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— SHORT COMMUNICATION —

Microscopic evidence supports the hypothesis of high cellulose degradation capacity by the symbiotic fungus of leaf-cutting ants

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Leaf-cutting ants (Hymenoptera, Formicidae) use fresh leaf fragments to cultivate a symbiotic fungus (Basidiomycota, Agaricales) as their food source. There are three hypothetical propositions for the degree of cellulose degradation capacity of this symbiont: (i) that it is high, rendering this polymer an important energy source, as originally purposed by Martin and Weber; (ii) that it is very small, and only facilitates the symbiont to use other cell nutrients and, (iii) that this fungus is metabolically inept against this polymer. The two latter proposals are more recent than the first one but are based on *in vitro* or highly indirect evidence. Consequently, we carried out a new evaluation of the degradation capability of this fungus, utilizing as realistic an approach as possible by assessing the microscopic effect of fungus cultivation on the leaf anatomy of the grass *Paspalum notatum* within colonies of the grass-cutting ant *Atta capiguara*. We observed a complete degradation of the most abundant leaf cells (the non-lignified ones). On the other hand, since lignin-rich structures presented only slight damage, the leaf format was maintained. Therefore, this *in vivo* study corroborates Martin and Weber's hypothetical proposition: that cellulose is highly degraded by the leaf-cutting ant symbiont, thus serving as an important energy source.

Key words: leaf-cutting ant, Attini, leaf anatomy, plant cell wall.

INTRODUCTION

Nutrition in leaf-cutting ants, genera *Atta* and *Acromyrmex* (Hymenoptera, Formicidae, Attini), comes mostly from the ingestion of its symbiotic fungus (Basidiomycota, Agaricales, Leucocoprini), which they grow using fresh plant fragments (Martin *et al*., 1969; Fowler *et al*., 1986). Such nutrition is supplemented with liquids from the plant during its cutting and preparation for fungus cultivation (Littledyke & Cherrett, 1976; Silva *et al*., 2003).

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It is recognized that the symbiotic fungus is capable of degrading several plant polymers including xylan, starch or pectin (Martin *et al*., 1969; De Siqueira *et al*., 1998; Schiott *et al*., 2008), which is used in its metabolism. The fungus is rich in nutrients for the ant diet, and the most abundant soluble components are simple carbohydrates (sugars) such as glucose (Martin *et al*., 1969; Silva *et al*., 2003), a very important component in the ant diet (Silva *et al*., 2003).

However, the remaining controversy as to the extent to which the symbiont degrades cellulose, the most abundant plant polymer (Popper, 2008), has given rise to three hypothetical propositions: *(i)* that the degree is high, rendering this polymer an important

energy source (Martin & Weber, 1969); *(ii)* that such degradation is very small and only permits the symbiont hyphae to penetrate into the nutrient-rich cytoplasm (De Siqueira *et al*., 1998) or to facilitate the demolition of the cell walls (Erthal *et al*., 2009); and, *(iii)* that such fungus is metabolically inept against this polymer (Abril & Bucher, 2002).

The two latter hypotheses are based mainly on cultivation assays of symbiotic fungus in culture medium containing cellulose as the sole carbon source (*in vitro* evidence), which resulted in little (De Siqueira *et al*., 1998; Erthal *et al*., 2009) or no cellulose degradation (Abril & Bucher, 2002). Additionally, Abril & Bucher (2002) presented indirect *in vivo* evidence: *(i)* the lignin:cellulose ratio of depleted plant fragments (garbage) was < 1, which, according to these authors, indicates non-degradation; and *(ii)* in a particular group of leaf-cutting ants, the grass-cutting ants, the fact that the grass leaf format is preserved after degradation by the fungus was considered a suggestion of non-degradation hypothesis.

However, Martin & Weber (1969), reported that: *(i)* the symbiotic fungus grows well in medium with cellulose as the only carbon source (evidencing high degradation capacity); and that *(ii)* the cellulose:ash ratio greatly decreased from garden to garbage fragments, indicating that 45% of the cellulose is degraded. According to these authors, this is an underestimation because the fragments in the fungus garden are in an intermediate stage of degradation.

Thus, taking in account that cellulose degradation is a very complex process (Dix & Webster, 1995), it can be stated that the main recent data (De Siqueira *et al*., 1998; Abril & Bucher, 2002; Erthal *et al*., 2009), due to being basically *in vitro*, are only indicative for inferring the degree of degradation that really occurs *in vivo*.

Consequently, to obtain a better understanding of this issue, new evaluations of degradation capability of the symbiotic fungus, are desirable to improve this specific knowledge. The objective of this study was to evaluate the *in vivo* cellulose degradation capacity of the symbiotic fungus of the grass-cutting ant *Atta capiguara* Gonçalves, 1944, by making an anatomical analysis of the leaf tissues of *Paspalum notatum* Flugge in their various stages of degradation by the fungus.

MATERIALS AND METHODS

Ant colonies

Small colonies of *A. capiguara* were collected 4 months after their foundation from *P. notatum* pastures located in the cities of Bofete (23°06′08″S and 48°15′28″W) and Santa Cruz do Rio Pardo (22°53′53″S and 49°37′57”W), SP, Brazil. The colonies were maintained in a laboratory environment, at 24±1°C and about 60% RH, in clear plastic jars. The jar containing the fungus garden had 1 cm gypsum layer at the base to keep moisture high, and was interconnected by means of clear plastic tubes with the foraging arena, where plant fragments were provided daily, and with the garbage chamber, where the workers disposed the fragments after they were used by the symbiont.

Anatomical analysis of leaf blades at various degradation stages by the symbiotic fungus

Plant fragments were sampled from four *A. capiguara* colonies that received only *P. notatum* leaves. Collections were made from the foraging, garbage, and fungus chambers, at four different stages: Stage 1 – leaf fragments before being transported by workers into the fungus garden (typically only cut, unprocessed, leaf pieces); Stage 2 – fragments processed by workers, removed from the top part of the fungus garden, containing freshly incorporated hyphae; Stage 3 – fragments already partially used by the fungus, removed from the base of the fungus garden; Stage 4 – degraded fragments recently discarded by workers into the garbage chamber.

The leaf fragments collected at each stage were fixed in FAA 50 (formaldehyde + glacial acetic acid + 50% ethyl alcohol) and preserved in 70% alcohol (Johansen, 1940). These fragments were then infiltrated in glycol methacrylate resin, according to Guerrits (1991), sectioned into 8 μm thick slices with a rotary microtome, stained with 0.05% toluidine blue, which colors the cellulose with blue and the lignin with green (O' Brien *et al*., 1964) and mounted between slides and coverslips with synthetic resin. The anatomical structure was analyzed and photomicrographed, in an attempt to monitor the degradation of leaf tissues found at the various collection stages.

RESULTS

The four classification stages of *P. notatum* leaf fragments cut by *A. capiguara* workers used in the cultivation of the symbiotic fungus are presented in Figure 1A-D.

Figure 1A shows a leaf blade of *P. notatum* with all its structures intact [without any degradation or injury on the epidermis, sclerenchyma, endodermis

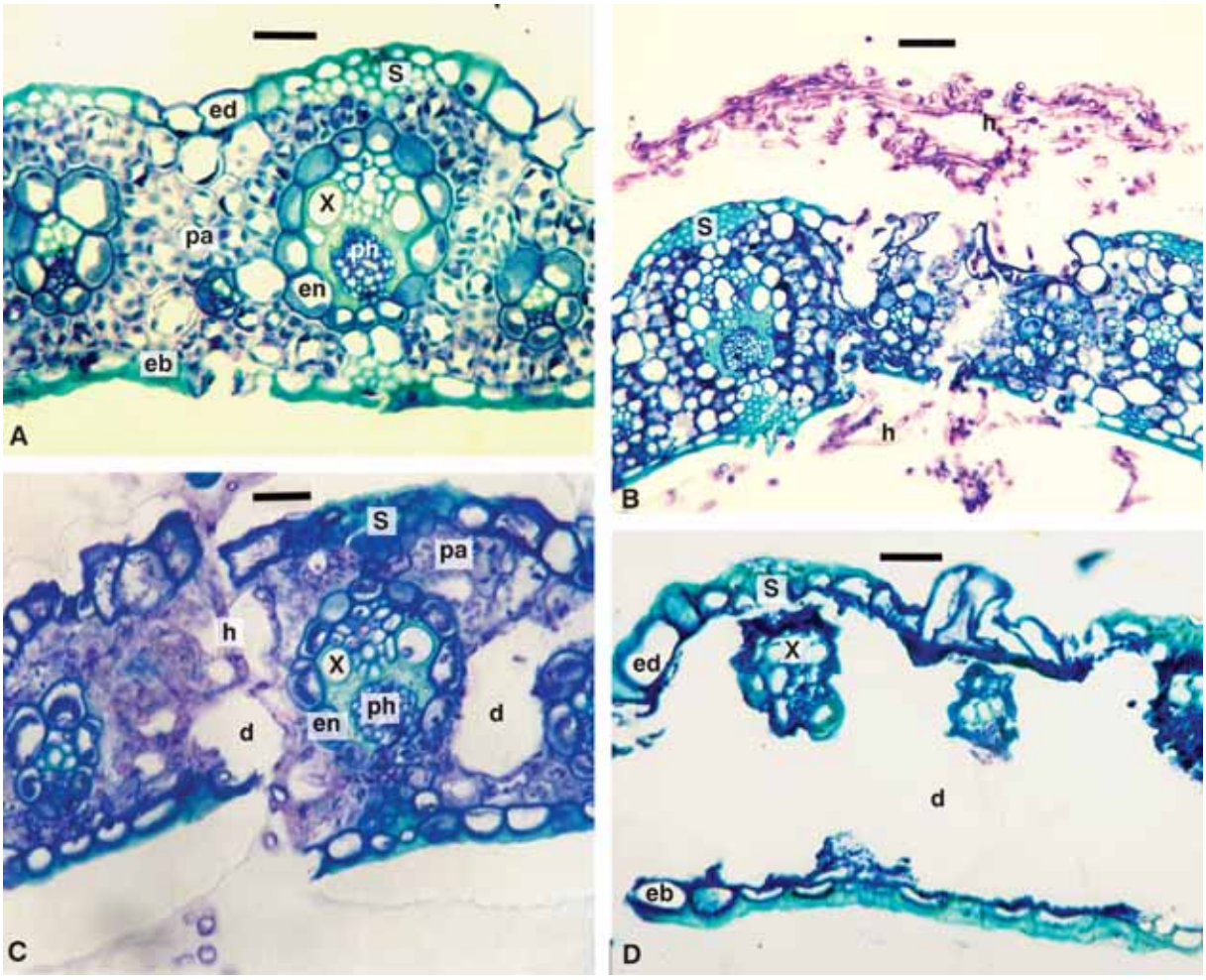

FIG. 1. Anatomical features of leaf blade fragments of *Paspalum notatum*, cut by *Atta capiguara* workers to grow their symbiotic fungus, in transverse section view. (A) Stage 1: fragment prior to processing for fungus cultivation, with intact leaf structure (40×). (B) Stage 2: fragment after processing, collected from the top part of the fungus garden, showing clusters of hyphae on the external surface and hyphal penetration through the cuts produced by the ants on the leaf surface (20×). (C) Stage 3: fragment collected from the bottom part of the fungus garden, showing penetration of hyphae through the cuts produced by the ants on the leaf surface, presence of hyphae inside the leaf blade, and degradation areas in the mesophyll (40×). (D) Stage 4: fragment collected from the garbage, showing lack of hyphae and partial degradation of the epidermis and complete degradation of the parenchyma, endodermis (vascular bundle sheath) and phloem (40×). d: degradation area of parenchyma cells in the mesophyll. eb: epidermis of the abaxial surface. ed: epidermis of the adaxial surface. en: endodermis (vascular bundle sheath). h: hyphae. pa: parenchyma. ph: phloem. s: sclerenchyma. x: xylem. Bars = 25 μm (A, C, D) and 50 μm (B).

(vascular bundle sheath), phloem, and xylem], since it had not yet been inoculated with the fungus, nor was it was prepared for this (Stage 1).

The presence of a mantle of hyphae is observed in Figure 1B, both on the adaxial and the abaxial surfaces of the leaf blade. Some of the hyphae are penetrating into the leaf through injuries caused by the cutting and substrate-processing behavior performed by the workers (Stage 2), which is a similar characteristic as the one observed by Mohali (1998). The presence of some hyphae dispersed within the leaf chlorenchyma can also be seen, as well as the occurrence of initial cell degradation in the tissue, whose walls consist primarily of cellulose.

An increase in the number of hyphae present in the parenchyma can be observed in Figure 1C, as well as some areas where the mesophyll has been degraded, especially in the parenchymatous tissue located around the endodermis (Stage 3).

Figure 1D is characteristic of Stage 4, with several mesophyll tissues completely degraded, such as the parenchyma, the phloem, and the endodermis, as in those tissues the walls are basically constituted of cellulose, and consequently are the first tissues to un-

dergo degradation by the symbiotic fungus because of their higher amounts of cellulose. The epidermis is in a state of partial degradation, due to depositions in its walls. The cuticle is almost intact, which contributes towards this tissue being less degraded. At this stage, the xylem and the sclerenchyma have not undergone degradation, as these tissues show intense lignification in their cell walls, therefore possessing a greater quantity of lignin (which was very poorly degraded), which probably makes cell degradation by the symbiotic fungus more difficult.

DISCUSSION

Our results indicate that, the most of the plant cells, that are lignin free, such as parenchyma and phloem cells, are completely degraded, indicating that the hypothesis proposed by Martin & Weber (1969) that the symbiotic fungus is capable of high (probably of more than 45%) cellulose degradation, is valid. This, allows us to conclude that cellulose is an important energy source for the fungus. In addition, it can be stressed that, as De Siqueira *et al*. (1998) and Erthal *et al*. (2009) stated, the degradation of the cell wall components, as cellulose, is important for the fungus to access the nutrient rich cytoplasm. On the other hand, the hypothesis of low-magnitude cellulose degradation (De Siqueira *et al*., 1998; Erthal *et al*., 2009) and non degradation (Abril & Bucher, 2002), does not fit well with our results. The anatomical analysis of leaf blades clearly showed the occurrence of cellulose degradation, revealing that the symbiotic fungus is the agent that causes leaf fragment degradation. It was evidenced that the more advanced the leaf colonization by the fungus, the larger the degradation areas found in the leaf mesophyll (fragment still in the fungus garden), ending with the almost complete degradation of tissues whose cell walls were rich in cellulose (fragment freshly discarded into the garbage chamber). Most cellulose degradation occurred at the basal (older) portion of the fungal sponge, which is compatible with the findings of Schiott *et al*. (2008), who observed higher cellulolytic activity precisely in this layer. Only very lignified structures remained in the fragments discarded into the garbage, such as the sclerenchyma and the xylem. It should be pointed out that these leaf fragments retained the aspect of their format; therefore, the consideration made by Abril & Bucher (2002) is inaccurate: the apparent robustness of the discarded fragments, is not indicative of non-degradation of cellulose.

Abril & Bucher (2002) showed that lignin:cellulose ratios in degraded substrates are low (0.10 to 0.28), and stated that, in substrates degraded by typically cellulose-digesting microorganisms, it is > 1. However, in *A. sexdens rubropilosa* garbage, Sousa-Souto *et al*. (2007) found that this ratio was 1.0 or 1.2 depending on the substrate. Given that leaf-cutting ants forage for a wide variety of plant species (Fowler *et al*., 1986), they probably carry leaves with different initial lignin:cellulose ratios, so that a given substrate with a higher initial ratio than another would maintain its greater ratio after decomposition, given the low or no degradation of lignin. Additionally, based on the original data of Martin & Weber (1969) for *A. colombica tonsipes*, we proceeded to calculate this ratio: it was low in the garbage (0.34), but even lower (0.20) in the gardens of the same colonies, indicating high cellulose degradation. Furthermore, since our results show complete degradation of the majority of the leaf cells, including their walls, we can conclude that Martin & Weber (1969) proposal of high cellulose degradation is greatly reinforced.

This high degradation rate (Martin & Weber, 1969; present work) is not very compatible with the evidence for little degradation obtained by De Siqueira *et al*. (1998) and Erthal *et al*. (2009), but this is not so surprising in the context of comparing *in vivo* vs. *in vitro* data. *In vitro* studies, being more isolated than *in vivo* variables, are useful for specific purposes. For example, the qualitative study of Bacci *et al*. (1995), indicated that the fungus possesses the capacity to degrade cellulose (degree not evaluated), and reinforced Martin & Weber (1969) proposal. The divergent result of Abril & Bucher (2002) is probably due to the use of an unfavorable high temperature (28-30°C). The fungus is relatively temperature-sensitive and the ideal condition for its growth is between 20 and 25°C (Bollazzi & Roces, 2002, and references included).

Abril & Bucher (2002) indicated that Attini (fungus growing ants) probably domesticated fungi that are mycorryzical, thus presenting little or no cellulose degradation capacity from the start until the present. However, we propose that the evolutionary enzymatic evidence presented by De Fine Licht *et al*. (2010) provides additional support for cellulose degradation capacity. It was indicated that high degradation ability is present in the highly coevolved symbiont of leaf-cutting ants, in the less coevolved symbionts of the basal Attini (non leaf cutters) and in free living saprophytic fungi. Therefore, it is probable that a high cel-

lulose degradation capacity was present in all fungi that were domesticated by the Attini ants, and that this ability was maintained in subsequent ant-fungus coevolution because of its high adaptive value.

In addition, according to Silva-Pinhati *et al*. (2004), the leaf-cutting ants probably cultivate some related lineages that belong to a single species (*Leucoagaricus gongylophorus*). Consequently, it is highly probable that the symbiont lineages, associated not only with the grass-cutting ant *Atta capiguara* but with all leaf-cutting ants, are capable of high cellulose degradation.

Therefore, the cumulative evidence has reinforced the statement of Martin & Weber (1969) that the symbiosis between fungus and leaf-cutting ants is a biochemical partnership in which the former produces a pool of enzymes that degrade the major part of leaf organic matter, including cellulose. Consequently, the fungus releases leaf nutrients, which would otherwise be inaccessible to the ants. In addition, by this high degree of cell-wall degradation, the fungus certainly gains access to the cytoplasmic nutritional content.

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