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ORIGINAL ARTICLE

Growth and enzymatic activity of *Leucoagaricus gongylophorus,* a mutualistic fungus isolated from the leaf-cutting ant *Atta mexicana,* on cellulose and lignocellulosic biomass

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Significance and Impact of the Study: According to the best of our knowledge, this is the first report about the growth of *Leucoagaricus gongylophorus,* isolated from the colony of the ant *Atta mexicana,* on semisolid medium with cellulose and solid-state cultures with lignocellulosic materials. The maximum CO_2 production rate on grass was three times higher than on sugarcane bagasse. Endoglucanase activity was detected and it was possible to recover glucose from the fungal gongylidia. The cellulolytic activity could be used to process lignocellulosic residues and obtain sugar or valuable products, but more work is needed in this direction.

Keywords
Atta mexicana, cellulose, *Leucoagaricus gongylophorus*, lignocellulosic biomass, solid-state culture.

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Abstract

A mutualistic fungus of the leaf-cutting ant *Atta mexicana* was isolated and identified as *Leucoagaricus gongylophorus.* This isolate had a close phylogenetic relationship with *L. gongylophorus* fungi cultivated by other leaf-cutting ants as determined by ITS sequencing. A subcolony started with ~500 *A. mexicana* workers could process 2 g day^{-1} of plant material and generate a 135 cm^3 fungus garden in 160 days. The presence of gongylidia structures of ~35 μm was observed on the tip of the hyphae. The fungus could grow without ants on semi-solid cultures with α-cellulose and microcrystalline cellulose and in solid-state cultures with grass and sugarcane bagasse, as sole sources of carbon. The maximum CO_2 production rate on grass (V_{max} = 17·5 mg CO_2 L_g^{-1} day^{-1}) was three times higher than on sugarcane bagasse (V_{max} = 6·6 mg CO_2 L_g^{-1} day^{-1}). Recoveries of 32·9 $mg_{glucose}$ $g_{biomass}^{-1}$ and 12·3 $mg_{glucose}$ $g_{biomass}^{-1}$ were obtained from the fungal biomass and the fungus garden, respectively. Endoglucanase activity was detected on carboxymethylcellulose agar plates. This is the first study reporting the growth of *L. gongylophorus* from *A. mexicana* on cellulose and plant material.

Introduction

Trophic mutualism involves complementary interactions between different species to obtain energy and nutrients from the environment. Among these, the fungus-farming ants originated around 50 million years ago with the cultivation of a diverse subset of fungal species followed by further transitions to the farming of a single fungal species, probably around 10 to 15 million years ago, resulting in complex associations in which the leaf-cutting ants cultivate fungal gardens for food (Schultz and Brady 2008; Nygaard *et al.* 2016). Plant material is transformed into the feed source for ant colonies that would otherwise be unable to access the nutrients present in plant biomass. The microbial role among this complex mutualistic association that allows the availability of nutrients and the survival of ant colonies is of high relevance. Leaf-cutting ants can be easily identified as they form long lines and

carry pieces of plant material to their nests which consist in complex underground tunnels and fungus chambers housing thousands of individuals.

Mexico hosts the species *Atta mexicana* (Smith), *A. cephalotes* (Linnaeus) and *A. texana* (Buckley), where the first one has the biggest presence, from Texas and Arizona in the North to Guatemala and Belize in the South (Byars 1949; Mueller *et al.* 2011). In some regions, queen ants, traditionally called Chicatanas, have gastronomic value and are therefore captured when they emerge from the nest to begin their nuptial flight. It is calculated that a mature colony of leaf-cutting ant can process up to 0·5 ton of plant material per year (De Fine and Boomsma 2010). Leaf-cutting ants colonies are therefore also considered plagues as they affect agricultural systems by attacking crops. In particular, *A. mexicana* ants are frequently responsible for significant losses in citrus, cacao, coffee, maize, ornamental plants and Amaranth cultivations (Vázquez-Franco *et al.* 2014).

The fresh foliar material collected by the ants is used to cultivate the fungus *Leucoagaricus gongylophorus* (Möller) Singer, establishing a mutualistic relationship, where the fungus provides the necessary enzymes for the degradation of the structural polymers of the plant material (De Siqueira *et al.* 1998; Silva *et al.* 2006). The fungus covers its nutrient and energy requirements consuming a fraction of the carbohydrates released and produces specialized hyphal swellings called gongylidia, which contain a mixture of carbohydrates, amino acids, proteins, lipids, and vitamins, that serve as food to the adult ants and larvae. Gongylidia also contain a set of enzymes involved in the degradation of polysaccharides, plant toxins, and proteins. The ants eat the gongylidia and carry fresh leaves they chew in small pieces and inoculate with faecal droplets that vector the same enzymes present in the fungal gongylidia. The plant material is therefore pretreated with the same enzymes that the fungus expresses in the gongylidia and that the ants eat (Rønhede *et al.* 2004; Aylward *et al.* 2015). The leaf pieces are stacked into the nest chambers, leaving behind void spaces that allow airflow to maintain gas exchange and a temperature around 26°C. The fungus garden has a pH ~5·0, which altogether with the antibiotics secreted by bacterial exosymbionts hosted by the ants as *Streptomyces* sp. and the cleaning labour of the ants, generate favourable conditions to control the proliferation of opportunistic micro-organisms (Seipke *et al.* 2011).

The fresh foliar material incorporated into the top strata of the fungus garden is progressively degraded forming a vertical gradient of biomass degradation products and nutrients throughout all strata up to the lower level. It has been shown that *L. gongylophorus* produces distinct enzymatic cocktails throughout these different strata (Aylward *et al.* 2013; Huang *et al.* 2014). The relation between leaf-cutting ants and *L. gongylophorus* is the product of millions of years of evolution and has been optimized to provide sufficient and continuous supply of energy sources and nutrients both for the ants and for the fungus from lignocellulosic substrates. This symbiotic association has therefore been considered as a model for the fundamental understanding of plant biomass deconstruction in natural ecosystems and also a potential source of new enzymes for lignocellulosic biomass hydrolysis.

The ability of *L. gongylophorus* to degrade lignocellulosic materials has not been clearly demonstrated. Some authors suggest that yeasts and bacteria that live in the nest have an important role in the degradation of the vegetal material (Richard *et al.* 2005; Silva *et al.* 2006; Lange and Grell 2014). Other authors report that the fungus does not metabolize cellulose under laboratory conditions nor in the nest (Abril and Bucher 2002). Studies supported on classic enzymatic assays and 'Omics' tools have shown that *L. gongylophorus* can produce Carbohydrate-Active Enzymes (CAZymes) such as cellulase, xylanase, glucosidase, rhamnosidase and β-Galactosidase and Fungal Oxidative Lignin-degrading enzymes (FOLymes) such as laccase and glyoxal oxidase (De Siqueira *et al.* 1998; Erthal *et al.* 2004; Schiøtt *et al.* 2008; Bacci *et al.* 2013). Aylward *et al.* (2013) have published the first draft of the genome sequence of *L. gongylophorus*, symbiont of *A. cephalotes* ants from Panama. They found 145 genes associated to lignocellulosic biomass degrading enzymes, including carbohydrate hydrolases and laccases, using genomics and metaproteomics tools. Taken together these results show, on the one hand, the ability of the cultivated fungi to provide food and energy for ant colonies from lignocellulosic material and, on the other hand, the potential of the fungus as a source of enzymes with possible biotechnological applications.

Here, the symbiotic fungus of the leaf-cutting ant *A. mexicana* was isolated from a laboratory fungal garden and identified as *L. gongylophorus*. The ability of the isolated fungus to grow under laboratory conditions (i) on cellulose as sole source of carbon in semisolid medium and (ii) in solid state cultures on fresh foliar material and lignocellulosic biomass was tested and demonstrated. Additionally, endoglucanase activity was detected and glucose production quantified.

Results and discussion

Development of *Atta mexicana* laboratory fungal gardens

The *A. mexicana* ants transported and deposited fresh foliar material on the top of the garden leaving void

spaces that allowed gas interchange. The fungal garden obtained in the laboratory had a sponge-like structure and white fungal mycelium was visible over the plant material. The subcolony with ~500 workers was able to process 2 g of plant material per day and the nest container was 90% full after 160 days corresponding to ~135 cm^3 of fungus garden (see Fig. S1). Laboratory colonies of leaf-cutting ants have been developed and maintained at ~25°C and ~70% relative humidity by supplying different feeds including rose petals and leaves, apple, orange peel and oat (Weber 1957; Grell *et al.* 2013). These constructed laboratory colonies are useful to elucidate different aspects of the ant- fungus symbiosis. For example, Schiøtt *et al.* (2010) used *Acromyrmex echinatior* (Forel) laboratory colonies and proteomics to describe the life cycle of ant faecal droplet proteins that are initially produced in the fungus gongylidia, passed through the ant digestive system and released onto the new plant substrate to form a leaf pulp manure that the ants add to the fungus garden. Aylward *et al.* (2013) conducted metaproteomic studies on garden material collected from *A. cephalotes* and *Acromyrmex echinatior* laboratory colonies showing that *L. gongylophorus* produces distinct sets of lignocellulolytic enzymes throughout the different stages of biomass degradation in the fungus garden.

Isolation and molecular identification of the *Atta mexicana* fungal mutualistic symbiont

Pure cultures of the fungus were obtained both in potato dextrose agar (PDA) and modified malt extract agar (MEA-LP). Fungal growth was visible from day 5, and the white colonies reached a diameter of around 5 mm after 12 days (see Fig. 1). Fungal growth was slightly more abundant in MEA-LP than in PDA reaching average diameters of 15 mm and 13 mm in these media after 28 days, respectively. As expected, the observed colony morphology was similar to fungi isolated from other leaf-cutting ant, showing white irregular colonies with filamentous structures and umbonate elevation with some white spots around the periphery of the colony (Lugo *et al.* 2013). The isolated fungus formed an abundant and thin mycelium (length ~3 μm) and produced clusters of hyphal tip swellings called gongylidia of about ~35 μm in diameter, these structures are typical from ants fungal symbionts. Masiulionis *et al.* 2014 have reported that gongylidia are morphologically very similar among colonies of diverse leaf-cutting ant species, but could differ significantly in their size, depending on the developmental stage of the colony, the amount of available nutrients and the geographic locations. According to these authors, the diameter of gongylidia in *Trachymyrmex fuscus* (Emery) gardens is ~55 μm, *A. laevigata* (Smith) ~40 μm, *A. sexdens* (Linnaeus) ~30 μm, and the gongylidia-like structures of *Mycocepurus smithii* (Forel) have a diameter of ~20 μm. Several media have been reported for the isolation and maintenance of *L. gongylophorus*, such as PDA, malt extract agar, MEA-LP, Pagnocca A and B, and yeast nitrogen base using glucose as carbon source with and without chloramphenicol (Weber 1957; Silva-Pinhati *et al.* 2004; Miyashira *et al.* 2010). A comparison of radial growth rate of the mutualistic fungus of *A. sexdens* in MEA-LP and Pagnocca B media did not detect any significant difference in these two media (Miyashira *et al.* 2010). Lugo *et al.* (2013) reported greater growth of a *L. gongylophorus* isolate obtained from *Acromyrmex lobicornis* (Emery) on malt extract agar compared to PDA with colony diameters of 53 mm and 40 mm respectively after 3 months.

ITS sequencing was used to identify the isolated fungus named LEU18496. The PCR amplified fragment spanned the ITS1 and ITS2 region and the obtained fragment was approximately 750 bp long. The obtained sequence was registered in the GenBank database with accession number KJ419350.1. The comparison in the

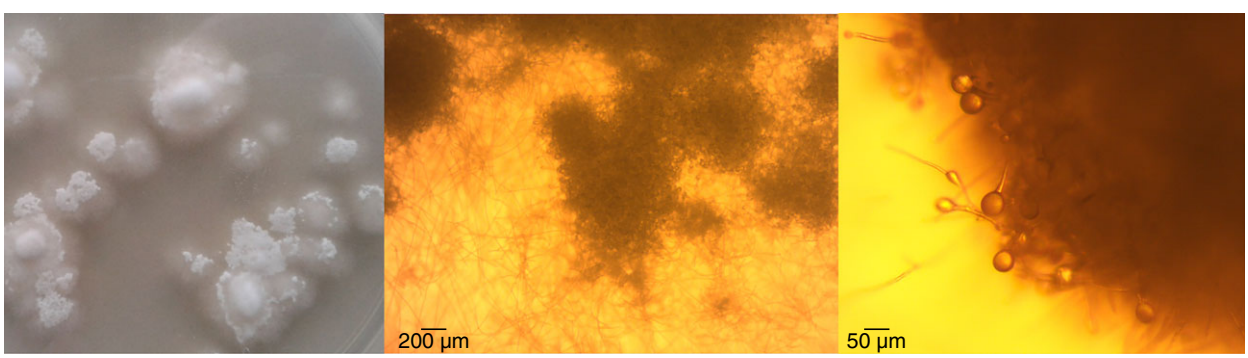

Figure 1 Growth of *Leucoagaricus gongylophorus* on semi-solid cultures of PDA (left), thin mycelium with cluster formation (center) and hyphal tips gongylidia accumulation of ~35 μm (right). [Colour figure can be viewed at wileyonlinelibrary.com]

NCBI database through BLAST showed 97% similarity with *L. gongylophorus* isolates cultivated by *A. cephalotes* and *Acromyrmex echiniator* ants from Panama. Cazin *et al.* (1989) have reported the isolation of the symbiotic fungus from an *A. mexicana* nest in Arizona and its growth on PDA medium and Sabouraud dextrose broth supplemented with different vegetable oils, but the isolate was not identified, and no ITS sequences could be found in public databases for this isolate. Mueller *et al.* (2011) used polymorphic microsatellite markers to study the coevolutionary patterns of the fungi cultivated by North American leaf-cutting ant populations (Mexico, Cuba, USA) and found no genetic difference between the fungi cultivated by *A. insularis* (Güerin) from Cuba and *A. mexicana* from Mexico, showing that fungi may disperse through long distances independently of the ant. In this work, the fungi were not isolated from the ants.

The phylogenetic tree (Fig. 2) based on ITS sequences showed that the LEU18496 isolate grouped with the other described *L. gongylophorus* symbionts. The mushroom *Leucoagaricus leucothites* (Vittad.) Wasser was chosen as the outgroup. *L. gongylophorus*, sometimes called *Leucocoprinus*, belongs to the phylum *Basidiomycota* and family *Agaricaceae* (Weber 1957; Silva *et al.* 2006). Silva-Pinhati *et al.* 2004 found that mutualistic fungus of leaf-cutting ant living in distant geographic sites have a low variation in ribosomal DNA and ITS, suggesting a single fungal species made up of closely related lineages of *L. gongylophorus*.

Growth of the fungal symbiont in semi-solid and solid-state cultures

Figure 3 shows the growth of *L. gongylophorus* in semi-solid cultures using α-cellulose or microcrystalline cellulose as carbon source. After 20 days, fungal biomass was twice higher in the cultures supplemented with peptone and yeast extract in addition to α-cellulose or microcrystalline cellulose as compared to those cultures containing only α-cellulose or microcrystalline cellulose in mineral medium. On the other hand, no significant difference was observed for the biomass in the cultures that contained α-cellulose or microcrystalline cellulose. De Siqueira *et al.* (1998) have shown that *L. gongylophorus* metabolizes plant polysaccharides, xylan, starch, pectin and cellulose; however, the latter is poorly degraded and assimilated by the fungus. Cellulose may therefore not be the central carbon source for the symbiosis between leaf-cutting ants and their fungi.

The experiments in microcosms showed that *L. gongylophorus* could grow in solid-state culture using either grass or sugarcane bagasse as substrate, added with mineral medium. The growth of *L. gongylophorus* after 20 days was visibly better in the cultures in grass compared to sugarcane bagasse (see Fig. S2). Due to the difficulty in separating the fungal biomass and the plant material, growth was determined by monitoring the CO_2 production as indirect estimation of the fungal growth. These experiments were conducted in closed flasks, where the CO_2 accumulation was monitored. Figure 4 shows the

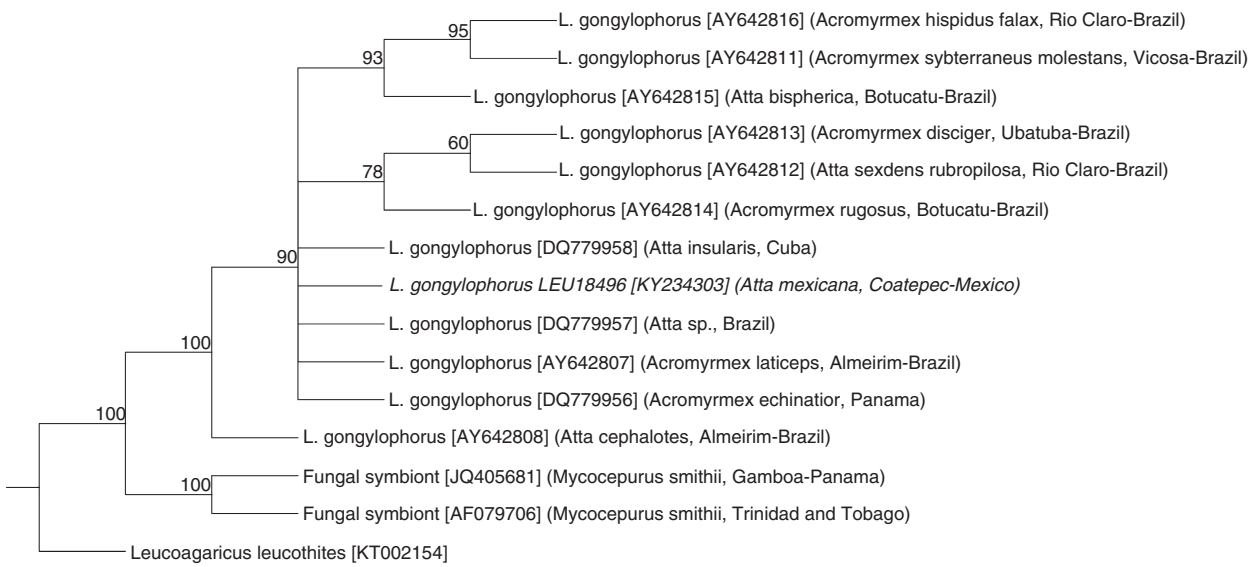

Figure 2 Phylogenetic tree based on ITS sequences showing phylogenetic relationship between *Leucoagaricus gongylophorus* LEU18496 and other symbionts from different leaf-cutting ants (the corresponding sequences were taken from GenBank). Numbers on branches indicate bootstrap support values.

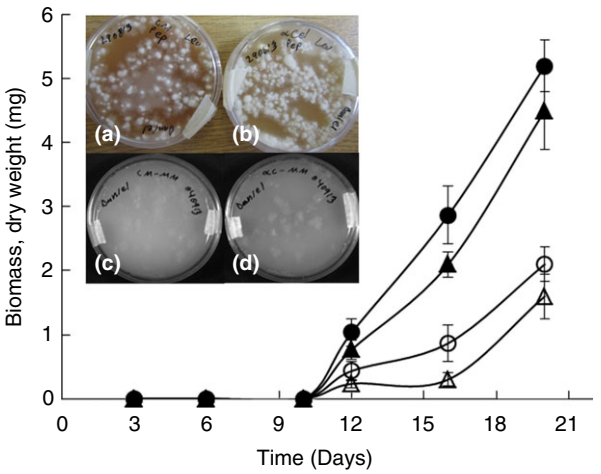

Figure 3 *Leucoagaricus gongylophorus* semi-solid cultures after 20 days of growth in the presence of microcrystalline cellulose (a and c; triangles) or α-cellulose (b and d; circles) as sole source of carbon; with peptone and yeast extract enriched medium (a and b; closed symbol) or mineral medium (c and d; open symbol). Bars represent standard error of the mean. [Colour figure can be viewed at wileyonlinelibrary.com]

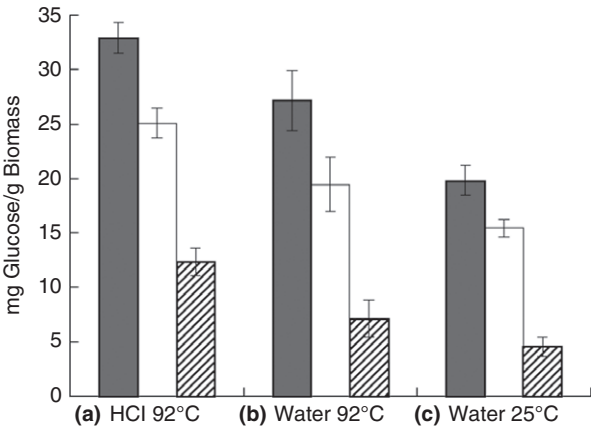

Figure 5 Glucose recovery from fungal biomass produced in the MEA-LP medium. MEAN2: fungal biomass pulverized with liquid N_2 and freeze-dried; MEA: fungal biomass produced by freeze drying only; GardenN2: fungal biomass from the fungus garden pulverized with liquid N_2 produced. Incubation conditions for the recovery of glucose: (a) 0·6 mol l^{-1} HCl at 92°C; (b) ultrapure water at 92°C; or (c) ultrapure water at 25°C. Error bars represent standard error of the mean. ■ MEAN2, □ MEA, ▨ GardenN2.

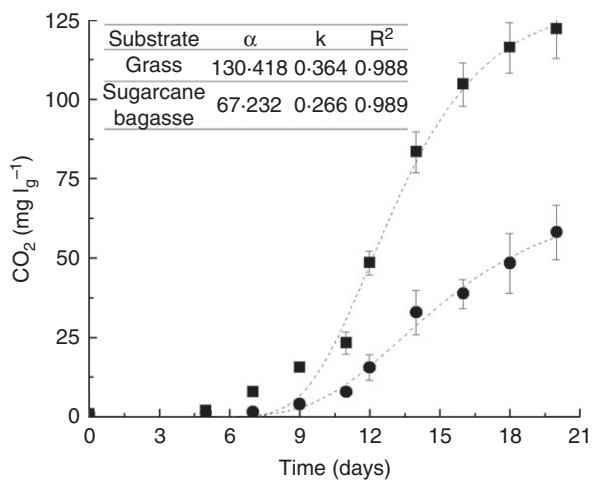

Figure 4 CO_2 production by *Leucoagaricus gongylophorus* in solid state cultures using either grass (square) or sugarcane bagasse (circles) as substrate. The dashed lines correspond to the Gompertz model fit. Bars represent standard error of the mean.

kinetics of CO_2 production, the maximum production rate of CO_2 in grass (V_{max} = 17·5 mg CO_2 L_g^{-1} day^{-1}) was almost threefold that obtained with sugarcane bagasse (V_{max} = 6·6 mg CO_2 L_g^{-1} day^{-1}). The CO_2 concentration increased from day 5, reaching values of 58·1 and 122 mg CO_2 L_g^{-1} on day 20, for sugarcane bagasse and grass respectively. This variation could be attributed to the difference in composition of these plant materials. While grass contains around 5% lignin and other nutrients, such as other polysaccharides, proteins and vitamins from the cytoplasmic material; sugarcane bagasse has less available nutrients and a higher lignin content (~15%) (Sun and Cheng 2002). CO_2 evolution is often used for the indirect estimation of fungal growth in solid-state cultures (Auria *et al.* 1993). Here, the CO_2 production of *L. gongylophorus* was proportional to the biomass production when grown on glucose, which means that the CO_2 production correlates with the fungal symbiont growth rate (Fig. S3). These results indicate that it is possible to cultivate *L. gongylophorus* on lignocellulosic substrates in solid-state cultures without the presence of ants. Natural fungal gardens could be considered as efficient solid-state bioreactors. Somera *et al.* (2015) confirmed that the grass-cutting *A. bisphaerica* (Forel) fungus garden operates as a biphasic solid-state mixed bioreactor that converts plant biomass to polyols.

Glucose recovery and endoglucanase activity

Glucose accumulates in the gongylidia in the form of glycogen. Figure 5 shows the glucose recovery from fungal biomass using different extraction protocols. Pulverization with liquid N_2 and hydrolysis with 0·6 mol l^{-1} HCl at 92°C allowed the recovery of 32·9 ± 1·4 $mg_{glucose}$ $g_{biomass}^{-1}$ for the fungus cultivated in MEA-LP. Using the same treatment on biomass extracted from the fungus garden, 12·3 ± 1·3 $mg_{glucose}$ $g_{biomass}^{-1}$ were recovered. However, it should be noted that this sample not only contained fungal biomass but some plant material as

well. Silva *et al.* (2003) reported that fungus garden material from *A. sexdens* nests and laboratory cultures of the fungus *L. gongylophorus* CCT 6414 contained an average of $27{\cdot}7 \pm 5{\cdot}3$ $mg_{glucose}\ g_{biomass}^{-1}$ and $4{\cdot}3 \pm 0{\cdot}9$ $mg_{glucose}\ g_{biomass}^{-1}$, respectively. These authors disrupted the biomass with liquid N_2 and extracted with ultrapure water, however their results were reported as wet weight, making it difficult to compare with our results determined as dry weight. *L. gongylophorus* has the ability to store glycogen in gonglydia, which serves to feed the ants. Powell and Stradling (1986) have shown that the type of substrate, pH, and temperature affect the size and production of gongylidia on semi-solid cultures.

Concerning endoglucanase activity, hydrolysis halos corresponding to carboxymethyl cellulase activity were found in *L. gongylophorus* LEU18496 enzymatic extracts obtained after growth on different carbon sources. The clear zones attributed to the hydrolysis of CM-cellulose were visible in all the samples (see Fig. S4) while the greater hydrolysis halos were observed in the sugarcane bagasse extracts ($30 \pm 0{\cdot}4$ mm diameter) and grass ($37 \pm 0{\cdot}3$ mm diameter). The determination of hydrolytic activities in plates is a fast, economic and sensible method that allows to qualitatively verify the presence of different types of enzymatic activities. Different substrates such as CM-cellulose, pectin, xylan and lignin have been reported as adequate to test by this method (Badel *et al.* 2011).

According to the best of our knowledge, this is the first report of the growth of *L. gongylophorus* without ants on solid-state cultures with lignocellulosic biomass (grass or sugarcane bagasse). The metabolic ability of this fungus to degrade plant materials could be used to process lignocellulosic residues and obtain sugars or valuable products.

Materials and methods

Development of *Atta mexicana* laboratory fungal gardens

The leaf-cutting ant colonies were initiated from fertilized *A. mexicana* queens captured in Coatepec, Veracruz (México). Each queen was placed in 150 cm^3 containers using sterile perlite soaked in water (64% w/v). The samples were kept in the dark at 26°C. When the worker ants emerged, the 150 cm^3 recipients were placed open in 1000 cm^3 containers to allow foraging. The colonies were fed twice a week with fresh rose leaves. A sub-colony without queen was prepared by extracting ~15 cm^3 of the developed fungus garden together with ~500 individual working ants and introduced into 150 cm^3 containers protect from light and connected to a second container ($22 \times 16 \times 6$ cm) via a tube. The second container served as foraging area, where the rose leaves and vials with water were provided once a week. These systems were maintained at 26°C with alternating 12 h dark-light periods.

Isolation and molecular identification of the *Atta mexicana* fungal mutualistic symbiont

Pellets collected from the fungus garden developed by the sub-colony were inoculated on PDA and MEA-LP. The MEA-LP medium contained (g l^{-1}): malt extract 20, bacteriological peptone 5, yeast extract 2 and agar 20, and the pH was 5·0 (Miyashira *et al.* 2010). Cultures were incubated at 26°C in the dark. The isolated white fungus was propagated on PDA at 26°C. Fungal suspensions were obtained by adding a few millilitres of a 0·1% (v/v) Tween 80 solution and scrapping off the agar surface with glass beads. The biomass present in fungal suspensions was determined by determining the total protein by the Bradford method, and the inoculum was standardized to contain ~2 mg protein ml^{-1}. The strain was maintained at −80°C in filter paper strips cultures recovered from PDA medium and soaked in glycerol 20% (v/v). Microscopy images of the fungus were obtained with a Carl Zeiss (Güttingen, Germany) microscope equipped with an AxioCam ERc 5s camera operated with ZEN software (Jena, Germany) and using a 10× dry lens objective.

Genomic DNA was extracted from the isolated white fungus using the ZR Fungal/Bacterial DNA MiniPrep kit (Orange, CA, USA). The ITS1 and ITS4 primers were used to amplify the internal transcribed spacers (ITS) regions of the fungal ribosomal DNA (http://sites.biology.duke.edu/fungi/mycolab/primers.htm). The PCR products from two different reactions were cloned into the pGEM-T Easy vector. The ITS regions were re-amplified from isolated plasmids, purified and sent out for sequencing at the Instituto de Biotecnología-UNAM. The CLUSTAL W software was used to perform the alignment of multiple sequences and a phylogenetic tree was constructed using the Tamura-Nei evolution model under neighbour joining criteria as implemented in the Geneious 10.0.2 software (Auckland, New Zealand) with 1000 bootstrap replications.

Growth of the fungal symbiont in semi-solid and solid-state cultures

The mineral medium contained (g l^{-1}): $NaNO_3$ 6, KH_2PO_4 1·3, $MgSO_4{\cdot}7H_2O$ 0·38, $CaSO_4{\cdot}2H_2O$ 0·25, $CaCl_2$ 0·055, and 4 ml l^{-1} of solution of trace elements containing (g l^{-1}) $FeSO_4{\cdot}7H_2O$ 0·015, $MnSO_4{\cdot}7H_2O$ 0·012, $ZnSO_4{\cdot}7H_2O$ 0·013, $CuSO_4{\cdot}7H_2O$ 0·0023, and $CoCl_2{\cdot}6H_2O$ 0·0015. The pH of medium was 5·0. The semi-solid cultures contained noble agar (20 g l^{-1}) and

mineral medium or peptone-yeast extract (5 or 2 g l^{-1} respectively). Two different carbon sources were tested in the mineral medium: α-cellulose (CAS 9004-34-6) and microcrystalline cellulose (CAS 9004-34-6), both at 20 g l^{-1}. Petri dishes were inoculated with 100 μl of fungal suspension, and incubated at 26°C.

Solid-state cultures were performed in microcosms in 125-ml flasks sealed with inert valves, containing 2 g of grass or sugarcane bagasse and 5 ml of mineral medium. After sterilization at 121°C for 15 min, the flasks were inoculated with 1 ml of standardized fungal suspension (1·88 mg protein ml^{-1}), and the cultures were incubated at 26°C. Control cultures were carried out with glucose (20 g l^{-1}), and without carbon source. All experiments were performed in triplicate with the same fungal isolate (technical replicates). The CO_2 concentration was monitored by gas chromatography and the maximum CO_2 production rate in the microcosms was calculated with the integrated Gompertz model, $V_{max} = 0{\cdot}368\ \alpha\ k$, where α = maximum CO_2 concentration (mg CO_2 L_g^{-1}); k = CO_2 production rate constant ($days^{-1}$). The parameters of the model were calculated using the OriginPro 8SR0 software (Northampton, MA, USA). The CO_2 concentration was determined by injecting 200 μl of headspace with a precision syringe into a GOW MAC 580 gas chromatograph equipped with a thermal conductivity detector and a Poropack column. The operating conditions were; injector at 50°C, oven at 40°C, detector at 115°C, and a flowrate of 4·4 ml min^{-1} (Vigueras *et al.* 2014). All assays were performed in duplicate.

Recovery of glucose and endoglucanase activity

The recovery of glucose from fungal biomass was extracted and quantified according to modified protocols (Silva *et al.* 2003; Lanham *et al.* 2012). After 30 days, a first fungal biomass sample (MEA) produced in MEA-LP agar was collected and freeze-dried. A second sample (MEA N2) also taken from MEA-LP agar was pulverized in a mortar with liquid N_2 before freeze-drying. Additionally, a fungal biomass sample (Garden N2) was also taken from the fungus garden and treated as above. Glucose was recovered by incubation at different temperatures for 1·5 h in an orbital shaker at 400 rev min^{-1} from ~50 mg of dried biomass suspended in 1 mL of either: (i) 0·6 mol l^{-1} HCl at 92°C, (ii) ultrapure water at 92°C, or (iii) ultrapure water at 25°C. The samples were then placed in an ice bath and centrifuged (8000***g*** for 5 min at 4°C). The glucose content was determined in the supernatant with a YSI 2900 Biochemistry Analyzer (Yellow Springs, OH, USA). All experiments and assays were performed in triplicate with the same fungal isolate (technical replicates), and controls without biomass were also included.

Endoglucanase activity was determined on CM-cellulose agar plates using Gram's iodine as described by Kasana *et al.* (2008). Extracts from semi-solid cultures were obtained by pressing into a syringe and centrifuged (8000***g*** for 10 min at 4°C). The obtained supernatant was then lyophilized and reconstituted with citrate buffer pH 5·3. 10 μl of enzymatic extract were spotted onto CM-cellulose agar plates containing (per litre): 2 g CM-cellulose, 1 g $NaNO_3$, 1 g K_2HPO_4, and 15 g of agar. The agar plates were incubated at 37°C under static condition for 12 h. The zones of hydrolysis (halos) were revealed with a Lugol solution containing 1 g of iodine and 2 g of potassium iodine per 100 mL. All assays were performed in triplicate.

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Conflict of Interest

The authors have no conflict of interest to declare.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Time course development of the laboratory fungus garden cultivated into 150 cm^3 containers by *Atta mexicana*, ~500 worker ants, fed twice a week with rose leaves.

Figure S2. Photograph of solid state cultures of *Leucoagaricus gongylophorus* after 20 days of growth using two different substrates grass (left) and sugarcane bagasse (right) added with mineral medium.

Figure S3. Correlation between CO_2 production and fungal biomass production in control cultures grown with glucose (20 g l^{-1}). All experiments were performed in triplicate. The CO_2 production from cultures without carbon source was subtracted.

Figure S4. Hydrolysis halos of endoglucanase activity on CM-Cellulose from extract of *Leucoagaricus gongylophorus* grown on solid state cultures with different carbon sources (a) microcrystalline cellulose; (b) α-cellulose, (c) sugarcane bagasse y (d) grass. Bars represent standard error of mean.