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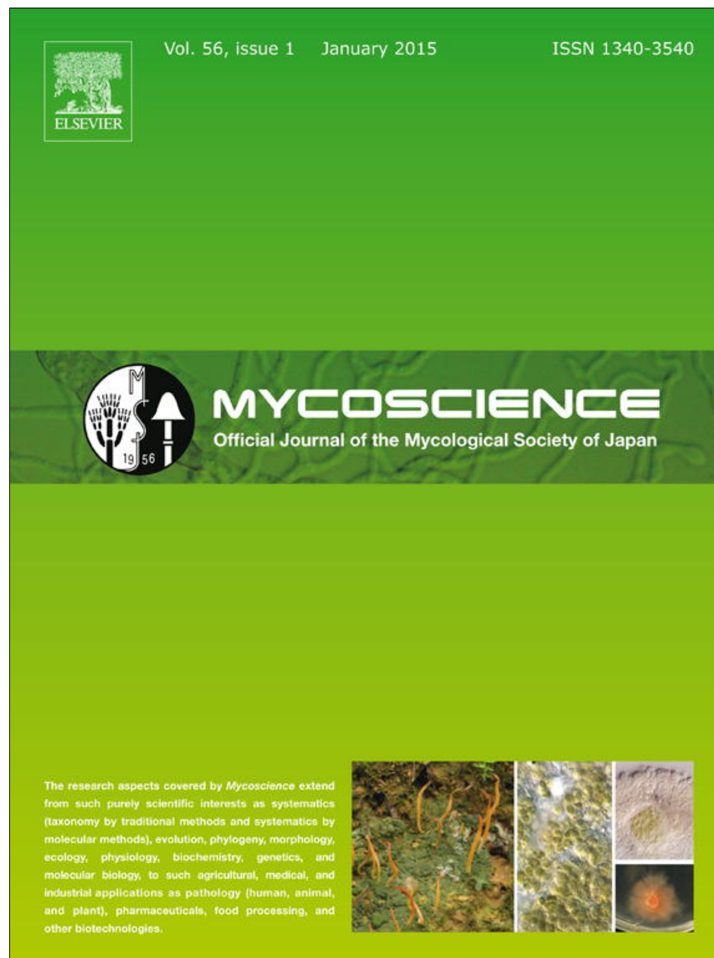

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Full paper

Growth and oxidative enzymatic activity of in-vitro cultures of *Ciliochorella buxifolia*

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ABSTRACT

To get a better insight into the physiological capabilities of *Ciliochorella buxifolia*, the most frequent fungus occurring on *Scutia buxifolia* leaf-litter in a native forest from Argentina, its in-vitro ability to use 10 carbon sources and to produce extracellular enzymes, including its response to tannic acid and to the addition of a water-soluble fraction of *Scutia buxifolia* leaf-litter, was analyzed. Growth, colony morphology and extracellular enzyme activity as well as differentiation of pycnidia were a function of the C substrate. The fungus responded to the presence of tannic acid in a range between 0.001 and 0.1% (w/v), by increasing growth, but higher phenol concentrations like 0.5% were inhibitory. The activity of extracellular oxidative enzymes increased with the concentration of tannic acid. Furthermore, the fungus showed extracellular laccase and peroxidase activity, being the former increased by water-soluble fraction in association to pycnidia development. Based on these results, *Ciliochorella buxifolia* is a fungus growing on *Scutia buxifolia* leaf-litter that is able to metabolize soluble phenolic compounds, which triggers the synthesis of extracellular oxidative enzymes possibly involved in sporulation and detoxification reactions.

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1. Introduction

One of major pools of organic matter in forest soils is leaf-litter, which is recycled by microorganisms and becomes an enormous source of nutrients, regulating, in this way, forest productivity (Osono 2007; Valášková et al. 2007; Wurzburger and Hendrick 2007). Although the soil microflora, as a whole, is involved in organic matter transformation, fungi play a major role in mineralization, mainly during the early stages of decay (Dighton 2003; Lensing and Wise 2007; Paul 2007; Valásková et al. 2007; Osono et al. 2008). However, richness, abundance and activity of litter-degrading fungi are a function of substrate quality, as well as of their competitive and reproductive ability, which are additionally modified by abiotic and biotic interactions (Cooke and Rayner 1984; Coleman and Whitman 2005; Hättenschwiler et al. 2005).

Allegrucci et al. (2005, 2007) and Cabello and Arambarri (2002) as well as Elíades et al. (2010, 2011) found a complex assemblage of fungi associated with leaf-litter of *Scutia buxifolia* Reiss (Rhamnaceae, Rhamnales). This perennial tree grows in a native temperate forest of Argentina that is characterized by alkaline calcareous (Rendolls) soils (Arturi et al. 1996). The debris from this tree, which is of a dark-brown color, is recalcitrant to degradation due to its content in polyphenols such as tannins (1.1%) and lignin (41.7%; Saparrat et al. 2008, 2010). This conditions the colonization and abundance of fungi (Saparrat et al. 2007b, 2008, 2010; Allegrucci et al. 2011). Among them, *Ciliochorella buxifolia* is the only fungus that grows exclusively in *S. buxifolia* leaf-litter and because of this, is the more frequent one (Allegrucci et al. 2005, 2007; Elíades et al. 2010). While analyzing the role of *C. buxifolia* LPSC 847 on *S. buxifolia* leaf-litter at the early stages of degradation we found that the fungus had a low saprotrophic ability on cell wall polymers, low levels of β-1,4 endoglucanase activity and lack of oxidative enzymes related to lignin degradation (Saparrat et al. 2010), unless the fungus was cultured in liquid medium (Troncozo et al. 2008).

These findings raised some questions about the eco-physiological characteristics of *C. buxifolia*, which might be related to degradation of *S. buxifolia* leaf-litter. The working hypotheses were: 1. *Ciliochorella buxifolia* has higher saprotrophic ability on soluble phenolic compounds than on other C sources, which leads to the specific colonization and high frequency on *S. buxifolia* leaf-litter. 2. Growth of this fungus is modulated by tannin. 3. Soluble phenolic compounds like those released by *S. buxifolia* leaf-litter in water induce in *C. buxifolia* the synthesis of laccases and peroxidases. Therefore, the aim of this work was to know further about the physiological capabilities that allow this fungus to use C sources that might be or are released by leaf-litter and to produce extracellular degrading enzymes. Furthermore, the response of the fungus to tannic acid, a model phenolic compound, and to the addition of a water-soluble fraction (WSF) of *S. buxifolia* leaf-litter, on its growth and the synthesis of extracellular oxidative enzymes, also was analyzed.

2. Materials and methods

2.1. Fungal isolate and inoculum source

Ciliochorella buxifolia LPSC (Culture collection of the La Plata Spegazzini Institute) 847 strain is a type specimen. It was isolated from leaf-litter of *S. buxifolia* of a natural dry forest at the Biosphere Reserve "Parque Costero del Sur" (MABUNESCO), located in eastern Buenos Aires province, Argentina (35°11′S, 57°17′W; Allegrucci et al. 2011). Stock cultures were maintained as slants on malt extract agar (MEA) medium at 4 °C.

2.2. Carbon substrates as sources for fungal growth

The utilization of organic substrates as the sole C source in association to the enzyme's activity involved in their degradation was evaluated by cultivating the fungus on a basal agar (2%, w/v) mineral salts medium (5 g $NH_4H_2PO_4$, 2.5 g K_2HPO_4,

Table 1 – Organic compounds used in this study, their concentration and fungal enzymes involved in their degradation.

C substrate type[a]	Organic compound	Concentration (%, w/v)	Major enzymes involved in degradation
Readily available C	Glucose	1	Hexokinase (EC 2.7.1.1)
	Sucrose	1	Invertase (EC 3.2.1.26)
Moderately recalcitrant ('slow') C	Apple-pectin	0.1	Pectin lyase (EC 4.2.2.10) and polygalacturonase (EC 3.2.1.15)
	Birch-wood xylan	0.2	1,4-β-Xylan xylanohydrolase (EC 3.2.1.8)
	Soluble starch	1	α-Amylase (EC 3.2.1.1)
Highly recalcitrant C	Gallic acid	0.05	Catechol oxidase (EC 1.10.3.1), laccase (EC 1.10.3.2) and monophenol monooxygenase (EC 1.14.18.1)
	Humic acid	0.05	Manganese peroxidase (EC 1.11.1.13)
	Indulin-AT (Kraft lignin)	1	Laccase (EC 1.10.3.2), lignin peroxidase (EC 1.11.1.14), manganese peroxidase (EC 1.11.1.13) and versatile peroxidase (EC 1.11.1.16)
	Sodium-carboxy-methylcellulose (CMC)	0.5	Endo-(1,4)-β-d-glucanase (EC 3.2.1.4)
	Tannic acid	0.05	Catechol oxidase (EC 1.10.3.1), monophenol monooxygenase (EC 1.14.18.1) and tannin acyl hydrolase (EC 3.1.1.20)

[a] Based on its degree of degradability due to their molecular weight, structure, and the need for enzymatic digestion prior to assimilation (Cooke and Whipps 1993; Mondini et al. 2006; Goldfarb et al. 2011; Hoeksema and Classen 2012).

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1 g $MgSO_4 \cdot 7H_2O$, 20 mg $Ca(NO_3)_2 \cdot 4H_2O$, 2 mg $FeCl_3 \cdot 6H_2O$, 0.5 mg H_3BO_3, 0.1 mg $CuSO_4 \cdot 5H_2O$, 0.1 mg KI, 0.4 mg $MnSO_4 \cdot 5H_2O$, 0.4 mg $ZnSO_4 \cdot 7H_2O$, 0.2 mg Na_2MoO_4 and 0.1 mg $CoCl_3$ per liter of deionized water; Kreisel and Schauer 1987) supplemented with a known concentration of 10 organic compounds that differ in their resistance to degradation (Table 1). Plates filled with the described medium and without C amendment (control) were inoculated with a 6-mm diameter mycelial plug of a culture grown on MEA medium at 25 ± 2 °C for 7 days and were incubated in the dark at 25 ± 2 °C for 21 days (Saparrat et al. 2008). Growth was estimated by measuring the diameter of the colonies and the rate of growth (K_r) was calculated according to Baldrian and Gabriel (2002). Colony morphology was estimated according to Elíades et al. (2010). The developmental stage of colonies was characterized by the presence/absence of reproductive structures (pycnidia). Both morphology and the developmental stage of colonies were associated with the C substrate, which was analyzed by contingency tables. Extracellular amilolytic, cellulolytic, pectinolytic and xylanolytic enzyme activity of the fungus was evaluated according to the clearing-halo method on media supplemented with the desired substrate (Magnelli and Forchiassin 1999). Extracellular ligninolytic enzyme activity was determined by the appearance of a chromophore halo below colonies of the fungus grown on medium with indulin-AT for 21 days (Fisher et al. 1983). Oxidative activity was determined by the appearance of a chromophore halo on media supplemented with both gallic and tannic acid (Davidson et al. 1938; Saparrat and Hammer 2006). All enzyme activities were expressed as the ratio of clearing or chromophore halo to colony diameter (Choi et al. 2005). The experimental design was completely at random and the number of replicates per treatment was five plates. A one-way ANOVA and Tukey's test ($P \leq 0.01$) were used to contrast means of treatments.

2.3. *Growth and extracellular oxidative activity under a range of tannic acid concentration*

Growth and extracellular oxidative activity was determined on a basal glucose agar medium (20 g agar, 10 g glucose, 2 g $(NH_4)_3C_4H_4O_6$, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 1 g yeast extract (Difco), 0.1 mg $Na_2B_4O_7 \cdot 10H_2O$, 0.07 mg $ZnSO_4 \cdot 7H_2O$, 0.05 mg $FeSO_4 \cdot 7H_2O$, 0.01 mg $CuSO_4 \cdot 5H_2O$, 0.01 mg $MnSO_4 \cdot 4H_2O$ and 0.01 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ per liter of deionized water; Saparrat et al. 2002) amended at five concentrations of tannic acid (0.0001, 0.01, 0.05, 0.1, and 0.5% w/v). Plates were inoculated as described before. Five replicates per treatment, including control plates free of tannic acid, were incubated in the dark at 25 ± 2 °C for 14 days. Growth was estimated by measuring the diameter of the colonies grown on the basal medium and in the presence of known concentrations of tannic acid for 7 and 14 days (Saparrat et al. 2007a). Extracellular oxidative activity was determined and expressed as described before. The intensity of the dark-brown-colored halo around colonies generated by the oxidative reaction also was expressed by means of a relative scale (Davidson et al. 1938). Data were analyzed by means of an ANOVA and means of the treatments were contrasted by the Tukey Test ($P \leq 0.01$).

2.4. *Effects of amendment of water-soluble fraction on growth and extracellular oxidative enzyme activity*

A WSF was obtained by adding 50 ml of distilled water to 10 g of *S. buxifolia* leaf-litter, which was heated at 121 °C for 20 min.

Table 2 – Growth, colony morphology, enzyme activity and differentiation of pycnidia by *Ciliochorella buxifolia* on a basal agar-mineral salts medium supplemented with several C sources.

Treatment	K_r[a] (mm/day)	Colony morphology	Reproductive structures	Ratio halo to growth
Basal medium	1.97 (0.98)[b] a[c]	+[e]	1[f]	–[g]
+Labile C				
Glucose	0.27 (0.84) cd	+++	1	–
Sucrose	1.31 (0.93) ab	+++	1	–
+Moderately recalcitrant C				
Apple-pectin	*[d]			
Birch-wood xylan	0.45 (0.95) cd	++	2	6.49 ± 0.49[h] a[c]
Soluble starch	0.73 (0.92) bc	++	2	4.36 ± 1.73 ab
+Highly recalcitrant C				
Gallic acid	0.37 (0.91) cd	++	2	6.99 ± 1.98 a
Humic acid	1.75 (0.94) a	++	2	nd[i]
Indulin-AT	0.79 (0.97) b	+	1	nd
CMC	0.54 (0.84) bd	++	2	4.90 ± 1.14 a
Tannic acid	2.41 (0.97) a	++	2	1.65 ± 0.20 b

[a] Radial increase rate.
[b] Correlation coefficient.
[c] Data followed by the same letter are not significantly different (Tukey test, $P < 0.01$).
[d] No growth.
[e] Colony morphology rating: +, poorly visible inconspicuous mycelium; ++, conspicuous mycelium; +++, mycelial mats.
[f] Pycnidia: 1, absent; 2, present.
[g] Not tested.
[h] Mean ± S.D. of five replicates.
[i] Not detected.

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Table 3 – Contingency table (3 × 3)[a] for the association between morphology of colonies of *Ciliochorella buxifolia* and the C substrate type.

		Colony morphology			
		Inconspicuous mycelium	Conspicuous mycelium	Mycelial mats	Total
C substrate type	Readily available C	0[b]	0	10	10
	Moderately recalcitrant C	0	10	0	10
	Highly recalcitrant C	5	20	0	25
	Total	5	30	10	45

[a] χ^2 (0.01, 4) = 48.36.
[b] Observed frequency.

Then the suspension was centrifuged at 10,000 *g* for 30 min and the resulting supernatant was filtered through a 0.2 μm pore membrane and analyzed for pH (5.34) and phenolic compounds (0.04 g/100 ml) according to the Folin–Ciocalteu method (Osono and Takeda 2001). The WSF was conserved at 4 °C until use.

The fungus was grown at 150 rev/min and 25 ± 1.5 °C for 17 days in the basal agar free glucose medium (BM; see Section 2.3), which was supplemented with the WSF at 0.5% (v/v), after 3 days of incubation. Controls lacking WSF were incubated under identical conditions. Homogenized pellets from 7-day-old BM-cultures were used as inoculum (Saparrat et al. 2002). Six replicate cultures were carried out for each treatment (BM and BM + WSF). Laccase (EC 1.10.3.2) and peroxidase (EC 1.11.1.7) activity was analyzed on aliquots of the supernatants collected at different time intervals (Saparrat et al. 2007a). Pearson's correlation coefficients were calculated for linear relationships between laccase and peroxidase activities from cultures on BM and BM + WSF (Sokal and Rolf 1995). Seventeen days after inoculation the mycelium was pelleted by centrifugation at 20,000 *g* for 10 min at 4 °C, dried overnight at 90 °C and weighed to determine biomass (mg/100 ml). The biomass data were analyzed by means of a *T*-test ($P \leq 0.01$). Some pellets were stained by means of cotton blue and were observed in the microscope to look for the presence of reproductive structures (pycnidia and/or conidia).

3. Results

Growth, colony morphology and extracellular enzyme activity as well as differentiation of pycnidia by *C. buxifolia* were all affected by the C source provided in the culture medium (Table 2). While growth, measured as the increase of colony radius, was at a rate of 1.97 mm/day on basal medium, the addition of 6 C substrates resulted in a reduction of fungal growth rate ($F_{9,\ 174} = 60.78$, $P < 0.01$). Within readily available C sources like sucrose and glucose, the latter one provoked a more severe reduction. Also moderately recalcitrant C substrates such as starch and xylan as well as highly recalcitrant ones such as gallic acid, indulin-AT and CMC provoked a reduction in fungal colony size. Apple-pectin totally inhibited colony growth. Only two recalcitrant C compounds, humic acid and tannic acid, proved to have no effect on the rate of fungal growth. Except indulin-AT C substrates also provoked changes in the colony density as compared to the inconspicuous mycelium developed on basal medium. When the fungus was cultured on glucose or sucrose, colonies were made of a dense mycelium that gave them a mat appearance, while on moderately or highly recalcitrant C sources the mycelium was conspicuous (Table 3).

The development of reproductive structures by *C. buxifolia* also was dependent on the C source (Table 4). The fungus developed pycnidia only when CMC, gallic acid, humic acid, starch, tannic acid and xylan were added to the medium.

The ratio between enzyme activity halo and colony diameter is an index of extracellular enzyme activity. While the ratio between hydrolytic activity halo and colony diameter was similar when *C. buxifolia* was cultured in the presence of CMC, starch or xylan, the oxidative activity halo was larger in the presence of gallic acid than tannic acid ($P < 0.01$).

Tannic acid enhanced growth of *C. buxifolia* in-vitro within concentrations ranging from 0.001 to 0.1% (w/v), whether the incubation period was 7 ($F_{4,\ 20} = 40.13$, $P < 0.01$) or 14 ($F_{4,\ 20} = 52.33$, $P < 0.01$) days (Table 5). However, a concentration of 0.5% inhibited growth. The reaction-test of Davidson et al. (1938) showed that the extracellular oxidative enzyme activity turned the media to a brown color, which became more intense as the phenol concentration increased. Interestingly, the fungus responded differentially to tannic acid concentration after 7 ($F_{6,\ 13} = 137.09$, $P < 0.01$) and 14 ($F_{2,\ 12} = 179.15$, $P < 0.01$) days, increasing the ratio between the brown halo and the colony diameter over 0.05%. Although 0.5% of tannic acid inhibited growth of *C. buxifolia*, it induced high levels of extracellular oxidative activity after 14 days of incubation.

Fungal growth remained the same in liquid media supplemented or not with the WSF (*T*-test, $P > 0.05$), which was not the case of oxidative activity. Laccase and peroxidase activities were detected in supernatants from cultures grown in

Table 4 – Contingency table (2 × 3)[a] for the association between reproductive structures of *Ciliochorella buxifolia* and the C substrate type.

		Reproductive structures		
		Presence	Absent	Total
C substrate type	Readily available C	0[b]	10	10
	Moderately recalcitrant C	10	0	10
	Highly recalcitrant C	20	5	25
	Total	30	15	45

[a] χ^2(0.01, 2) = 27.19.
[b] Observed frequency.

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Table 5 – *Ciliochorella buxifolia* growth and enzyme activity in the presence of tannic acid after 7 and 14 days of incubation.

Tannic acid (%)	Effect on growth (%)[a,b]		Extracellular oxidative activity			
			Reaction-test[d]		Ratio halo to growth[b]	
	7	14	7	14	7	14
0.001	9.92 ± 4.71 b[c]	19.10 ± 4.20 bc	–	–	0.00 ± 0.00 c[c]	0.00 ± 0.00 c
0.01	16.53 ± 7.95 ab	23.60 ± 6.55 ac	+	–	0.33 ± 0.08 b	0.00 ± 0.00 c
0.05	29.75 ± 7.51 a	32.58 ± 6.55 a	++/+++	++	1.17 ± 0.12 a	0.83 ± 0.05 b
0.1	28.51 ± 3.06 a	28.54 ± 3.69 ab	++/+++	++	1.17 ± 0.09 a	0.82 ± 0.03 b
0.5	−9.47 ± 2.94 c	−9.79 ± 4.15 d	+++	+++	1.34 ± 0.02 a	1.19 ± 0.03 a

[a] Growth on increasing concentrations of tannic acid compared to that on basal medium (control) using the formula: [(diameter from cultures supplemented with a certain concentration of tannic acid – diameter from control cultures on basal medium)/diameter from control cultures] × 100.
[b] Mean ± S.D. of five replicates.
[c] Data for each incubation time followed by the same letter are not significantly different (Tukey test, $P < 0.01$).
[d] Relative scale: –, no brown coloration of medium; +, zone of the medium with a brown color beneath the inoculum plug that is visible on the reverse of the plate; ++, zone of the medium with a brown color beneath the most of the colony but not extending to its margin and that is visible on the reverse of the plate; +++, zone of the medium with a brown color which is extended to a short distance beyond the margin of the fungal colony and that is visible on the top of the plate.

both media (Fig. 1). On BM, laccase activity was detected after 6 days of incubation, peaked 10 days later and decreased thereafter. Peroxidase activity was detected after 13 days of incubation and increased thereafter. The addition of WSF of *S. buxifolia* leaf-litter had opposite effects on these enzymes activity. It altered laccase activity which was increased though its pattern along the incubation period remained the same. However, WSF provoked an earlier synthesis of peroxidases, whose activity remained at the same level compared to that detected on cultures grown in BM. Laccase activity was negatively correlated with that of peroxidases both on BM and BM + WSF, though such relationship became more negative when WSF of *S. buxifolia* leaf-litter was amended to BM ($R = -0.92$ on BM + WSF versus $R = -0.60$ on BM, $P < 0.01$). In addition to this, while on BM fungal cultures did not differentiate any stroma-like hyphal aggregation, they developed pycnidia on BM + WSF (data not shown).

4. Discussion

According to Osono and Takeda (2006) and Song et al. (2010) fungi that grow on litter may be divided into three functional groups, sugar fungi, cellulose decomposers and lignocellulose ones. Our results raised the question to which group does *C. buxifolia* belong to? While lignin degradation by white-rot basidiomycetes is known as a cometabolic process (Saparrat et al. 2008), many Ascomycota and their anamorphs use lignin as a sole C source and synthesize ligninolytic enzymes (Bi et al. 2012; Saparrat et al. 2013). This is not the case of *C. buxifolia* that had no ligninolytic activity on indulin-AT and developed an inconspicuous fine mycelium, which might be an ecological strategy of the fungus to survive when nutrient resources are scarce (Cooke and Rayner 1984; White and Boddy 1992).

In-vitro, *C. buxifolia* nurses itself from several C substrates other than indulin-AT. Therefore, the fungus might be either a cellulose decomposer or a sugar fungus according to categories defined by Osono and Takeda (2006) and Song et al. (2010). However, the cellulolytic ability of *C. buxifolia* was low compared to that of *Ulocladium botrytis* LPSC 813 (Saparrat et al. 2007b, 2010). Osono and Takeda (2001) attributed the reduced ability of several fungi to degrade certain types of leaf-litter to the barrier role of lignin. So the low saprotrophic ability of *C. buxifolia* might be related to the cellulose to lignin ratio and the cell wall architecture of the litter (Saparrat et al. 2008; Jurado et al. 2011). Therefore, probably the lignin content of *S. buxifolia* litter might be acting against degradation of cell wall

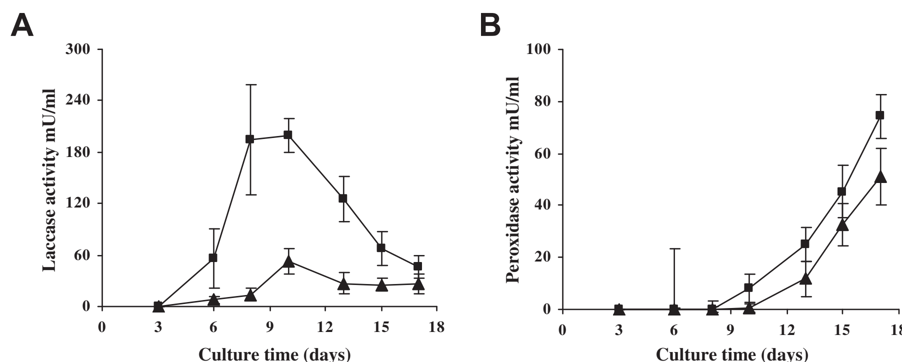

Fig. 1 – Time course of extracellular (A) laccase and (B) peroxidase activity of *Ciliochorella buxifolia* grown on basal medium (▲) and medium supplemented with WSF (■). Mean ± S.D. of six replicates were used.

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polysaccharides, preventing the fungus from feeding on many C sources other than soluble ones. This might be related to mat-like growth of the fungus, which only was developed in the presence of readily available sources of C such as glucose or sucrose. Furthermore, pectin inhibited growth of *C. buxifolia* even at low concentration, probably due to the matric effect of colloids (Kaufmann 1970; Panchev et al. 2010).

Based on the model of Berg and McClaugherty (2008), at the early stages of decay those fungi that use soluble compounds prevail mostly on fresh shed litter and are the primary colonizers that hardly have extracellular depolymerizing enzyme activity (Paul 2007). These fungi usually grow on low-molecular-mass compounds such as sugars, simple phenolics, hydrocarbons, and glycerides (Berg and McClaugherty 2008). *Ciliochorella buxifolia* had a reduced cellulolytic activity and grew in-vitro on media with soluble and low-molecular-mass compounds such as gallic acid, glucose, sucrose and tannic acid, which suggests a key role of this fungus in early degradation of the soluble fraction of shed litter that is driven at a high rate (Berg and McClaugherty 2008). However, our findings like those of Song et al. (2010) suggest no direct relationship between the frequency of isolation and the decomposing ability of fungi, particularly if they can use other substrates (McClaugherty 1983).

Ciliochorella buxifolia differentiated pycnidia only when the media had moderately to highly recalcitrant C sources. These compounds and/or the low metabolic activity of *C. buxifolia* on them might have triggered, as a survival strategy, sporulation, a process that might be initiated by a threshold concentration of signaling molecules and by the exhaustion of available resources (Friedl et al. 2008). Casas-Flores et al. (2006) found that carbohydrate metabolism, its response and/or the presence of certain C sources in the medium were intimately associated with fungal sporulation. Such relationship was found in *Trichoderma reesei*, where cellulose was a key modulator of the differentiation of pycnidia through a signaling pathway regulated by the C source sensed (Zhang et al. 2012). However, *C. buxifolia* was unable to obtain energy and nutrients (C) from indulin-AT and even though the fungus developed mycelia profusely in media with glucose or sucrose, these cultures hardly sporulated. So it appears that fungal sporulation is a complex event, where mycelium initiates a differentiation process only under define conditions that include medium components (Dahlberg and Etten 1982), being at least, in the case of *C. buxifolia*, conidiation not restricted to the lack of energy. Future studies should be aimed at clarifying conidiation induction in *C. buxifolia* and its physiological significance.

Our data and those from Saparrat et al. (2008, 2010) demonstrated that soluble compounds from *S. buxifolia* litter, including free phenolic ones, are metabolized by *C. buxifolia*, though probably at a rather slow rate, as sources of C and energy. However, this might lead either to a reduction in growth or to a change in growth pattern probably due to the reduced pool of enzymes (Bending and Read 1996). *Ciliochorella buxifolia* is unique, considering that it responded to low levels of phenolics such as tannic acid, by increasing growth. Therefore, depending upon the available concentration, on *C. buxifolia* tannic acid played different roles, like a source of C or an ecological allelochemical inhibitor of growth. This ability of *C. buxifolia* to tolerate and use phenolics, which regulates enzyme activity, might have implications in phenol-rich litter colonization (Saparrat et al. 2008, 2010). Colpaert et al. (2004) suggested that the place of collection of fungal isolates conditions their functional diversity and so their tolerance to factors that trigger stresses. *C. buxifolia* cultured on tannic acid polymerized phenol around the colonies by means of extracellular oxidative enzymes as a potential mechanism of detoxification (Saparrat et al. 2008; Díaz et al. 2010; Saparrat et al. 2010), which in the presence of high levels of phenol might have a cost for fungal growth. Since here we analyzed only the response of *C. buxifolia* to 0.001–0.5% of tannic acid, additional experiments should be aimed at evaluating the effect of higher concentrations of water-soluble tannins from *S. buxifolia* litter and their relation with ecologically allelochemical interactions of litter-*C. buxifolia*.

Among extracellular oxidative enzymes, laccases and peroxidases are those mainly related to transformation of phenolics (Crowe and Olsson 2001; Saparrat et al. 2010). *Ciliochorella buxifolia* released both enzymes, whose level of activity was similar when the fungus was grown on liquid BM supplemented with glucose. However, laccases peaked earlier than peroxidases, which most probably reflected the sequence of events leading to detoxification of phenolic compounds and generation of oxygen active species (Gómez-Toribio et al. 2009). The amendment of WSF of *S. buxifolia* leaf-litter to cultures of *C. buxifolia* triggered differentiation of reproductive structures and induced laccases as well as peroxidases, though the former ones at higher levels. Haars et al. (1981) and Tsioulpas et al. (2002) also found that laccases were induced by soluble phenolic compounds in in-vitro cultures of *Fomes annosus* and *Pleurotus* spp. Unlike other chemical inducers, such as copper and paraquat, which reduced fungal growth (Crowe and Olsson 2001), the WSF of *S. buxifolia* did not reduce *C. buxifolia* biomass such as reported for *Trametes* spp. using mandarin peelings as C source (Osma et al. 2007). All these findings together suggest that the laccase activity of *C. buxifolia* played a key role in morphogenesis and detoxification of phenolics, like those available in *S. buxifolia* litter (Saparrat et al. 2007a). Additional studies remained to be done to elucidate the role of the oxidative enzymes of *C. buxifolia* in nature.

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