.1 (0.65)	1000
Mercury (mg kg ^{−1} DM)	0.06 (0.001)	1	0.86 (0.06)	10
Nickel (mg kg ^{−1} DM)	45.3 (0.17)	50	16.5 (0.23)	200
Lead (mg kg ^{−1} DM)	43.1 (0.26)	100	57.3 (2.53)	800

DM: Dry Mass. FM: Fresh Mass.

1.5 ml min^{−1}. Ergosterol eluted after 9 min and was detected at 282 nm; peak identity was checked on the basis of retention times of commercial ergosterol purchased from Fluka® (>98% purity).

In the fine soil organic fraction, some physical–chemical parameters were determined using French standard analysis procedures (AFNOR, 1999). Cationic exchange capacity (CEC) was measured by percolation with an ammonium acetate solution. Organic C was determined using sulphuric–chromic oxidation and spectrophotometry (Cary 50 VARIAN). Total nitrogen (N) was determined by dry combustion and thermic conductimetry (FP 428 LECO). Available P₂O₅ was determined in a sodium hydrogenocarbonate solution using spectrophotometry (Olsen et al., 1954) (Cary 50 VARIAN). To measure total phosphorus concentrations, samples were digested in *aqua regia* and analysed using plasma emission spectrophotometry (VARIAN VISTA Axial). Moisture content was determined by oven drying samples at 60 °C for 3 days.

Total phenolic compounds

The method of extraction of total phenolic content of leaves was based on the work of Peñuelas et al. (1996): 500 mg per sample of dry leaf litter or green leaves were extracted with

20 ml of 70% aqueous methanol (v/v) acidified with some concentrated HCl drops. The mixture was left at ambient temperature for an hour and a half, and then filtered. Quantification of the total phenols was done by colorimetric reaction using Folin–Ciocalteu reagent (Folin and Denis, 1915). After 1 h, the reaction was completed and measured at 720 nm on a Phillips® PU 8620 spectrophotometer. The quantitative results were expressed with reference to gallic acid as in Peñuelas et al. (1996).

Statistical analyses

Two-way ANOVAS combined with Tukey tests were used to make comparisons of the different parameters (ergosterol, physical–chemical parameters) according to season and compost amendment. If any interaction occurred between the two studied factors (compost rate, date), one-way Anova were performed at each sampling date to study compost rate effect. The comparisons of mean phenolic content as a function of studied species were processed by one-way ANOVA followed by Tukey test (Zar, 1984). Previously, normality and homocedaticity were verified by Shapiro–Wilks and Bartlett tests, respectively (Zar, 1984). Significant relationships between the fine soil organic fraction parameters and ergosterol were assessed using Pearson correlation. The software

Statgraphics plus (version 2.1: Statistical Graphics Corporation®, Copyright 1994–1996) was used.

Results

Effects of compost amendment and season on the fine soil organic fraction

Temperature and rainfall, between January 2002 and April 2003, showed marked seasonal changes

(Fig. 1), with maximum rainfall in May, September, November 2002 and in January and April 2003. Maximum temperature occurred in June, July and August 2002.

Soil cationic exchange capacity (Fig. 2D) and moisture content (Fig. 2G) varied according to the season (Table 2), while organic matter (Fig. 2A), total nitrogen (Fig. 2B), C/N ratio (Fig. 2C) and total phosphorus (Fig. 2E) varied significantly with compost rate (Fig. 2, Table 2). Available phosphorus varied according to season and rate of compost (Fig. 2F, Table 2), and the compost effect was

Figure 2. Dynamics of (A) organic matter content, (B) total nitrogen content, (C) C/N ratio, (D) cationic exchange capacity, (E) total phosphorus content, (F) available P₂O₅ content, and (G) moisture content, of the fine soil organic fraction, according to season and rate of compost (0: non-amended plots, 50: amended plots with 50 Mg ha⁻¹ and 100: amended plots with 100 Mg ha⁻¹ of compost). Bars denote ±95% confidence limit (N = 4). ANOVA: *P < 0.05; **P < 0.01; ***P < 0.001. Results of the comparison are given by a letter: values that do not differ at the 0.05 level are indicated with the same letter (a < b < c).

Table 2. Results of the variance analysis with two factors (rate of compost amended and season) on fine soil organic fraction parameters

	Anova	Tukey
Organic matter	Rate: $F = 4.49$; $P = 0.02$ Season: $F = 1.98$; $P = 0.16$ Rate $\times$ Season: $F = 0.3$, $P = 0.88$	0 ^a 50 ^{ab} 100 ^b — —
Cationic exchange capacity	Rate: $F = 1.92$; $P = 0.17$ Season: $F = 19.86$; $P < 0.001$ Rate $\times$ Season: $F = 0.64$, $P = 0.64$	— Apr. 02 ^b –Jul 02 ^a –Oct 02 ^c —
C/N	Rate: $F = 13.04$; $P < 0.001$ Season: $F = 1.34$; $P = 0.28$ Rate $\times$ Season: $F = 0.43$, $P = 0.78$	0 ^b 50 ^a 100 ^a — —
Total nitrogen	Rate: $F = 39.90$; $P < 0.001$ Season: $F = 1.91$; $P = 0.17$ Rate $\times$ Season: $F = 1.46$, $P = 0.24$	0 ^a 50 ^b 100 ^b — —
Total phosphorus	Rate: $F = 418.11$; $P < 0.001$ Season: $F = 1.41$; $P = 0.67$ Rate $\times$ Season: $F = 1.25$, $P = 0.31$	0 ^a 50 ^b 100 ^b — —
Available phosphorus (P ₂ O ₅)	Rate: $F = 431.6$; $P < 0.001$ Season: $F = 12.64$; $P < 0.001$ Rate $\times$ Season: $F = 5.26$, $P = 0.003^*$	0 ^a 50 ^b 100 ^b Apr. 02 ^b –Jul 02 ^b –Oct 02 ^a —
Moisture content	Rate: $F = 0.40$; $P = 0.67$ Season: $F = 96.36$; $P < 0.001$ Rate $\times$ Season: $F = 3.88$, $P = 0.001^*$	— Apr. 02 ^a –Jul 02 ^b –Oct 02 ^c Dec 02 ^d –Feb 03 ^d –Apr. 03 ^c

Values that do not differ at the 0.05 level are indicated with the same letter (a < b < c). 0: non-amended plots; 50: plots amended with 50 Mg ha⁻¹ of compost and 100: plots amended with 100 Mg ha⁻¹ of compost.

*See Fig. 2 for the one-way ANOVA and Tukey test results.

significant at each sampling date (Table 2). Compost amendment increased soil moisture content in July 2002.

Compost amendment led to an increase in the organic matter content, total nitrogen, total and available phosphorus, while C/N ratio decreased with compost rate (Tukey test, $P < 0.05$). However, there was no significant difference between the two rates of compost.

Influence of exogenous factors on ergosterol content of *Q. coccifera* leaf litter

Ergosterol content of *Q. coccifera* leaf litter varied from 103.8 $\mu\text{g g}^{-1}$ DM (plot with 100 Mg of compost per ha in April 2002) to 265.5 $\mu\text{g g}^{-1}$ DM (plots without compost in March 2003) (Fig. 3). Ergosterol content of leaf litter changed significantly according to season but did not increase significantly on amended plots (Two-way ANOVA, season factor, $F = 14.69$, $P < 0.001$; rate factor, $F = 1.63$, $P > 0.05$; season $\times$ rate, $F = 0.85$, $P > 0.05$). Ergosterol content of leaf litter showed higher values between October 2002 and

Figure 3. Dynamics of ergosterol concentrations associated with leaf litter of *Quercus coccifera* in non-amended plots (0), plots amended with 50 Mg ha⁻¹ (50) and plots amended with 100 Mg ha⁻¹ (100) of compost. Bars denote $\pm 95\%$ confidence limit ($N = 4$).

March 2003 (Tukey test, $P < 0.05$), and did not change significantly from 1 year to another (April 2002–April 2003, Tukey test, $P < 0.05$; Fig. 3).

We observed a significant positive linear relationship between ergosterol content of leaf litter and moisture content of the fine soil organic fraction

($r = 0.60$, $P < 0.05$). In addition, a significant positive correlation between ergosterol content of leaf litter and cationic exchange capacity of the fine organic soil fraction was observed for April 2002 and July 2002 ($r = 0.58$ and 0.54 , respectively, $P < 0.05$).

We did not find a significant relationship between ergosterol content and the other chemical parameters (organic matter, C/N ratio, total N, total P, available P; $0.16 < r < 0.39$, $P > 0.05$).

Influence of an endogenous factor on ergosterol content of leaf litter: Total phenolic compound content

Total phenolic compound leaf content varied from 55 to 120 mg gallic acid g^{-1} DM (dry mass) of green leaves and from 10 to 40 mg gallic acid g^{-1} DM of leaf litter (Fig. 4), and showed significant differences between plant species for green leaves (one-way ANOVA; $F = 876.19$, $P < 0.001$) and for leaf litter (one-way ANOVA; $F = 26.58$, $P < 0.001$). For each of the seven plant species, total phenolic compound content was significantly higher in green leaves than in leaf litter (t -test; $P < 0.05$). When we excluded *Q. coccifera* species, whose litter had a

low total phenolic content although that of green leaves was very high, we observed a significant positive linear regression between total phenolic content of green leaves and total phenolic content of litter leaves ($y = 0.4129x - 12.121$; $r = 0.76$, $P < 0.05$).

For all the seven plant species it was possible to establish a significant negative linear regression between total phenolic compound and ergosterol contents of litter leaves ($y = -7.3163x + 367.54$; $r = 0.8$, $P < 0.05$).

Discussion

Improving knowledge on litter degradation under Mediterranean climate is necessary for understanding the functioning of Mediterranean ecosystems. Litter constitutes an important source of carbon and energy supply for microbial communities (Pascual et al., 2000). In extensive areas of the Mediterranean regions, the natural vegetation is exposed to the harsh climatic conditions (Pascual et al., 2000). Therefore, humidity and soil nutrients are limiting factors in these ecosystems (Rapp et al., 1999).

Figure 4. Total phenolic compound concentrations (in mg of gallic acid g^{-1} DM) of green leaves, total phenolic compound and ergosterol concentrations (μg^{-1} DM) of leaf litter of the seven species studied. Bars denote $\pm 95\%$ confidence limit ($N = 3$).

As the soil at our site presented a low level of organic matter and a very weak moisture content, we had assumed that ergosterol content of leaf litter would be enhanced significantly by compost amendment. Previous studies have investigated how microbial and particularly fungal populations are reactivated after organic matter input to soil (Caravaca et al., 2002). It has also been proved that organic amendment is a source of carbon and energy for the soil microbiota and that it increases fungal diversity (Acea and Caballas, 1996). Likewise, Pascual et al. (2000) achieved an increase in the microbial biomass, by means of organic matter. In the present study, even if total organic matter, nitrogen and phosphorus contents of soil significantly increased with compost, we did not find any significant effect of the compost on the ergosterol content of *Q. coccifera* leaf litter. However, our study was carried out under natural conditions and we examined ergosterol associated with leaf litter whereas the studies cited above focused on the soil. Moreover, compost rates used were low, in accordance with authorized French limit values, and it was very mature, containing only 28 per cent of sludge from the purification of urban waste water. According to Caravaca et al. (2002), this part of organic matter is easily assimilated by microorganisms, and little is known about the effect of mature compost on microbial communities (Borken et al., 2002). In our study, cationic exchange capacity did not increase on amended plots, showing that the incorporation of the compost organic matter into the soil has not been achieved (Gobat et al., 2003). Our results indicate that there is a need for longer time-scale surveys, especially in the case of mature compost that decomposes slowly.

In contrast, soil moisture strongly improved leaf litter colonization by fungi. Ergosterol content was positively correlated to soil moisture content. This result is in accordance with previous works on Mediterranean ecosystems, where fungal biomass and enzymatic activity reach maximum values under moist conditions (Criquet et al., 2000; Fioretto et al., 2000, 2001; Barajas-Aceves et al., 2002).

So in our experimental site, soil moisture is more important than organic matter content for litter colonization by fungi, and thus for the recycling of organic matter in this shrub ecosystem. However, other environmental parameters could explain ergosterol dynamics in leaf litter and then decomposition, such as temperature, plant cover (Garcia et al., 2002; Ballini, 1997) and mesofauna diversity (Cortet et al., 2003).

Another important factor improving leaf litter colonization by fungi is litter quality (Albers et al., 2004). In the present study, although ergosterol and

phenol concentrations that we obtained are within the same range as those obtained in other Mediterranean ecosystems (for ergosterol: Barajas-Aceves et al., 2002; Pascual et al., 2000; Cortet et al., 2003; for phenols: Peñuelas et al., 1996; Castells et al., 2002; Pasqualini et al., 2003), significant differences were observed between plant species.

Pooling the seven species studied, we could observe a significant negative relationship between ergosterol and phenol concentrations of litter leaves. This result confirms that total phenolic compounds act as inhibitors of microorganisms involved in litter decomposition process (Anderson, 1973; Isidorov and Jdanova, 2002). On the other hand, total phenolic compound content of green leaves is significantly correlated with that of litter leaves. Therefore, different plant communities promote variations in litter quality and decomposability (Koukoura et al., 2003). As a consequence, fungal colonization of leaf litter with high phenolic content may be lower than for leaf litter with low phenolic content. This relationship acts as a feed-back control on nutrient availability in ecosystems (Aerts, 1997). Nevertheless in our work, this positive relationship only exists when we exclude *Q. coccifera*. Green leaves of this species contain high concentrations of total phenolic compounds while litter leaves show low concentrations, in contrast to the other species studied. This particular feature could be explained by the fact that senescent leaves of *Q. coccifera* remain on the tree for a long time before falling (Floret et al., 1989), and therefore may lose a large proportion of their phenolic compounds by leaching (Hättenschwiler and Vitousek, 2000). On the other hand, there is also a relationship between the litter initial nutrient content and litter decomposition. High N litter content especially has been shown to enhance leaf litter colonization by fungi (Berg and Söderström, 1979) and leaf litter decomposition (Van Wesemael, 1993; Ballini, 1997; Gallardo and Merino, 1999; Gartner and Cardon, 2004). The initial N litter content is very variable among plant species (Van Wesemael, 1993; Gallardo and Merino, 1999). On the basis of this observation, nutrient concentrations could probably control ergosterol concentration as much as total phenolic compound content.

Conclusion

In conclusion, these data on ergosterol dynamics associated with decomposed *Quercus coccifera*

leaves in a Mediterranean shrub ecosystem show that leaf litter colonization by fungi is not affected by compost amendment but is closely linked to soil humidity and total phenolic concentrations of leaf litter. These findings suggest that nutrient release from decomposing litter should vary according to climatic conditions and plant species. Therefore, it would be of great interest to study leaf litter breakdown of the main Mediterranean species using litter bags in order to determine the relative importance of the factors controlling the process.

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