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Metabolism of Plant Polysaccharides by *Leucoagaricus gongylophorus*, the Symbiotic Fungus of the Leaf-Cutting Ant *Atta sexdens* L.

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***Atta sexdens* L. ants feed on the fungus they cultivate on cut leaves inside their nests. The fungus, *Leucoagaricus gongylophorus*, metabolizes plant polysaccharides, such as xylan, starch, pectin, and cellulose, mediating assimilation of these compounds by the ants. This metabolic integration may be an important part of the ant-fungus symbiosis, and it involves primarily xylan and starch, both of which support rapid fungal growth. Cellulose seems to be less important for symbiont nutrition, since it is poorly degraded and assimilated by the fungus. Pectin is rapidly degraded but slowly assimilated by *L. gongylophorus*, and its degradation may occur so that the fungus can more easily access other polysaccharides in the leaves.**

Leaf-cutting ants in the genera *Atta* and *Acromyrmex* are assumed not to feed on solid plant material (16, 20) but to utilize instead the liquid nutrients gathered from both the leaves they cut (3, 13, 14) and the fungus, *Leucoagaricus gongylophorus*, that they cultivate in their nests (4, 21, 26, 27). The fungus metabolizes cellulose (1, 15), which is the most common plant polysaccharide (12). Thus, current opinion is that the symbiosis is based on the ability of ants to feed on cellulose through the fungus (8). However, besides cellulose, vegetation contains xylan, pectin, and starch, in amounts up to 60% of the leaf dry weight (6, 12). This abundant source of nutrients could be assimilated by the fungus and thus utilized by the ants.

To test this hypothesis, we have studied the metabolism of plant polysaccharides by *L. gongylophorus*. The fungus was cultured in single carbon sources, such as cellulose, xylan, pectin, starch, and the hydrolysis products of some of these polymers. After cultivation, substrate assimilation and polymer degradation were evaluated. Our results show that *L. gongylophorus* is able to mediate the assimilation by *A. sexdens* of all plant polysaccharides. However, contrary to current thought, cellulose seems not to be the most important carbon source for symbiont nutrition.

MATERIALS AND METHODS

Organism and culture conditions. Strains CCT1, CCT2, and CCT3 of *L. gongylophorus* (formerly called *Leucocoprinus gongylophorus*) have been isolated from different nests of *A. sexdens* L. and are deposited in the Tropical Culture Collection, Campinas, SP., Brazil, under the accession numbers CCT 6414, CCT 6415, and CCT 6416. The isolates were kept at 25°C in the dark in culture medium A (18). Cells (41 ± 14 mg [dry weight]) from 30-day cultures were transferred to 250-ml Erlenmeyer flasks containing 50 ml of yeast nitrogen base (YNB; Difco) medium (pH 6.0) and 0.25 g of a single carbon source. After 30 days of incubation at 25°C in the dark, the metabolic ability of the fungus was evaluated by determining the cell mass variation, degradation of polysaccharides, and efficiency of growth on polysaccharide hydrolysis products.

Cell mass variation. Flasks with culture medium were weighed before and after cell transfer, and the inoculum dry weight (I) was estimated as 5% of the net weight. After incubation, fungal mycelia were collected, dried at 70°C for 24 h, and weighed, and the final cell mass (F) was calculated. Cell mass variation (V) was estimated by the equation $V = F/I$. The growth rate is constant for 50 days when cells are incubated on glucose or starch and for over 60 days when they are incubated on cellulose or carboxymethylcellulose (data not shown). The cell mass variation was calculated after 30 days of cultivation to estimate fungal ability to assimilate different carbon sources.

Sugar determination. Reducing sugars were assayed by the method of Miller (17). The concentration of cellulose or starch hydrolysis products was calculated with glucose as the standard; galacturonic acid and xylose were used as standards for the quantitation of pectin and xylose hydrolysis products, respectively.

Growth efficiency. Growth efficiency ($Y\%$) was calculated by the equation $Y\% = 100\,X/M$, where X is the cell mass production (in milligrams [dry weight]) and M is the substrate consumed (in milligrams).

Enzyme assay. After the fungus was cultured, the culture medium (50 ml) was filtered through a 0.45-μm-pore-size Millipore filter and the proteins were precipitated in an ice-cold bath with 40 mg of ammonium sulfate and diluted in 50 mM phosphate buffer, pH 6.0. Enzyme activity was determined at 25°C in the same buffer containing 2% (wt/vol) of the substrate (xylan, pectin, starch, microcrystalline cellulose, carboxymethylcellulose, or acid-swollen cellulose) and 0.10 mg of protein/ml. The reaction mixture was incubated on a reciprocating shaker (100 rpm); samples were collected every 15 min of incubation, boiled for 1 min, and centrifuged, and the concentration of reducing sugars in the supernatant was determined. The sugar concentration increased linearly during incubation (1 h for xylan, pectin, or starch and 4 h for microcrystalline cellulose, carboxymethylcellulose, or acid-swollen cellulose), so linear regression was used to calculate the enzyme activity, which was expressed in micromoles of hydrolysis products per gram of cells (dry weight) per hour.

Substrates. The substrates used were purchased from Sigma (St. Louis, Mo.) and Merck (Darmstadt, Germany), as well as the Brazilian companies Reagen, Synth, and Polyfarma (São Paulo). Xylan from birchwood (Sigma catalog no. X-0502), soluble starch (Reagen catalog no. 8412), pectin from citrus (Sigma catalog no. P-9135), cellulose (Sigma catalog no. C-6288), microcrystalline cellulose (Merck catalog no. 2331), carboxymethylcellulose (Polyfarma catalog no. 1221), acid-swollen cellulose (obtained from microcrystalline cellulose [Merck catalog no. 2331] as described previously [28]), maltose (Sigma catalog no. M-5885), D-cellobiose (Sigma catalog no. C-7252), D-glucose (Synth catalog no. 32850), D-xylose (Sigma catalog no. X-1500), L-arabinose (Sigma catalog no. A-3256), and D-galacturonic acid (Sigma catalog no. G-2125) were used.

Statistics. Each analysis was carried out four to nine times. The results are expressed as means ± standard deviations and were subjected to the Kruskal-Wallis test followed by the nonparametric Dunn's test for multiple-column comparisons or to the Mann-Whitney test for two-column comparisons (30).

RESULTS

We used several polysaccharides as sole carbon sources for *L. gongylophorus* CCT 6414. Depending on the substrate added to YNB medium, the cell mass variation was 1.02 to 1.77 (Table 1). Growth was significantly faster on xylan or starch than on pectin or different sources of cellulose. In a control experiment with YNB alone, the cell mass variation was 0.65,

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TABLE 1. Cell mass variation and reducing sugars and enzymes produced after 30 days of cultivation of *L. gongylophorus* CCT1 on polysaccharides

Carbon source	Cell mass variation [mean (SD)][a]	Reducing sugar production (mg) [mean (SD)][a]	Enzyme activity[b] (μmol/g/h) [mean (SD)][a]
Xylan	1.8 (0.13)[1]	47 (5.8)[1,2]	190 (56)[1]
Starch	1.7 (0.15)[1]	44 (26)[1,2]	620 (250)[1]
Pectin	1.3 (0.25)[1,2]	190 (25)[1]	4,600 (1,100)[2]
Cellulose	1.0 (0.06)[2]	ND[c]	ND
Acid-swollen cellulose	1.1 (0.28)[2]	ND	ND
Microcrystalline cellulose	1.0 (0.13)[2]	1.4 (1.1)[2d]	23 (5.7)[1d]
Carboxymethylcellulose	1.1 (0.11)[2]	29 (3.0)[2d]	120 (90)[1d]
None	0.65 (0.10)		

[a] Values followed by distinct numbers are significantly different from each other (Dunn's test).
[b] Enzyme activities assayed were xylanase after growth on xylan, amylase after growth on starch, pectinase after growth on pectin, CMCase after growth on carboxymethylcellulose, and cellulase after growth on acid-swollen cellulose or microcrystalline cellulose.
[c] ND, not detected.
[d] Detected in 60-day cultures but not in 30-day cultures.

probably because of the consumption of stored fungal nutrients.

After cultivation, reducing sugars were detected in culture media with xylan, starch, or pectin but not in culture media with different sources of cellulose. However, reducing sugars could be detected in culture media with microcrystalline cellulose or carboxymethylcellulose, in which cultivation was extended for 60 days (Table 1).

Enzyme activity was also determined in culture media after 30 days of cultivation (Table 1). After cell growth on pectin, pectinase production was significantly higher than amylase or xylanase production after culture on starch and xylan, respectively. Cellulase or CMCase were not detected after 30 days of cultivation on cellulose or its derivatives but could be estimated after 60 days of cultivation.

Further information on *L. gongylophorus* metabolism was obtained by culturing the fungus in some of the polysaccharide hydrolysis products, the consumption of which was 57 to 170 mg (Table 2). Cell mass variation and growth efficiency on these substrates varied, with high mean values for glucose and xylose, intermediate values for maltose and cellobiose, and low values for arabinose and galacturonic acid.

Cellulose metabolism was also evaluated in other strains of *L. gongylophorus*. As was observed with CCT1, CCT2 and CCT3 grew significantly more slowly on cellulose than on glucose (Table 3) and failed to produce cellulase or detectable amounts of reducing sugars after 30 days of cultivation on cellulose.

DISCUSSION

Leaf-cutting ants must feed on their symbiotic fungus to survive. Their dependence is such that the insect population size is affected by the fungal growth rate (24). Thus, there must be a means to transfer carbon from the vegetation to the fungus and then to the ants.

Cellulose is often thought to be the central carbon source for this integration (8). However, our results showed that *L. gongylophorus* CCT 6414 grows poorly on cellulose, acid-swollen cellulose, microcrystalline cellulose, or carboxymethylcellulose (Table 1). Strains CCT 6415 and CCT 6416 also grew poorly on cellulose (Table 3), indicating that a poor ability to metabolize this polysaccharide is characteristic of the fungus symbiotic with *A. sexdens* L. Microbial degradation of cellulose involves endoglucanase, which randomly cleaves the polysaccharide; exoglucanase, which breaks cellulose ends to originate cellobiose and glucose; and β-glucosidase, which hydrolyzes cellobiose (25). Since the fungus grows well on cellobiose and glucose but slowly on cellulose (Tables 1 and 2), poor cellulose assimilation is secondary to low glucanase production. Inefficient metabolism of cellulose from various sources by *L. gongylophorus* suggests a different scenario from that drawn by Martin and Weber (15), who credit the symbiotic fungus of *Atta colombica tonsipes* with the consumption of at least 45% of the cellulose from leaves during vegetation processing in the ants' nest.

Unlike cellulose, starch and xylan are likely to be rapidly consumed from vegetation by *L. gongylophorus*. The fungus could efficiently hydrolyze these polysaccharides (Table 1) and assimilate the resulting xylose, maltose, and glucose (Table 2). Cell mass production from xylan or starch was at least 13 times faster than that from cellulose. A high growth rate on xylan and starch may be important for the fungus to outcompete other microorganisms in ant nests, such as bacteria (2, 19), yeasts (7), and other fungi (11), some of which are able to metabolize leaf polysaccharides (2, 22, 23). Leaves may contain up to 10% starch (6) and 22% xylan (12), which could support fungal growth in ant nests. Since ant nutrition relies on the fungus,

TABLE 2. Cell mass variation, substrate consumption, and growth efficiency after 30 days of cultivation of *L. gongylophorus* CCT1 on polysaccharide hydrolysis products

Carbon source	Cell mass variation [mean (SD)][a]	Consumption (mg) (mean [SD])[a]	Growth efficiency [mean (SD)][a]
Glucose	1.9 (0.32)[1]	170 (26)[1]	12 (3.1)[1]
Xylose	1.8 (0.41)[1]	170 (42)[1]	12 (4.3)[1]
Maltose	1.5 (0.29)[1,2]	150 (21)[1,2]	4.7 (1.4)[1,2]
Cellobiose	1.4 (0.12)[1,2]	150 (21)[1,2]	4.4 (0.9)[1,2]
Arabinose	1.1 (0.08)[2]	57 (17)[2]	3.4 (2.1)[2]
Galacturonic acid	1.1 (0.05)[2]	130 (23)[1,2]	0.8 (0.6)[2]

[a] Values followed by distinct numbers are significantly different from each other (Dunn's test).

TABLE 3. Cell mass variation after 30 days of cultivation of different *L. gongylophorus* strains on glucose or cellulose

Carbon source	Cell mass variation [mean (SD)][a]		
	CCT 6414	CCT 6415	CCT 6416
Glucose	1.9 (0.32)[1]	1.9 (0.22)[1]	1.9 (0.18)[1]
Cellulose	1.0 (0.06)[2]	1.1 (0.18)[2]	1.1 (0.05)[2]

[a] Values followed by distinct numbers are significantly different from each other (Mann-Whitney test).

xylan and starch are probably key carbon sources for insect survival as well.

We were surprised at the high levels of pectinase that we found and the efficient hydrolysis of pectin by the fungus (Table 1). The levels of pectinase secreted were more than 7 times higher than those of amylase, 24 times higher than those of xylanase, 38 times higher than those of CMCase, and 200 times higher than those of cellulase. Nevertheless, pectin did not support rapid fungal growth (Table 1), a fact possibly explained by the low efficiency of growth on galacturonic acid (Table 2), which is the major pectin component (12).

Pectin is located in the intercellular space and acts as a cement to aggregate leaf cells (29). Pectin degradation separates plant cells from each other (5, 10) and has been suggested as essential for fungal invasion of plant tissue (5, 9). Thus, the high levels of pectinase and pectin degradation may be primarily used by *L. gongylophorus* to macerate leaf tissue and access its nutrients. Pectin from leaves, however, is probably poorly assimilated by the fungus.

Once *L. gongylophorus* degrades and assimilates cellulose, xylan, pectin, and starch, it is able to mediate the transferring of carbon from leaves to the ants. This metabolic integration may provide ants with solid plant material not otherwise available to them. It is important to note that there are factors that may influence the ants' utilization of leaf polysaccharides through the fungus, including the kind of vegetation cut by the ants, the modulation of fungal metabolism by the ants, compounds liberated during leaf maceration, or competition by other microorganisms for the leaf polysaccharides. However, if *L. gongylophoprus* behavior in laboratory cultures typifies the fungal role in the symbiosis, then xylan and starch rather than cellulose would be the main leaf polysaccharides supporting ant nutrition.

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