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Tropical stream microcosms of isolated fungal species suggest nutrient enrichment does not accelerate decomposition but might inhibit fungal biomass production

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Abstract

Terrestrial leaf litter is an essential energy source in forest streams and in many tropical streams, including Cerrado, litter undergoes biological decomposition mainly by fungi. However, there is a limited understanding of the contribution of isolated fungal species to in-stream litter decomposition in the tropics. Here we set a full factorial microcosms experiment using four fungal species (*Aquanectria penicillioides*, *Lunulospora curvula*, *Pestalotiopsis submerses,* and *Pestalotiopsis sp.*) incubated in isolation, two litter types (rapid and slow decomposing litter) and two nutrient levels (natural and enriched), all characteristics of Cerrado streams, to elucidate the role of isolated fungal species on litter decomposition. We found that all fungal species promoted litter mass loss but with contributions that varied from 1% to 8% of the initial mass. The fungal species decomposed 1.5 times more the slow decomposing litter and water nutrient enrichment had no effect on their contribution to mass loss. In contrast, fungal biomass was reduced by nutrient enrichment and was different among fungal species. We showed fungal contribution to decomposition depends on fungal identity and litter type, but not on water nutrients. These findings suggest that the identity of fungal species and litter types may have more important repercussions to in-stream decomposition than moderate nutrient enrichment in the tropics.

Keywords: aquatic hyphomycetes, litter breakdown, functional traits, nutrient pollution, Cerrado, leaf litter quality

Introduction

Anthropogenically nutrient enrichment of streams from organic inputs and agriculture run-off is one of the major causes of deterioration of fresh waters (Vörösmarty et al. 2010). The pace of stream nutrient loading is likely to continue and intensify in the future due to intensification of agricultural activities, waste water disposal and atmospheric nitrogen deposition (Galloway et al. 2008). This is concerning because nutrient enrichment has the potential to alter key stream ecosystem processes such as plant litter decomposition (Woodward et al. 2012, Ferreira et al. 2015), which is intrinsically related to the amount of carbon and nutrients stored in organism biomass and sediments, and emitted to the atmosphere.

Plant litter decomposition is a critical process specially to forested streams, which are light limited ecosystems and dependent on the carbon and nutrients from terrestrial plant litter (Neres-Lima et al. 2017). Although both microorganisms and litter-consuming detritivores have their own roles in litter decomposition in streams, there is compelling evidence of the lower relevance of detritivores activity—due to the absence, lower abundance and/or omnivore diet of some detritivores—at lower latitudes (Boyero et al. 2011a,b). This is the case of Cerrado streams—located in the Brazilian Central Plateau with a tropical wet-dry climate and draining through corridors of evergreen forests known as gallery forests—that harbor a diverse fauna of aquatic invertebrates but few of them are litter-consuming detritivores (Gonçalves et al. 2006, Gonçalves et al. 2007). Consequently, a large proportion of biological litter decomposition is assumed to be performed by microbial decomposers, which include bacteria but mostly fungi that often account to more than 90% of microbial biomass growth on litter (Pascoal and Cássio 2004).

Aquatic hyphomycetes (hereafter fungi) produces filaments that grow on the surface and interior of litter and secretes extracellular enzymes to break down complex compounds of litter (e.g. cellulose and lignin) (Chamier 1985). Fungi can also take up nutrients, especially in inorganic forms (e.g. nitrate and phosphate), from the water for their growth (Findlay 2010). As a result, fungi growth and reproduction tend to be stimulated by water nutrient enrichment up to certain threshold as well as by nutrient-rich litter resources (e.g. lower lignin to nitrogen mass ratios), which in turn accelerate litter decomposition (Ferreira et al. 2015). However, despite the prominent role of fungi to biological litter decomposition at several regions of the world (especially in tropical ones), it is surprising the scarcity or even absence of information in the tropics on how different fungal species are able to decompose lit-

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ter and respond to stressors of global importance, such as nutrient enrichment.

Here, we take advantage of isolates of aquatic fungi from a tropical region (i.e. Cerrado) to perform a laboratory experiment using four fungal species (in single-species treatments) subjected to two water nutrient concentrations (i.e. natural or enriched) and two litter types (i.e. lower- or higher-quality). We thus assessed the interactive effect of water nutrient enrichment and litter types of different nutritious quality on fungal-mediated litter decomposition as well as on production of fungal mycelial biomass by each species. We specifically hypothesized (i) that water nutrient enrichment would enhance fungal-mediated decomposition and biomass production because the low dissolved nutrient concentrations found in Cerrado stream waters (Gonçalves et al. 2007) limit fungal activity and growth; and (ii) that the positive nutrient enrichment effect on decomposition and on biomass production would be greater on lower-quality litter because it is more nutrient limited. Although we might expect different performances of fungal species under nutrient enrichment and on litter types, we lack support from the literature to anticipate specific responses of species.

Methods

Fungal Species

We selected four fungal species commonly found on decomposing litter in Cerrado streams (Fiuza et al. 2017): *Aquanectria penicillioides* (Ingold) (hereafter *A. penicillioides*), *Lunulospora curvula* (Ingold) (heareafter *L. curvula*), *Pestalotiopsis submersus* (Steyaert) (hereafter *P. submersus*) and a species from the *Pestalotiopsis* (Steyaert) genera (hereafter *Pestalotiopsis sp.*). Fungal species were collected in foams (Descals 2020) formed in pristine headwater streams in the state of Bahia, Brazil. Subsequently, in the laboratory, species were isolated under a microscope. The spores of the isolated species were then inserted into an isolation medium (0.1% malt extract, 2% agar, and 1gL-1 of antibiotic). The isolation medium was transferred to the growth medium (2% malt agar) and the Castellani method was performed for colony preservation (Marvanová 2020). Fungal species were deposited in the fungal collection of the Herbário Alexandre Leal Costa at Federal University of Bahia. Fungal colonies of each species were grown up in sterilized Petri dishes (Ø 100 mm x 15 mm height) in 2% malt extract agar during 1–2 weeks before the experiment starts. Each microcosm (250 ml glass flasks, previously autoclaved for 20 min) was inoculated with five agar plugs (10 mm in diameter) collected from Petri dishes with fungal mycelium of each fungal species. Agar plugs were collected from the center to the edge of Petri dishes to take into account the radial growth of fungi and to standardize the biomass of fungal species per microcosm.

Litter types

We selected litter from two common tree species found in riparian forests of Cerrado biome to represent litter of contrasting nutritious quality: *Tapirira guianensis* Aubl (hereafter *Tapirira*) and *Protium spruceanum* (Benth.) (hereafter *Protium*) (Tonin et al. 2021). The content of nitrogen (N), phosphorus (P), and lignin was used as a proxy for the nutritious quality of leaf litter (Zhang et al. 2019); N, P, and lignin were determined, respectively, using a CHN elemental analyzer (Leco Corporation—TruSpec Micro CHN628), by the ascorbic acid method (Flindt et al. 2020) and gravimetrically through the acid-detergent fiber method (Goering and Van Soest 1970).

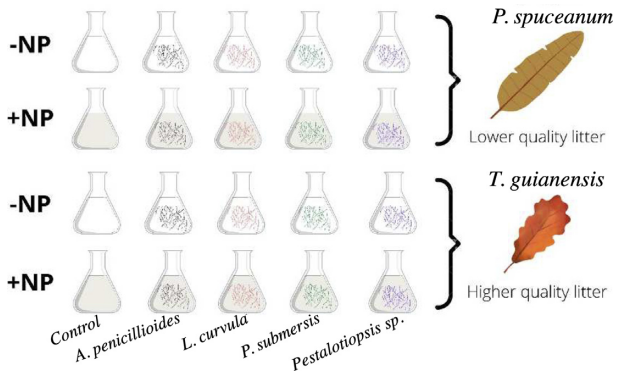

Figure 1. Setup of one replicate of experimental microcosms showing the four fungal species (*Aquanectria penicillioides*, *Lunulospora curvula*, *Pestalotiopsis submersus* and *Pestalotiopsis sp.*; and a control without fungi) and two litter types (*Protium spruceanum* Benth. and *Tapirira guianensis* Aubl; lower- and higher-quality litter) on two nutrient water concentrations (-NP, natural; +NP, enriched).

Leaf litter of *Tapirira guianensis* Aubl (hereafter *Tapirira*) was used as a higher-quality litter to fungal decomposers because of higher content of nitrogen (despite similar concentration of phosphorus) and lower lignin to N mass ratio than of *Protium spruceanum* (Benth.) (hereafter *Protium*) leaf litter, which was used as a lower-quality litter (*Tapirira* vs. *Protium*: 0.82% vs. 0.64% of N, 0.07% vs. 0.07% of P and 41 vs. 61 of lignin: N mass ratio). Recently fallen litter was collected from the soil in a preserved riparian forest (Ecological Station of Botanical Garden of Brasília) and air-dried at ambient temperature (~ 20ºC). Discs from each litter type were cut with a 12 mm diameter cork borer, freeze-dried (48 h), and weighted in portions of eight discs to the nearest 0.1 mg. Discs were then sterilized in ultraviolet radiation for 48 h before the experiment.

Experimental set-up

We assessed the effects of nutrient enrichment (both N and P) on litter mass loss and fungal biomass using a microcosms approach. The experiment included four fungal species, two water nutrient concentrations (natural or enriched), and two litter types (lower- or higher-quality litter). Each microcosm was replicated five times (including controls), resulting in 100 microcosms (Fig. 1). The microcosms contained five discs of one of the four fungal species (except control microcosms, which have no fungal addition), eight discs of one type of litter, and 50 mL of water (natural or enriched).

During the experimental period, the microcosms were conditioned on a refrigerated incubator shaker at 80 rpm and kept in the dark at a temperature of 20ºC to simulate the conditions found in Cerrado streams (Aida Campos et al. 2021). Ten days before the start of the experiment, we supplied all microcosms with 50 mL of natural water (see below) to allow fungal colonization on litter. After this period, half of the microcosms (50) were replaced with natural water (hereafter natural) and the other half (50) with a nutrient enriched water (hereafter enriched). The natural water was ultrapure water (from Milli-Q® System Direct 8/16) supplemented with $NaNO_3$ and K_2HPO_4 to obtain nutrient concentrations similar to reference streams of Cerrado (Gomes et al. 2016). The enriched water was supplemented to obtain a nutrient enrichment of five times the nutrient concentrations of the natural water (natural: 8 μg N L^{-1} and 0.9 μg P L^{-1}; enriched: 41 μg N L^{-1} and 4 μg P L^{-1}). The concentration of the enriched treatment was based on nutrient levels generally found in non-pristine streams of Cerrado

which are impacted by agricultural and/or urban activities (Aida Campos et al. 2021). Both natural and enriched waters were previously autoclaved for 20 min and supplemented with $CaCl_2$ and $MgSO_4.7H_2O$ to have salts found in streams (Gomes et al. 2016, Aida Campos et al. 2021). Water was replaced every five days until the experiment was terminated after 30 days.

Response variables

At the end of the experiment, all litter material (> 1 mm; not including fine particulate organic matter) was freeze-dried (for 48 h) and weighed to determine litter dry mass. We quantified the decomposition mediated by fungi (i.e. that resulted exclusively from fungal activity) as the relative litter mass loss (LML) after correcting for mass loss in control microcosms (that is, LML = 0.138 for *Protium* and LML = 0.262 for *Tapirira*), which encapsulated losses by leaching, handling and/or from other sources. This mass loss correction was done by multiplying initial dry mass by a correction factor derived from control microcosms, separately for *Protium* and *Tapirira* litter. LML was then quantified as:

$$\left[\frac{DMi - DMf}{DMi}\right] \text{x } 100$$

where DM_i was the control-corrected initial litter dry mass and DM_f the final litter dry mass in a microcosm.

To estimate fungal biomass on litter from each replicate we quantified ergosterol content (Gessner 2005). Leaf discs were freeze-dried (48 h), weighed (±0.01 mg) before analysis, and immersed in 8 mg KOH L^{-1} methanol. Lipids were extracted at 80°C for 30 min and purified with solid-phase extraction (Vac RC tC18 cartridges, 500 mg; Waters Sep-Pak®, Waters Corp., Milford, MA, USA) using a vacuum system. Ergosterol was eluted in isopropanol and quantified by high-performance liquid chromatography (HPLC, Dionex ICS Series PDA, Sunnyvale, CA, USA; detection wavelength, 282 nm; flow rate, 1.5 mL s^{-1}; column temperature, 33°C; injection volume, 20 μL). We measured extraction efficiency by running ergosterol standards in parallel (Ergosterol ≥ 95% [HPLC], Sigma®). Fungal biomass was expressed as μg ergosterol per gram of litter dry mass.

Data Analysis

Data analysis was conducted in R v. 4.0.1 (R Core Team 2021) using the packages nlme (Pinheiro et al. 2017) and boot (Davison and Hinkley 1997, Canty and Ripley 2017), and figures were prepared using ggplot2 (Wickham 2016). We examined our experimental hypotheses (i.e. water nutrient effect and its interaction with litter quality on fungal-mediated decomposition and species biomass) using linear models and confidence intervals. Models were fitted using the gls function and restricted maximum likelihood (REML) method with three categorical predictors: water nutrient enrichment (natural and enriched) litter type (lower and higher-quality), fungal species (four species) and their two-way interactions; fungal-mediated decomposition or fungal species biomass was the response variable. The residual spread was allowed to vary in relation to litter types and fungal species using a constant variance function structure (varIdent). This model structure was chosen after comparing models with different random structures using the Akaike information criterion. Visual exploration of residuals indicated no violation of model assumptions. When significant effects of fungal species on decomposition or biomass were demonstrated, we tested species differences by calculating ordinary non-parametric bootstrapped 95% confidence intervals with non-overlapping intervals supporting statistical differences (Canty and Ripley 2017). Confidence intervals were calculated with the BCa method based on 1000 bootstrap replicates. Finally, we also tested whether higher biomass of fungi increases litter mass loss using a linear model (fitted as explained above) with fungal biomass as a continuous predictor and litter types and fungal species as categorical predictors as well as their two-way interactions.

Results

Fungal-mediated litter decomposition was responsible for 3.31% ± 0.37% (average ± SE) of litter mass loss after 30 days of the experiment. Nutrient enrichment did not enhance overall litter decomposition (natural vs. enriched, 3.32% ± 0.59% and 3.31% ± 0.50%, respectively; $F_{1,75} = 1.99$, $P = 0.163$) nor decomposition mediated by each fungal species (i.e. interaction between nutrients and fungal species: $F_{3,75} = 1.76$, $P = 0.165$) or decomposition of different litter types (i.e. interaction between nutrients and litter types: $F_{1,75} = 1.47$, $P = 0.230$; Table 1). Due to the absence of nutrient enrichment effect on decomposition, data were pooled across natural and enriched treatments for further analysis.

Overall, fungal decomposition differed between litter types and among fungal species, but there was no interaction between fungal species and litter types, indicating a consistent role of fungal species in litter decomposition (independent of litter types) (Fig. 2A, Table 1). Decomposition was 39% higher on lower-quality litter than on higher quality litter, on average (*Protium* vs. *Tapirira*, respectively, 3.86% ± 0.52% and 2.78% ± 0.51% of litter mass loss). All fungal species showed litter mass loss higher than zero, indicating they were relevant to decomposition (Fig. 2A). Decomposition by different fungal species varied (on average) from 1.1% to 6.8%. Although three fungal species decomposed similar quantities of litter (consistently in both litter types), *P. submersus* decomposed 2–6 times more litter than the other species (Fig. 2A).

Fungal biomass—quantified as ergosterol content on the litter of each microcosm at the end of the experiment—was 30% reduced by nutrient enrichment (natural vs. enriched water, respectively, 613 ± 71 and 472 ± 54 μg ergosterol g^{-1} litter dry mass; $F_{1,64} = 5.4$, $P = 0.023$), but neither fungal species nor litter types were affected differently by water nutrients (i.e. non-significantly interactions; Table 1). Fungal species produced different quantities of biomass: *A. penicillioides* showed 2–3 times lower biomass than the other fungal species (Fig. 2B). Fungal biomass also depended on fungal species and litter type: i.e. *Pestalotiopsis sp.* for example, showed higher biomass than the other three species, but only when exposed to higher-quality litter (Table 1, Fig. 2C). However, we did not find support that higher fungal biomass enhances litter mass loss ($F_{1,64} = 0.27$, $P = 0.605$), suggesting that fungal biomass may not be a good predictor of species performance or even of their capacity to process litter carbon.

Discussion

Aquatic fungi are key organisms in detritus-based food webs and consequently, relevant for freshwater ecosystem functioning (Kubicek and Druzhinina 2007, Findlay 2010). However, there still is a lack of knowledge on the importance of different fungal species to essential ecosystem functions, such as litter decomposition, especially in tropical regions (Tam et al. 1998, Grossart and Rojas-Jimenez 2016). To our knowledge, this is the first study using fungal isolates from tropical regions to experimentally investigate ecosystem-level consequences. Here, we showed that fun-

Table 1. Summary of results from models testing for the effects of nutrient enrichment, litter types, fungal species and its interactions on fungal-mediated litter decomposition and fungal biomass. Total degrees of freedom for litter decomposition and fungal biomass models are, respectively, 67 and 60.

	df	F-value	P-value	Observation
Litter decomposition				
Litter Type (LT)	1	17.4	**< 0.001**	Low > high quality litter
Fungal Species (FS)	3	32.1	**< 0.001**	Fig. 2A
LT * FS	3	0.6	0.608	
Fungal biomass				
Nutrient enrichment (NE)	1	5.4	**0.023**	Natural > enriched water
Litter Type (LT)	1	0.5	0.465	
Fungal Species (FS)	3	30.8	**< 0.001**	Fig. 2B
NE * LT	1	0.9	0.344	
NE * FS	3	0.2	0.882	
LT * FS	3	3.5	**0.020**	Fig. 2C

gal species identity has a major role in explaining litter decomposition under microcosm conditions and using isolates of fungal species, with species presenting distinct performances to decompose litter. For instance, we found that one species (i.e. *P. submersus*) is capable to decompose at least two times more litter than other tested fungal species under microcosm conditions. Previous studies using fungal communities also pointed to the importance of species identity to litter decomposition, some as a result of complementary interactions between species, depending on the context tested (Bärlocher and Corkum 2003, Pascoal et al. 2010, Fernandes et al. 2011).

Our hypothesis that water nutrient enrichment would enhance fungal-mediated decomposition and biomass production was not supported. In contrast, water nutrient enrichment decreased fungal biomass production and did not influence fungal-mediated decomposition. This finding disagrees with the well-documented results for laboratory microcosms (Ferreira and Chauvet 2011, Fernandes et al. 2014, Tant et al. 2015), field experiments (Sridhar and Bärlocher 2000, Gulis and Suberkropp 2003), and meta-analysis which showed an increase in litter decomposition with nutrient enrichment, despite higher effects for laboratory than field experiments (Ferreira et al. 2015). It is generally expected an increase of litter decomposition in response to water nutrient enrichment, however, studies and data from temperate regions are overrepresented—even in meta-analysis (Ferreira et al. 2015)—which may hinder general conclusions that are not applicable to the tropics. Although the rate of nutrient enrichment used in our experiment (five times the concentrations found in pristine streams of Cerrado) are lower than those reported in the majority of studies (which often vary from 10 to 1000 times; (Woodward et al. 2012, Ferreira et al. 2015), it is unlikely that the absence of a nutrient effect on decomposition was due to a nutrient limitation of fungal activity. This argument is supported by previous similar results when using a 100-fold increase in dissolved nutrients. Another topic that could be addressed would be the intermediate disturbance theory, where decomposition would be negatively affected at both extremes, in oligotrophic environments such as the streams of Cerrado and in extremely eutrophic environments. In this context, even the 5-fold enrichment used in our experiment might took the species' performance from one extreme to the other of the gradient (Woodward et al. 2012). It is known that aquatic fungi are constrained by the supply of nutrients that can be taken up from water or C-rich resources for their growth and reproduction (Suberkropp 1998, Medeiros et al. 2010). Thus, increasing the concentration of dissolved nutrients in water would stimulate nutrient immobilization (Gulis et al. 2006), litter decomposition (Suberkropp 1998, Gulis and Suberkropp 2003), and enhance biomass production but results mostly come from cold and temperate waters (Medeiros et al. 2010, Ferreira et al. 2015). In contrast, results from tropical streams did not show stimulation of decomposition by water nutrient enrichment (Ferreira et al. 2015). A recent laboratory study using fungal communities demonstrated that litter decomposition is not altered by increasing the availability of dissolved nutrients (10 and 100 times higher than naturally found in streams), but fungal biomass production is inhibited in support of our findings (Biasi et al. 2017). Combined, these results suggest that fungal activity on decomposing litter is not nutrient-limited and that water nutrient increases—especially from anthropogenic sources, which can deliver high nutrient loads—can be most detrimental to fungal biomass production (at least in a short period of time) in tropical streams. The absence of nutrient limitation on decomposition may also suggest that fungi response to nutrient increases is context-dependent, such as observed by the modulation of nutrient effects by pH in naturally acidic and circumneutral streams (Stelzer et al. 2003).

Different performances of fungal species (i.e. species identity effect) on litter decomposition and biomass production, as reported in this study, were previously shown in microcosms conditions (Bärlocher and Corkum 2003, Gomes et al. 2018). In our study, these differences could be attributed to species traits that promote or retard the activity of species on decomposition rather than competition for resources with other fungal species, as observed in natural and experimental fungal communities (Gulis et al. 2006, Ferreira and Chauvet 2011). These results suggest (at least for microcosm conditions) that there is an interspecific difference in litter processing capacity of isolates of aquatic fungi. Although multiple fungal species do occur in decomposing litter within streams and species diversity effects may change the individual and overall community performance (Duarte et al. 2006), the novelty of this study is to test—for the first time in the tropics in microcosm conditions—the individual contribution of fungal species as a first step to understand whether and how fungal species differ in their potential to decompose litter.

Our results depict that fungal decomposition was higher on lower-quality litter (*Protium*), despite the higher concentration of lignin and lower concentration of N, which often increase fun-

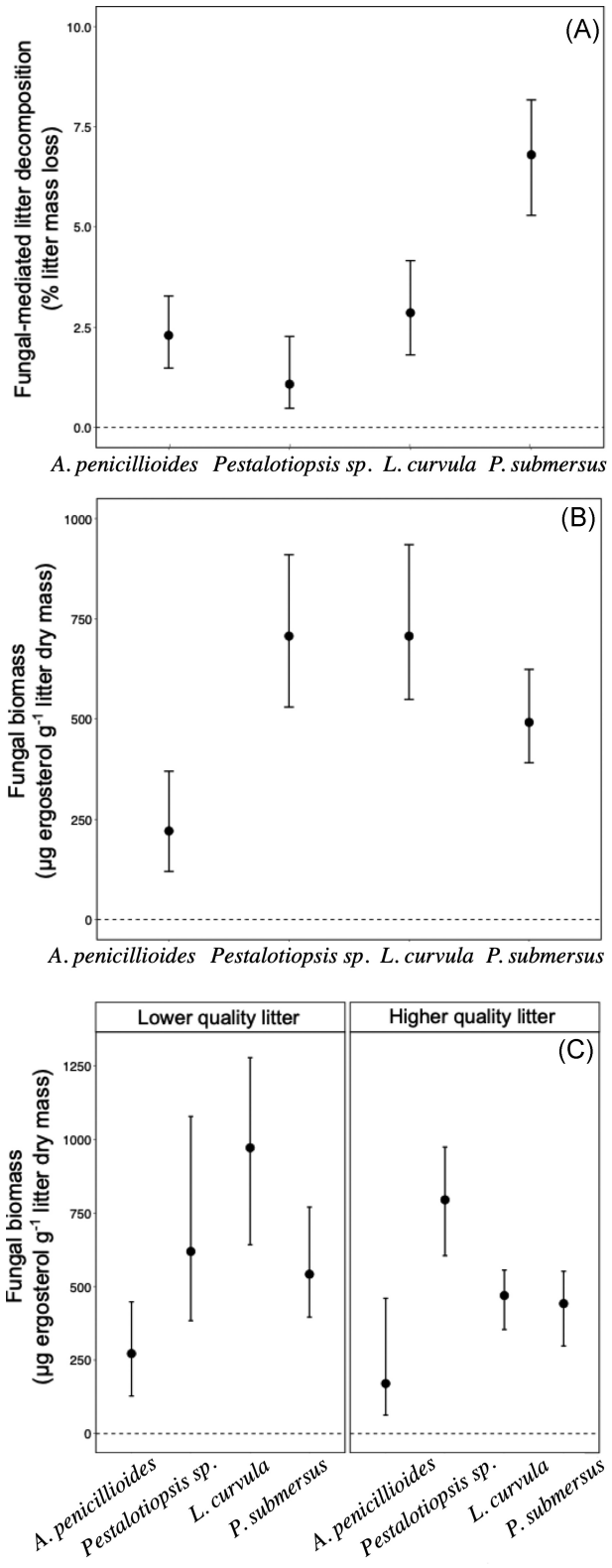

Figure 2. Fungal-mediated litter decomposition by the four fungal species (**A**); fungal biomass produced by the four fungal species independently of litter type (**B**) and on lower and higher-quality litter (**C**). Circles are means of microcosms data at the end of the experiment and vertical lines denote upper and lower limits of 95% non-parametric bootstrapped confidence intervals.

gal metabolic costs slowing down their growth and enhancing stoichiometric imbalances, respectively. On one hand, our finding suggests that the fungal species are adapted to decompose recalcitrant and nutrient-poor litter commonly found in Cerrado streams. On the other hand, it is possible that other litter compounds (i.e. micronutrients) not assessed in this study may be more correlated with decomposition or limit fungal activity (Mykrä et al. 2019). For instance, although three out of four fungal species showed similar biomass between litter types, *L. curvula* biomass was higher on lower-quality litter. This can be seen as evidence against the use of the term litter quality as it oversimplifies and does not consider that different organism may have different limitations (Chamier 1985). Previous studies showed that lower fungal-mediated decomposition in lower-quality litter (i.e. those with higher lignin and lower N concentrations) (García-Palacios et al. 2016) was due to lower fungal colonization (Fernandes et al. 2012). In contrast, we did not find a correlation between fungal biomass and decomposition in our study, as previously reported (Gomes et al. 2018), possibly because fungal species face a trade-off between growth, enzymatic investment (for resource acquisition), and reproduction (Zheng et al. 2020).

We provide evidence that nutrient enrichment of Cerrado streams—which are naturally oligotrophic (Gonçalves et al. 2006)—may not change litter decomposition (through alteration in fungal species performances to break down organic carbon), but may reduce fungal biomass production. Although, in our study, we did not detect a relationship between fungal biomass and decomposition, reductions in fungal biomass may impair decomposition and related processes (e.g. nutrient cycling and secondary production) in different time scales (from weeks to years) and/or spread to food webs, which depend on the conditioning of litter subsidies. We also showed that isolates of fungal species have consistently different capabilities to decompose litter under microcosm conditions, which are determined by litter quality or nutrient water concentration. These results are a key first step to enhancing the research of biodiversity-ecosystem functioning in the tropics, e.g. simulating fungal communities with different numbers, traits, and/or identities of species to understand the consequences of species loss. Also, these future studies could help to disentangle the consequences of species diversity effects on ecosystem functioning in the presence of multiple stressors (e.g. nutrient pollution, climatic change, forest conversion).

Conflict of interest statement. None declared.

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