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Fungal Community and Ligninolytic Enzyme Activities in *Quercus deserticola* Trel. Litter from Forest Fragments with Increasing Levels of Disturbance

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Abstract: Litter fungal communities and their ligninolytic enzyme activities (laccase, Mn-peroxidase, and lignin-peroxidase) play a vital role in forest biogeochemical cycles by breaking down plant cell wall polymers, including recalcitrant lignin. However, litter fungal communities and ligninolytic enzyme activities have rarely been studied in Neotropical, non-coniferous forests. Here, we found no significant differences in litter ligninolytic enzyme activities from well preserved, moderately disturbed, and heavily disturbed *Quercus deserticola* Trel. forests in central Mexico. However, we did find seasonal effects on enzyme activities: during the dry season, we observed lower laccase, and increased Mn-peroxidase and lignin-peroxidase activities, and in the rainy season, Mn-peroxidase and lignin-peroxidase activities were lower, while laccase activity peaked. Fungal diversity (Shannon-Weaver and Simpson indices) based on ITS-rDNA analyses decreased with increased disturbance, and principal component analysis showed that litter fungal communities are structured differently between forest types. White-rot Polyporales and Auriculariales only occurred in the well preserved forest, and a high number of Ascomycota were shared between forests. While the degree of forest disturbance significantly affected the litter fungal community structure, the ligninolytic enzyme activities remained unaffected, suggesting functional redundancy and a possible role of generalist Ascomycota taxa in litter delignification. Forest conservation and restoration strategies must account for leaf litter and its associated fungal community.

Keywords: oak litter; ligninolytic enzymes; forest litter degradation; fungal community

1. Introduction

Litter is a key component of nutrient dynamics in forest ecosystems that, upon its decomposition, provides available nutrients for the plant community [1]. Litter decay in forest ecosystems is a dynamic

process involving the participation of soil fauna and a complex microbial community producing extracellular lignocellulolytic enzymes [2]. Within litter biota, the fungal community performs roles in polysaccharide and lignin degradation [3], causing changes both in biotic and abiotic factors at different spatial and temporal scales [4]. The structure and function of fungal communities in the forest floor have patterns characteristic to forest types, with well-established differences between forests in temperate and tropical ecosystems [5] and between forests dominated by coniferous and broadleaf trees [6,7]. In addition, significant differences have been clearly documented among similar forest types along geographical gradients [8]. Furthermore, it has been noted that the principle significant difference in fungal communities in the same ecosystem generally occurs between communities in the litter and in the soil, rather than between the organic and mineral soil horizons [8]. Taking all this into account, the structure and dynamics of litter fungal communities in oak forests in tropical and Neotropical areas are not well understood, and, despite the key role of fungi in the process of litter decay in these ecosystems, knowledge regarding fungal community distribution, abundance, and spatial and temporal changes remains scarce.

The richness of oaks (*Quercus* L.; Fagaceae) has been estimated to be between 450 and 600 species worldwide [9,10], of which 160 to 165 are found in Mexico, making the country a hotspot for oak species [11,12]. Thus, at least 27% of the global species richness of *Quercus* is found in Mexico, across 5.5% of its territory, in ecosystems ranging from temperate humid to warm and arid climates [13]. Rural communities in Mexico exploit oak and oak-pine forests—mainly for the extraction of firewood and charcoal [14,15]—and some of these forests have also been converted to cropland and grazing areas [16]. As a result, the cover of oak forests in Mexico has been seriously reduced with additional impacts upon their structure and dynamics [17,18]. In this regard, García-Oliva et al. [19] reported that in oak-pine forests in central Mexico, uncontrolled wood extraction significantly reduced the carbon pools and disrupted soil nutrient dynamics. For these reasons, knowledge about how these forests respond to disturbance could contribute to developing strategies for their conservation and sustainable management [20,21], in particular changes in microbiological, biochemical, and nutritional components of oak forest litter due to anthropogenic disturbance [22,23], and their potential for recovery. Although Mexico is a hotspot of *Quercus* species diversity, studies of fungal communities in oak forests are limited [24], focusing on ectomycorrhizal species [25] but not other functional groups. Thus, evaluating the structure of the fungal community and the enzymatic profiles of lignin degradation in decomposing litter allows the characterization of spatial and temporal patterns of diversity and responses to environmental factors [23,26] and land use practices [27,28]. In turn, increased knowledge of forest soil and litter fungal communities will aid in identifying the consequences of changes in microbial diversity on ecosystem functions and services [29], as well as establishing restoration [30] and management [31] strategies.

This study aims to describe the ligninolytic enzyme activities and fungal communities present in *Quercus deserticola* Trel. litter in forests under different degrees of anthropogenic disturbance, and to evaluate differences associated with the dry and rainy seasons. Previous studies in *Quercus* spp. dominated ecosystems show that fragmentation significantly reduced the diversity of fungal communities, affecting their metabolic profiles [32]. Based on this and on the above cited works, we predicted that the *Q. deserticola* litter fungal communities would be less diverse in sites with increasing disturbance and that, independently of the degree of disturbance, at the end of the rainy season, the saprobic guild within basidiomycetes would predominate over mycorrhizal groups. Corresponding changes in ligninolytic enzyme activity of the fungal community may be observed, due to shifts in Basidiomycota functional guilds. Functional consequences of the observed patterns are discussed in the context of ecosystem use at the study site.

2. Materials and Methods

2.1. Study Area and Litter Sampling

Forest fragments studied are located in the Cuitzeo basin (19°32′ N, 101°18′ W), southeast of the city of Morelia in the Mexican state of Michoacán. The mean annual temperature in the study area is 15.5 °C and the mean annual precipitation is 1047 mm. The July-September period is the wettest period and the hottest period is April–June (http://smn.cna.gob.mx/). *Quercus deserticola* Trel. trees are dominant in the study area; they lose their foliage during the dry season from January to May and flush leaves in June at the start of the rainy season [24,33]. The region in which the study area is located has suffered forest losses due to logging for timber and charcoal extraction, agricultural expansion, and grazing [34–36].

Within the Cuitzeo basin, three sites with different levels of disturbance were selected: well preserved (WP), moderately disturbed (MD), and heavily disturbed (HD) (Table 1). In order to minimize differences in geography and climate between sites, forest fragments were located within 0.5 km of each other and the dominant soil type in all three sites was Acrisol. Because the disturbed sites showed evidence of tree extraction and no signs of burning, the conservation level of the three selected forest fragments was determined by a description of the vegetation structure and cover present in each site [37] using the method of Gentry [38]. Additionally, 100 m × 20 m plots were established in each of the three forest fragments and the diameters at breast height (DBH) ≥ 5 cm of all individual trees within the plots were measured (Table 1). Finally, aboveground biomass (crown and stem), litter biomass (Table 1), and plant species composition (Supplementary Table S1) were also used as indicators of disturbance/regeneration in each plot. The aboveground biomass was quantified from the DBH values using allometric equations proposed by Aguilar et al. [15]. The temperatures of the studied plots (Table 1) were measured with a VWR Traceable Logger-Trac RH/Temperature Datalogger (Radnor, PA, USA).

Table 1. Characteristics of the three forest sites with increasing levels of disturbance in central Mexico studied to examine fungal communities and ligninolytic enzyme activities.

Coordinates	Well Preserved (WP) 19°32′13.20″ N, 101°17′60.00″ W	Moderately Disturbed (MD) 19°32′18.24″ N, 101°17′56.40″ W	Heavily Disturbed (HD) 19°32′6.00″ N, 101°18′3.60″ W
Stand characteristics			
Number of *Quercus deserticola* Trel. trees	171	154	39
Mean tree DBH ± standard error (cm)	12.1 ± 0.3	12.3 ± 0.25	15.1 ± 0.6
Aboveground biomass (Mg ha^{-1})	42.7	46.3	27.4
Mean litter mass ± standard error (Mg ha^{-1})	1.5 ± 0.25	1.0 ± 0.15	1.1 ± 0.1
Temperature on sampling dates (°C) [1]	34.1, 25.6	34.3, 26.1	35, 26.4
Nutrient concentrations			
Litter [2]			
pH	5.9–6.1	6.0–6.3	6.1–6.2
Carbon (mg g^{-1})	417	391	473
Nitrogen (mg g^{-1})	10.3	8.9	10.4
Phosphorus (mg g^{-1})	0.34	0.42	0.54
C:N	40	44	45
C:P	1227	935	876
N:P	30	21	19
Soil [2]			
Carbon (mg g^{-1})	42.2	58.5	51.3
Nitrogen (mg g^{-1})	3.4	2.33	2.5
Phosphorus (mg g^{-1})	0.36	0.17	0.55
C:N	12	25	20
C:P	132	344	93
N:P	11	14	4

[1] Temperature data for the dry (June) and rainy (September) seasons separated by a comma. [2] Concentrations of nutrients were measured in a mixed sample from 20 soil subsamples and five litter subsamples as described in Materials and Methods.

Four transects of 1 m × 100 m of length were established within each plot to quantify nutrient and carbon contents in both the surface litter and the soil beneath it. Along each transect, a soil sample was taken from the top 20 cm with a soil-corer every 20 m. The 20 soil samples were then mixed and kept in a plastic bag. Five samples of litter were randomly collected from each plot within a polyvinyl chloride ring with a diameter of 160 mm. Soil and litter samples were transported to the laboratory in a cooler and placed in darkness at 4 °C until analysis [39].

For enzyme activities and fungal community composition, litter sampling was done directly beneath the crown of *Q. deserticola* trees to avoid heterogeneous litter composition in the degraded sites. In each plot, five oak trees were randomly selected and litter samples were collected in June 2015 (recent leaf-fall) and September 2015 using a 16 cm-diameter polyvinyl chloride ring, including the first 5–10 cm depth to avoid mineral soil layers. The two sampling dates allowed a comparison of the enzyme activity and fungal community between the driest (June) and the wettest (September) seasons of the year. Samples were immediately cooled to 4 °C and transported to the laboratory on the same day as collection. Once in the laboratory, each sample was subdivided in two: one subsample was stored at 4 °C for physicochemical analyses and enzyme assays, and the other was stored at −80 °C until the extraction of DNA for genetic analyses. Physicochemical analyses and enzyme assays were conducted within five days after sampling. A subsample was dried in an oven at 70 °C and the amount of litter dry matter was determined, with the litter mass estimated in grams per square meter (Table 1) [24].

2.2. Litter and Soil Nutrients Analyses

In the laboratory, total C, N, and P were analyzed for both soil and litter samples. For all three sites, 1 g of fresh litter was placed in a 50 mL beaker and 10 mL of distilled water was added. The mix was stirred for 30 min at 100 rpm, allowed to stand for 5 min, and the pH reading was performed with a potentiometer (Accumet basic AB15, Thermo-Fisher Scientific, Waltham, MA, USA). The litter samples were oven-dried at 70 °C for 72 h, subsequently ground with a mill (Retsch MM400, Haan, Germany), and sieved through a 40 mesh. Similarly, soil samples were oven-dried and ground. Total N and P were determined following acid digestion in a mixture of concentrated H_2SO_4 and K_2SO_4 plus $CuSO_4$, using the latter as a catalyst; N was determined by a micro-Kjeldahl method [40] and P by the molybdate colorimetric method following ascorbic acid reduction [41]. The extract was measured by colorimetry in an autoanalyzer (Bran-Luebbe; Nordestedt, Germany). Carbon was analysed in a total carbon analyzer (UIC 5012; Chicago, IL, USA) and determined by colorimetric detection [42].

2.3. Enzyme Activity Assays

The activity of ligninolytic enzymes in litter samples was determined in triplicate within five days of collection in a Nanodrop 2000c spectrophotometer (Thermo Scientific Inc., Waltham, MA, USA). Enzyme extracts were prepared from 0.5 g aliquots from each litter sample that were incubated for 15 min at room temperature in 30 mL of modified universal extraction buffer (MUB) [43] with continuous stirring. All reaction mixtures were incubated at 30 °C for 120 min. Enzyme activities were expressed in units (U), defined as μmoles of product formed from substrate per hour (μmol h^{-1}), per gram of soil (U g^{-1}).

2.3.1. Laccase (Lac; EC 1.10.3.2)

Lac determination was performed following the procedure of Nagai et al. [44], by measuring the oxidation of ABTS (2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) to its cation radical. The reaction mixture included 300 μL enzyme extract, 100 μL of 10 mM ABTS (Sigma-Aldrich, St. Louis, USA), 200 μL of 0.2 M sodium acetate buffer (pH 5), and 400 μL sterile distilled water. Absorbance determinations were made at 420 nm.

2.3.2. Lignin Peroxidase (LiP; EC 1.11.1.14)

The LiP assay was conducted using the method of Tien and Kirk [45], which is based on the principle that LiP uses H_2O_2 to catalyze the oxidation of veratryl alcohol to veratraldehyde. The reaction mixture included 300 µL of enzyme extract, 200 µL of 0.2 M sodium acetate buffer (pH 5), 200 µL of 10 mM veratryl alcohol (Sigma-Aldrich), 200 µL of sterile distilled water, and 100 µL of 0.4 mM H_2O_2 (Analytyka, Nuevo Leon, Mexico). Absorbance was measured at the absorption peak of veratraldehyde at 310 nm.

2.3.3. Manganese Peroxidase (MnP; EC 1.11.1.13)

The activity of MnP was measured using a method based on the oxidation of 2,6-dimethoxyphenol, as described by Martinez et al. [46]. The reaction mixture contained 300 µL of enzyme extract, 200 µL of 0.2 M sodium acetate buffer (pH 5), 200 µL of sterile distilled water, 100 µL of 0.1 mM $MnSO_4$ (Sigma-Aldrich), 100 µL of 10 mM 2,6-dimethoxyphenol (Sigma-Aldrich), and 100 µL of 0.4 mM H_2O_2 (Analytyka). Absorbance was measured at 469 nm.

2.4. Statistical Analyses of Enzyme Activity

Statistica v.9.0 software (StatSoft, Palo Alto, CA, USA) was used to conduct repeated-measures analysis of variance (RMANOVA) with the site as the between-subjects factor and season and the interaction site-season as the within-subject factor to test for differences in enzyme activity. When RMANOVA indicated significant factor effects, a mean comparison was performed with Tukey's multiple comparison test [47].

2.5. DNA Extraction, PCR Assays, Cloning, and Sequencing

For molecular analyses, genomic DNA was extracted from 5 g of litter using the FastPrep System with the FastDNA spin for soil kit (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions. The DNA obtained was purified with the DNA Clean and Concentrator-5 kit (Zymo Research, Irvine, CA, USA). The ITS region of the nuclear ribosomal unit was amplified using ITS1/ITS4 primers [48]; the total reaction mixture volume was 25 µL and it contained 50 ng DNA, 10 mM Tris-HCl (pH 8.5), 1.5 mM $MgCl_2$, 0.5 mM of each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), 0.5 µM of each primer, 2% bovine serum albumin (BSA; Thermo Scientific), and 0.5 U *Taq* DNA recombinant polymerase (Invitrogen, Carlsbad, CA, USA). The PCR conditions used were 94 °C for 5 min, 35 1-min cycles at 94 °C (denaturation), 62 °C for 1 min (annealing), and 72 °C for 1 min (extension), followed by 10 min at 72 °C. The amplification products were visualized on 2% agarose gel stained with SYBR® Safe (Invitrogen, Carlsbad, CA, USA). The amplification products were then cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Plasmids were recovered by alkaline lysis [49]. Finally, the cloned products were sequenced at Elim Biopharmaceuticals, Inc. (Heyward, CA, USA) with primer M13F (5′-GTAAAACGACGGCCAG-3′).

2.6. Bioinformatics and Fungal Community Analyses

Of the 1013 clone product sequences obtained, seven were identified as chimeras using UCHIME in de novo mode [50] and discarded, leaving a total 1006 sequences for analysis. Curated sequences were deposited in GenBank with accession numbers KT581643 to KT581949. DOTUR [51] was used to group the sequences obtained from the ITS region of rDNA into operational taxonomic units (OTUs) at 97% similarity. For each OTU, the longest sequence was selected and the closest hits were identified using the Blastn algorithm in GenBank (National Center for Biotechnology Information, NCBI, http://www.ncbi.nlm.nih.gov/). The following indices were also calculated with the same package: Shannon-Weaver (*H'*) and Simpson (1-D) [52] diversity indices, Chao1 richness estimator [53], and rarefaction analysis of each clone library. The library coverage values were calculated by [1-(n/N)],

where n is the number of OTUs representing a single clone (singleton) and N is the number of total OTUs representing the clones in the library [54]. The Jaccard index was calculated using the software SONS [55] to examine differences in the fungal community between sites and sampling dates. β-Diversity between fungal communities was estimated with the UniFrac package [56] by performing Principal Coordinates Analysis (PCoA) and Jackknife Cluster Environment analysis.

3. Results

3.1. Nutrients and Vegetation in Studied Plots

Litter mass decreased by around 30% in the disturbed plots (Table 1), but the C and N concentrations of litter were similar among all plots. However, the litter of the WP site had a lower P concentration, and therefore higher C:P and N:P ratios. The C concentration in the soil was lower in WP than in the MD and HD sites, whereas soil N concentration showed the opposite pattern. The P concentration was lower at the MD site where, therefore, higher C:P and N:P ratios were also found.

The presence of plant species characteristic of degraded sites including *Eysenhardtia polystachya* (Ortega) Sarg., *Loeselia mexicana* (Lam.) Brand, and *Croton* sp. was more conspicuous in the MD and HD sites, whereas all plant species found in the WP site were characteristic of undisturbed areas (Supplementary Table S1).

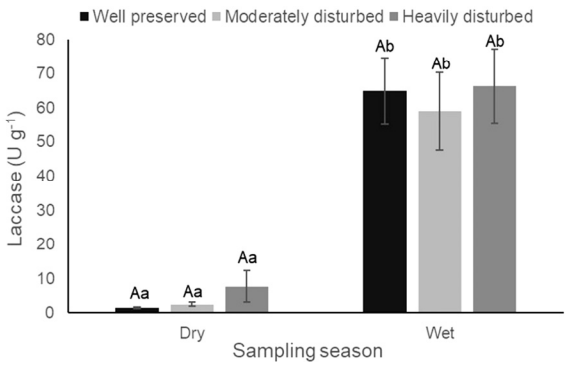
3.2. Enzyme Activity

The activity of the three enzymes was significantly affected by season but not forest disturbance (Table 2). At the three sites, Lac activity was five times higher in the rainy than in the dry season (Figure 1a), the activity of LiP decreased in the rainy season (Figure 1b), and MnP behaved in a similar way to LiP, with its activity being significantly higher in the dry than in the rainy season (Figure 1c).

Table 2. *F* (*p*) values from the RMANOVAs of enzymatic activity in *Quercus deserticola* litter in three forest sites with increasing levels of disturbance in central Mexico.

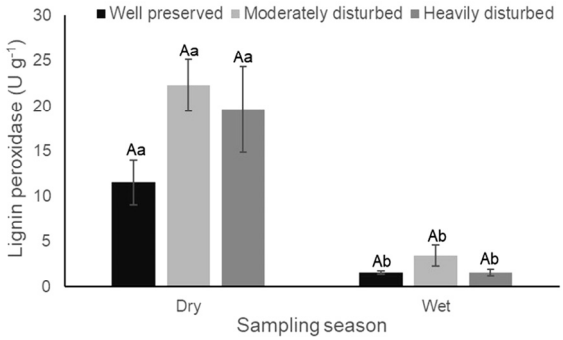
Enzyme	Between Subjects	Within Subjects	
	Site	Sampling Season	Interaction (Site:Season)
Laccase	0.28 (0.75)	106.42 (<0.0001)	0.12 (0.88)
Manganese peroxidase	1.65 (0.23)	24.37 (<0.0001)	2.42 (0.13)
Lignin peroxidase	3.55 (0.061)	52.73 (<0.0001)	1.76 (0.21)

Bold letters denote significant ($p \leq 0.05$) differences.

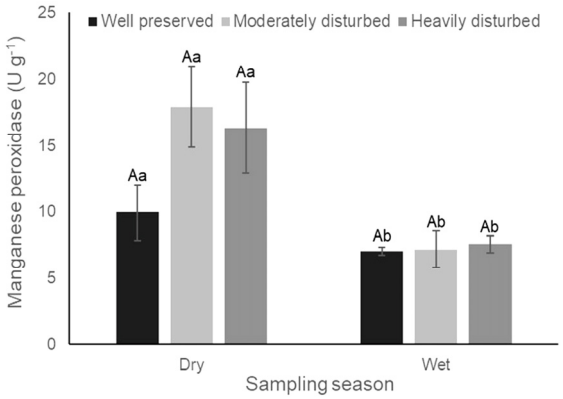


(**a**)

Figure 1. *Cont.*



(**b**)



(**c**)

Figure 1. Enzyme activities in litter samples from three forest sites with increasing levels of disturbance in central Mexico by sampling date. Enzyme activities for laccase (**a**), lignin peroxidase (**b**), and manganese peroxidase (**c**) are shown by site and season. Capital letters denote significant differences between sites; lowercase letters denote significant differences between sampling dates ($p \leq 0.05$).

3.3. Analysis of Fungal Communities

A total of 1006 sequences of the fungal ITS region were obtained over the two sampling periods: 502 during the dry season (175 from the WP site, 170 from the MD site, and 157 from the HD site; coverage values of 0.811, 0.823, and 0.892, respectively), and 504 during the rainy season (163 from the WP site, 173 from the MD site, and 168 from the HD site; coverage values of 0.816, 0.890, and 0.887, respectively). The coverage value of each library above 0.8 suggests that they represented the major fungal phyla present in the litter samples. In congruence with the coverage values obtained, rarefaction curves showed a tendency to slow their increase as the sampling effort increased (Figure 2).

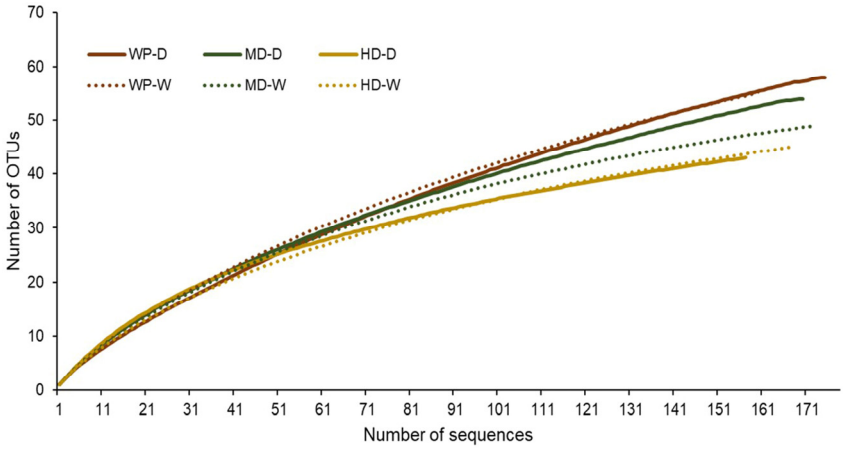


Figure 2. Rarefaction curves of a number of fungal OTUs found in samples of *Quercus deserticola* litter from forest sites with increasing levels of disturbance (well preserved: WP; moderately disturbed: MD; heavily disturbed: HD) in central Mexico in the dry (D) season and rainy (W) season.

When all samples were examined together, we observed a decrease in OTU richness with an increasing degree of disturbance: regardless of sampling date and using a 97% similarity threshold, the WP and MD sites showed a similar richness with 95 and 93 OTUs, respectively, while 80 OTUs were identified in the HD site (Table 3). When sequences were examined for each sampling date separately, in the dry season, the WP site showed the highest richness with 58 OTUs, followed by the MD site with 54 OTUs, and the HD site with 43 OTUs. For the rainy season samples, 56 OTUs were identified in the WP site, and 49 and 45 OTUs in the MD and HD sites, respectively. For both sampling dates, both diversity indices (Shannon-Weaver and Simpson) ranked the diversity of sites in the following order (from highest to lowest): WP, MD, and HD (Table 3). This ranking is also in agreement with the number of singletons identified for each site. In all three sites, the Chao1 index showed that the observed richness was lower than the estimated richness, but that a greater proportion of the estimated species had been found in the HD site and the MD site in the rainy season.

Table 3. Number of fungal OTUs, singletons, and diversity indices for three forest sites with increasing levels of disturbance in central Mexico in two contrasting seasons.

	Well Preserved (WP)		**Moderately Disturbed (MD)**		**Heavily Disturbed (HD)**	
	Dry	**Rainy**	**Dry**	**Rainy**	**Dry**	**Rainy**
No. of OTUs	58	56	54	49	43	45
No. of singletons	33	30	30	19	18	19
Sequences per group						
Ascomycota	145	116	113	124	83	133
Basidiomycota	17	29	21	37	25	28
Unidentified	16	19	39	12	49	7
Shannon-Weaver (*H'*)	3.39	3.51	3.16	3.24	3.16	3.12
Simpson (1-D)	0.96	0.93	0.94	0.93	0.90	0.92
Chao1	100	103	98	61	60	60

Independently of the abundance, some taxa were shared between sites: the WP site shared 15 taxa with the MD site and three taxa with the HD site, whereas the MD site shared 20 taxa with the HD site. Despite the similar number of OTUs identified in the WP and MD sites, there were differences in the order of the most abundant taxa at each site and in the abundance of shared taxa.

The majority of OTUs belonged to the phylum Ascomycota (71%) followed by Basidiomycota (16%); the remaining 13% were allocated to unidentified non-cultivated fungi. This pattern of many

more OTUs belonging to Ascomycota than to Basidiomycota was observed in the three sites and in both sampling dates (Table 3). In general, the most abundant taxa orders in the WP site were Capnodiales (18%), Hypocreales (17%), and Pleosporales (16%), whereas in the MD site they were Pleosporales (19%), Capnodiales (13%), and Agaricales (6%), and in the HD site they were Pleosporales (25%), Capnodiales (13%), and Thelephorales (6%). Taking sampling season into account, at the WP site, Hypocreales (30%) and Capnodiales (27%) were the most abundant orders in the dry season, while Pleosporales (23%) and Thelephorales (10%) dominated in the rainy season (Figure 3). In the MD site, Capnodiales (25%) and Hypocreales (7%) dominated in the dry season, and Pleosporales (34%) and Thelephorales (8%) were important in the rainy season. Finally, in the HD site, Capnodiales (23%) and Sordariales (13%) were dominant in the dry season, while Pleosporales (40%) and Tubeufiales (8%) dominated in the rainy season (Figure 3).

The Jaccard index was used to evaluate the similarity between sites in terms of fungal community composition (Table 4). The results revealed significant changes in the composition of the litter fungal community of a given site between the dry and rainy seasons. The WP site showed the highest between-dates similarity (J = 0.253) and the other two sites showed even larger differences between sampling dates, with Jaccard index values of 0.102 and 0.099 for the MD and HD sites, respectively.

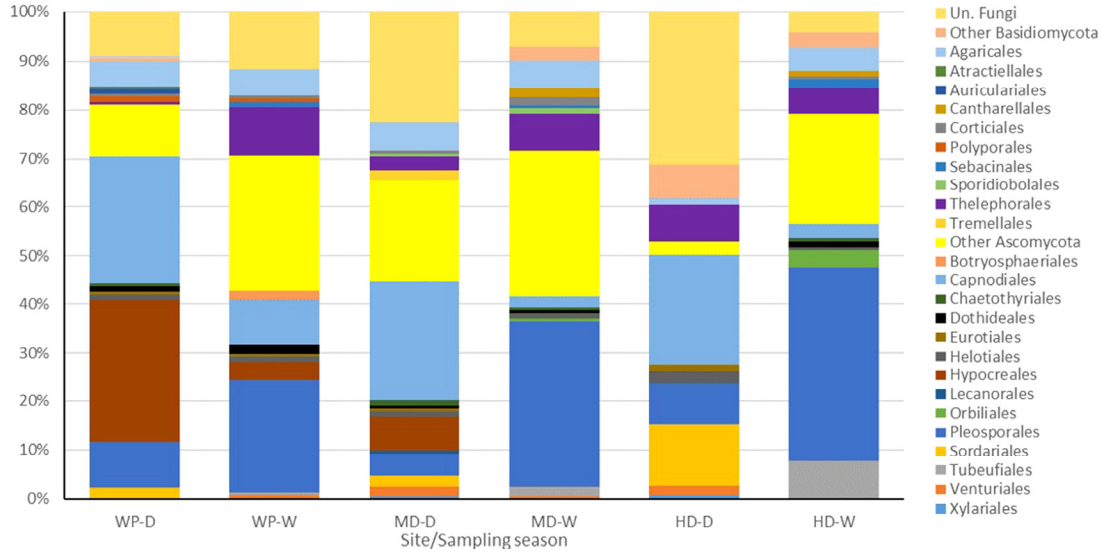


Figure 3. Abundance of different fungi orders in litter samples from forest sites with different levels of disturbance (well preserved: WP; moderately disturbed: MD; heavily disturbed: HD) in central Mexico in two contrasting seasons (dry season: D; rainy season: W). Un. = unidentified.

Table 4. Jaccard similarity index of fungal OTUs in forest sites with different levels of disturbance (well preserved: WP; moderately disturbed: MD; heavily disturbed: HD) in central Mexico in two contrasting seasons (dry season: D; rainy season: W).

	WP-W	**MD-D**	**MD-W**	**HD-D**	**HD-W**
WP-D	0.253	0.352	0.099	0.296	0.104
WP-W		0.160	0.301	0.133	0.304
MD-D			0.102	0.308	0.096
MD-W				0.106	0.667
HD-D					0.099

WP, well preserved site; MD, moderately disturbed site; HD, heavily disturbed site; D, dry season; and W, rainy season.

Although, in general, Basidiomycota were not among the most abundant taxa in the studied sites, the comparison of structural changes among communities is interesting because members of this

group are the main producers of laccase and peroxidases. The main orders of Basidiomycetes found in the three study sites were Thelephorales and Agaricales, but their abundance was different between study sites and sampling seasons. The abundance of Thelephorales increased during the transition between the dry season to the wet season in WP and MD sites from 2% to 35% and from 9% to 22%, respectively; in the HD site, the abundance of these taxa decreased from 23% in the dry season to 17% in the wet season. In the case of Agaricales, WP and MD sites maintained the abundance of this order between sampling dates (20% and 17%, respectively), but the abundance of Agaricales in the HD site increased from 4% in the dry season to 15% in the wet season. The orders Polyporales, Auriculariales, and Atractiellales were only found in the WP site, whereas the Tremellales and Sporidiobolales were only observed in the MD site. The HD site did not present an exclusive order, but there was a greater abundance of unidentified Basidiomycetes. The PCoA of the sequences obtained showed a clear separation between the dry and rainy season samples along the first component, which accounted for 35% of the total variance. Component 2 separated the WP site from the MD and HD sites on both sampling dates (Figure 4).

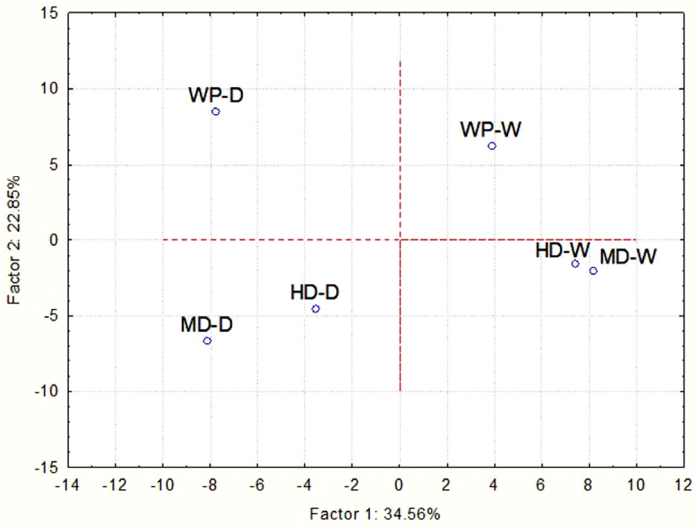


Figure 4. Principal coordinates analyses showing the relationships between fungal communities from forest sites with increasing levels of disturbance (well preserved: WP; moderately disturbed: MD; heavily disturbed: HD) in central Mexico in two contrasting seasons (dry season: D; rainy season: W).

4. Discussion

In this work, we compared the ligninolytic enzyme activities and the overall fungal richness of the litter community from three forest fragments dominated by *Quercus deserticola* with different disturbance levels and in two contrasting seasons (dry and rainy) in central Mexico.

The hypothesis of the C:N ratio being the major driver of litter decomposition in forest ecosystems [57] has been recently questioned [58]. Litter C:N ratios in the studied sites were similar to those reported for the litter from other oak species, including *Q. ilex* L. [58], *Q. petraea* (Matt.) Liebl. [59], and *Q. serrata* Thunb. ex Murray after one year of decomposition [60]. Most sites in this study had similar litter and soil nutrient contents and ratios, thus suggesting that decomposition rates might be similar between them. However, the slightly greater N in the soil of the well preserved site could promote differences in the litter decomposition rate in relation to the disturbed sites. It has recently been proposed that the decomposition rate in N-poor litter will increase when lying over N-rich soil, due to the translocation of N from soil to litter [58].

Each site had plant species characteristic of its disturbance status, thus creating different mixes of litter, which, due to differences in N availability, labile/recalcitrant C sources, and secondary metabolites [61], is a determining factor influencing the fungal litter community structure. Comparative

decomposition analysis involving oak species shows contrasting results; whereas some studies document that *Quercus* spp. litter has slower decomposition rates than the litter of other plant species [62], and some authors found higher decomposition rates in oaks [63,64]. *Quercus* spp. litter recalcitrance has been attributed to high tannin and phenol concentrations, a poorer quality of long-lived leaves, and sclerophyllous leaf properties [62]. However, synergistic interactions in mixed litter could translocate nutrients from the labile to the recalcitrant substrates in the mix [61]. In this regard, the litter of *Fraxinus uhdei* (Wenz.) Lingelsh is considered to be of high quality due to its high nutrient concentrations and low concentrations of lignin and soluble polyphenols, and because it harbors a microbial community that produces fewer enzymes involved in N and P acquisition and more enzymes involved in cellulose degradation [65]. Thus, *F. uhdei* in the well preserved site should provide resources to cellulo-hydrolytic Ascomycota that provide labile nutrients to sustain the litter decomposition of recalcitrant components. It has also been documented that the litter of *E. polystachya*—a woody plant species found in both disturbed sites studied—has a C:N ratio of 15.1, but its decay rate is slow compared to that of other tree species, with decomposition rates thought to be associated with microenvironmental conditions under its canopy, as well as with its lignin and polyphenol content [66]. These conditions suggest that plant species associated with the well preserved site might increase litter decomposition rates and that plants associated with the disturbed sites might slow it down; a possibility that needs to be further evaluated. To the best of our knowledge, there are no studies documenting litter ligninolytic enzyme activities of fungal communities related to *F. uhdei* and *E. polystachya*—or of other plants species associated with *Q. deserticola* in the studied sites, which requires further study linking mycobiota, enzyme activities, and nutrient cycles in litter.

Laccase activity in *Q. deserticola* litter samples was higher during the rainy than during the dry season, while peroxidase (LiP and MnP) activities showed the opposite pattern. In *Q. petraea* litter, Lac showed low activity in spring (May) and high activity levels in summer (July), while MnP exhibited a higher activity in spring than in summer [67]. A similar trend in Lac activity was observed for *Q. ilex* litter, but not for MnP [68]; however, for this same oak species, Kellner et al. [69] reported the higher MnP activity in litter subject to drought relative to control samples. Thus, our results are in agreement with previous findings of seasonal activity patterns of ligninolytic enzyme activities in oak forests. Changes in the activity of lignocellulolytic enzymes in forest soil and litter are correlated with environmental factors, including temperature, moisture, and pH [70]. However, Criquet et al. [68] found no correlation between the activity of Lac and MnP and abiotic factors such as temperature, humidity, and pH in *Q. ilex* litter. Thus, besides physical and climatic variables, chemical changes in litter composition [68], variations in fungal biomass [71], and changes in fungal community composition [72] can explain the changes in enzyme activity patterns. The lack of differences in enzyme activities between the sites studied could be attributed to functional redundancy in fungal communities: different species being capable of producing ligninolytic enzymes in different ecological contexts [73]. Recently, soil microbial community redundancy—also called functional convergence—has been described between under-canopy and open areas of *Q. ilex* forest fragments harboring different bacterial and fungal communities, but showing similar metabolic patterns [32]. Thus, it is quite possible that the functional redundancy of basidiomycetes could explain the similar ligninolytic activities found in the *Q. deserticola* litter along a disturbance gradient (see below).

The fungal community we described is highly consistent with previously studied fungal litter communities at different taxonomic levels. In our study, 70% of the sequences were associated with the phylum Ascomycota, 15% were Basidiomycota, and 15% remained as unidentified fungi. A small percentage (0.2%) of the sequences showed a relation with the former phylum Zygomycota, now invalid; however, because it was not possible to know if these sequences were within Glomeromycota or Mucoromycotina, we decided to group them with unidentified fungi. In a previous analysis conducted by the authors, we found the same proportions of Ascomycota and Basidiomycota [24], supporting the representativeness of our present data. These abundances agree with the relative percentages reported in previous investigations of mixed hardwood litter in which the

Ascomycota accounted for 62–85%, the Basidiomycota for 15–36%, and the Glomeromycota for up to 2% of the total fungal community [74]. In *Q. petraea* litter, the composition was different, with Ascomycota representing 42% and Basidiomycota 48% of the fungal community [67], the lowest abundances corresponding to Glomeromycota and Mucoromycotina. *Gibberella*, *Teratosphaeria*, and *Cladosporium* were among the most abundant genera during the dry season. Such results are consistent with previous findings for fungi colonizing *Quercus* spp. leaves and litter. *Cladosporium* spp. have been described as phyllosphere fungi in the early stages of litter decomposition in *Q. leucotrichophora* A. Camus [75], *Q. rotundifolia* Lam. [76], and *Q. petraea* [72] forests. *Teratosphaeria* species have been found in the phyllosphere of *Q. petraea* [72] and anamorphs of *Gibberella* (*Fusarium*) species have been isolated from senescent and early decomposition stages of *Q. rotundifolia* litter [76]; however, *Fusarium* has been found as a later colonizer of *Q. myrsinaefolia* Blume litter [77]. *Phoma* and *Pyrenochaeta* were the most abundant groups in the rainy season, with the former noted as phyllosphere fungi in *Quercus* spp. [72,77] and occurring in the early stages of litter decomposition [75,77]. All these genera within Ascomycota contribute as producers of hydrolytic enzymes acting on plant cell wall polysaccharides [78,79].

The fungal taxa associated with the degradation of lignin in litter are within Basidiomycota. Among the taxa of Basidiomycota we found in the studied sites, members of the family Thelephoraceae—particularly *Tomentella*—were the most abundant OTUs in rainy-season samples. Our previous analysis of litter of *Q. deserticola* found *Tomentella* sp. to be a prevalent taxon [24], which shows a wide distribution in *Q. deserticola* forests. Interestingly, it has been documented that *Tomentella sublilacina* forming ectomycorrhizal (ECM) associations with *Q. robur* L. increased the hydrolytic and laccase enzyme activities in a thinned tree stand, but not so in a disturbed stand of the same tree species [80]; this shows both that appropriate management will conserve fungal diversity and function, and that some of the fungal-plant biotrophic interactions might be used as indicators of forest performance.

Regarding the orders of Basidiomycota sampled here, genomic phylogenetic analysis has shown that white rot Auriculariales and Polyporales possess high numbers of MnP and LiP genes, Agaricales retained low gene numbers, and Tremellales, Atractiellales, and Sporidiobolales have lost the genes coding such enzymes [81]. Thus, our present results show that the litter of well preserved *Q. deserticola* forest sustains the highest diversity of well-identified ligninolytic fungi, mainly of the strongest lignin degraders. In the previous study conducted in *Q. deserticola* litter [24], Corticiales and Thelephorales were the only orders of Basidiomycota found, indicating the representativeness of the samples herein analyzed.

Despite the differences we observed in the structure of the ligninolytic fungal community, we found no differences in the enzyme activities of Lac and peroxidases among the three studied sites. It must be taken into account that both Agaricales and Thelephorales were found along the forest degradation gradient we evaluated, and that unidentified basidiomycetes were only found in the disturbed site. Although many members of the two former taxa are considered ECM, they may be contributing, along with the unidentified taxa, to sustain ligninolytic activities in the litter of disturbed sites. Recent genomic evidence shows that certain ECM taxa have retained genes for Lac and MnP enzymes from their saprotrophic ancestors [82,83]. However, it has been postulated that ECM fungi are not facultative saprotrophs using lignin as the principal source of metabolic C, but use the conservation of Lac and MnP activities for mobilizing N locked up in non-hydrolysable, recalcitrant organic matter complexes [84]. Despite this, the ligninolytic activity of ECM fungi might play a central role in the turnover and stabilization of organic matter, influencing the C and N dynamics of temperate forest ecosystems [83,84]. Thus, it is possible that ECM fungi replaced saprobic guilds in the perturbed forest sites, something that deserves detailed assessment in future studies.

We found that the fungal community of *Quercus deserticola* litter was influenced both by the sampling date and by the degree of forest disturbance. Seasonal changes of whole fungal soil and litter communities have been documented in *Quercus* spp. forests [67,74]. The composition of the fungal soil community in pine-oak and oak–hickory stands was found to be closely associated with changes

in soil nutrient status and specific changes in edaphic properties might explain the observed shifts in the microbial community [85]. Thus, previously noted seasonal changes in N content and the C:N ratio of the *Q. deserticola* litter partially explain seasonal changes in the fungal community structure. As stated above, the highest richness of fungal OTUs was found in the well preserved site, suggesting that conservation status affects the diversity of the fungal community in *Q. deserticola* litter.

Historically, pine-oak forest fragmentation in the state of Michoacán has been associated with agricultural expansion, grazing, and logging for wood and charcoal extraction [34–36], as is the case of the studied area. It has been previously documented that *Quercus* spp. forests management practices [62] and fragmentation cause changes in soil and litter fungal community structure [28]. On one side, in a *Q. ilex* forest, Richard et al. [86] documented a significant correlation between the species richness of macroscopic saprobic and ECM fungi and tree density. On the other side, Azul et al. [27,87] found that logging, soil tillage, and permanent grazing reduced the macroscopic (mainly ECM) fungal community in *Q. suber* ecosystems.

In the context of the socioeconomic needs of rural communities in developing countries like Mexico, a balance must be found between practices of forest use and conservation so that biodiversity is conserved while the energy and food requirements of the population are fulfilled [88]. In the state of Michoacán, the compromise between the use and conservation of forests has been analyzed in indigenous Purépecha artisanal and peasant rural communities [35]. However, forest management promoting conservation has largely ignored litter and fungi as vital components of forest functioning. Appropriate stand management of *Quercus* spp. forests—such as logging without soil tillage and grazing—has been documented to preserve fungal diversity [27,87]. Furthermore, within the studied area, models for sustainable charcoal extraction have been developed [36]. Additionally, our present results and those from previous work show that some of the orders and genera of fungi present in the *Quercus* spp. litter remain constant despite geographical distance and differences in climatic conditions. The data increases our understanding of the geographical range of fungi associated with *Quercus* spp. litter, and will enable the generation of successional models and increase our understanding of fungal community responses to management, restoration, and climatic change [89].

Further studies are needed to assess the interactions between environment and land use variables affecting the *Q. deserticola* litter fungal community. Knowledge derived from such studies will prove useful for designing better management practices so that the socioeconomic demands of the rural population are satisfied, in addition to preserving the functions and biodiversity of the forest ecosystem [31].

5. Conclusions

The fungal community structure and the decomposition process of *Q. deserticola* litter in Mexican forests are highly dynamic. The activity of three major ligninolytic enzymes showed similarities among them. Therefore, in the near future, it might be possible to formulate a model of fungal succession and enzyme dynamics in oak forest litter, as has been achieved by similar studies in different geographic areas. Such models can guide the formulation of appropriate management and conservation strategies for oak forests. In particular, further studies are needed to better understand the interactions taking place in *Q. deserticola* forests between forest management, litter chemistry, seasonal changes, the composition of fungal communities, and their enzyme activities.

Supplementary Materials: The following are available online at www.mdpi.com/1999-4907/9/1/11/s1, Table S1: Vegetation found in the study plots used as indicator of conservation or disturbance.

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R.A.-R. aided in selection and characterization of studied plots, and identified and described plant species in the plots; F.G.-O. performed litter nutrients determination and analyzed the data; M.S.V.-G. contributed reagents, materials, analysis tools, and ITS library construction; K.O., G.V.-M., and F.G.-O. wrote the paper.

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