Research article

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In vitro culture of *Leucoagaricus gongylophorus* (Agaricaceae), symbiont fungus of the leaf-cutting ant *Atta mexicana* (Hymenoptera, Formicidae), using solid and plant-supplemented culture media

Cultivo *in vitro* de *Leucoagaricus gongylophorus* (Agaricaceae), hongo simbionte de la hormiga cortadora de hojas *Atta mexicana* (Hymenoptera, Formicidae), utilizando medios de cultivo sólidos y suplementados con material vegetal

Dennis Adrián Infante-Rodríguez[1,6,8], Alberto Carlos Velázquez-Narváez[2,6,8], Juan Luis Monribot-Villanueva[4], Gloria Carrión[5], Klaus Mehltreter[6], Jean-Paul Lachaud[3], José Antonio Guerrero-Analco[4,7], Jorge E. Valenzuela González[6,7]

Abstract:

Background and Aims: The mutualism between leaf-cutting ants and the fungus *Leucoagaricus gongylophorus* is a remarkable instance of insect-fungus symbiosis. In this study, we aimed to make a molecular identification of the *L. gongylophorus* strain obtained from the fungal garden of *Atta mexicana*, to compare the mycelial growth of the strain in several culture media and PDA medium enriched with foliar material from several plants and perform a cost analysis for the *in vitro* maintenance of the symbiont fungus.

Methods: Seven solid culture media were compared for the in vitro growth of *Leucoagaricus gongylophorus* isolated from an *Atta mexicana* nest. In addition, we compared the fungal growth on Potato Dextrose Agar (PDA) medium enriched with selected foliar material from the leaves of six plants previously known to be either well-foraged or avoided by *Atta mexicana*.

Key results: Higher mycelial growth percentages were obtained on compost extract added with a mineral mixture (CE) (27.3±12.7 mm) and PDA media (25.3±1.15 mm) at 28 days of growth. Furthermore, this is the first study reporting the growth of a fungal symbiont of *Atta mexicana* on CE, complete basic medium (CBM), Pagnocca A, and B media. PDA media enriched with some plants did not show advantages for fungus growth. Lower mycelial growth percentages were obtained on PDA media enriched with *Rosa alba* (0.0±0.0 mm), *Coffea arabica* (5.3±0.66 mm), *Citrus reticulata* (3.0±1.0 mm), and *Psidium guajava* (2.0±1.15 mm) leaves, in comparison with the control treatment (PDA medium).

Conclusions: The use of culture media like CE and CBM might be a cost-effective alternative for *in vitro* culture of *Leucoagaricus gongylophorus*, even in the absence of ants. Leaves of some plant species inhibit *in vitro* growth of this fungus, in line with their status as plants avoided by *Atta mexicana*. The strong inhibition of the extract of *Rosa alba*, the best-foraged plant by *Atta mexicana*, suggests the existence of particularly effective detoxification mechanisms in natural conditions.

Key words: fungal culture, growth media, mutualism, mycelial growth.

Resumen:

Antecedentes y Objetivos: El mutualismo entre las hormigas cortadoras de hojas y el hongo *Leucoagaricus gongylophorus* es un ejemplo notable de simbiosis entre insectos y hongos. En este estudio, nuestros objetivos fueron realizar la identificación molecular de la cepa de *L. gongylophorus* obtenida del jardín de hongos de *Atta mexicana*, comparar el crecimiento del hongo en varios medios de cultivo y en medio PDA enriquecido con material foliar de varias plantas y realizar un análisis de costos para el cultivo *in vitro* del hongo simbionte cultivado por *A. mexicana*.

Métodos: Se compararon siete medios de cultivo sólidos para el crecimiento *in vitro* de *Leucoagaricus gongylophorus* aislado de un nido de *Atta mexicana*. Además, comparamos el crecimiento de hongos en medio PDA enriquecido con material foliar seleccionado de hojas de seis plantas que previamente se sabía que eran bien aceptadas o evitadas por *Atta mexicana*.

Resultados clave: Se obtuvieron porcentajes de crecimiento micelial más altos en medios CE (27.3±12.7 mm) y PDA (25.3±1.15 mm) a los 28 días de crecimiento. Además, este es el primer estudio que informa el crecimiento del simbionte fúngico de *Atta mexicana* en medios CE, CBM, Pag A y Pag B. Los medios PDA enriquecidos con algunas plantas no mostraron ventajas para el crecimiento de hongos. Se obtuvieron menores porcentajes de crecimiento micelial en medios PDA enriquecidos con hojas de *Rosa alba* (0.0±0.0 mm), *Coffea arabica* (5.3±0.66 mm), *Citrus reticulata* (3.0±1.0 mm) y *Psidium guajava* (2.0±1.15 mm), en comparación con el tratamiento control (medio PDA).

Conclusiones: El uso de medios de cultivo como CE y CBM podría ser una alternativa de bajo costo para el cultivo *in vitro* de *Leucoagaricus gongylophorus*. Las hojas de algunas especies de plantas inhiben el crecimiento *in vitro* de este hongo de acuerdo con su condición de plantas evitadas por *Atta mexicana*. La fuerte inhibición del extracto de *Rosa alba*, la planta mejor forrajeada por *Atta mexicana*, sugiere la existencia de mecanismos de desintoxicación particularmente efectivos en condiciones naturales.

Palabras clave: crecimiento micelial, cultivo de hongos, medios de crecimiento, mutualismo.

[1]Universidad de Guanajuato, Departamento de Biología, División de Ciencias Naturales y Exactas, Noria Alta S/N, Noria Alta, 36050 Guanajuato, Guanajuato, Mexico.
Continuation of the affiliation addresses on the next page.

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Introduction

The term "mutualism" can be more precisely defined as an interaction between individuals of different species that results in positive (beneficial) effects on *per capita* reproduction and/or survival of the interacting populations (Holland and Bronstein, 2008). As in other interspecific interactions, the degree of dependency of each mutualist upon the other can be classified as obligate or facultative. Consequently, mutualistic interactions can be classified as obligate-obligate, obligate-facultative, or facultative-facultative (Holland and Bronstein, 2008). Fungus-farming by leaf-cutting ants from the tribe Attini (hereafter referred to as LCA) is a highly specialized relationship involving species of the myrmicine ant genera *Acromyrmex* (Mayr, 1865) and *Atta* (Fabricius, 1805) and the fungus *Leucoagaricus gongylophorus* (A. Möller) Singer (Basidiomycota, Agaricales, Agaricaceae) (Miyashira et al., 2010). *Leucoagaricus gongylophorus* is the only recognized fungal species associated with LCA. This relationship is classified as an obligate mutualistic symbiosis, and such codependence requires millions of years of natural selection (Mueller, 2002). LCA are considered generalist and dominant herbivores in the Neotropics (Herz et al., 2007). They cultivate the mutualistic fungus as their main food source by cutting fragments of diverse plant species, mainly fresh leaves, flowers, dry plant tissues, and seeds (De Fine Licht and Boomsma, 2010) (Fig. 1).

LCA process vegetal material by chewing before depositing it into the fungal chambers at the top of their fungal gardens (Mueller, 2002). The plant substrate is infused with fecal fluids and enzymes that facilitate the degradation of certain compounds present in the plant base substrate (Rønhede et al., 2004; De Mattos-Shipley et al., 2016). The fungal garden operates as a bioreactor degrading efficiently plant biomass to simpler organic matter (Vigueras et al., 2017). Once the fungal mycelium grows, it produces gongylidia that are produced in bundles called staphylae (Mueller, 2002). Gongylidia are bulbous-structured hyphae containing high concentrations of lipids, free sugars, polysaccharides (Mueller, 2002), enzymes such as pectinases (Schiøtt et al., 2010), proteinases (Kooij et al., 2014), and laccase (De Fine Licht et al., 2013). They are used to feed the larvae and distributed throughout the colony to feed both the workers and the queen (Mueller, 2002; Rønhede et al., 2004).

To study *Leucoagaricus gongylophorus* under a controlled environment, a few culture media have been used for the isolation and growth of the mutualistic fungus. However, it is commonly reported that the mycelium has a slow growth rate in the absence of ant care (e.g. Borba et al., 2006; Miyashira et al., 2010; Lugo et al., 2013). Among the culture media commonly reported for *in vitro* culture of *Leucoagaricus gongylophorus* strains associated with LCA, the most effective ones are Pagnocca A (Pagnocca et al., 1990) and B media (Silva-Pinhati et al., 2005), Malt Extract Agar (MEA) (Miyashira et al., 2010) and Potato Dextrose Agar (PDA) (Espinoza et al., 2017; Vigueras et al., 2017; Infante-Rodríguez et al., 2020). Culture media supplementation with plant extracts has also been tested for growing some LCA fungal strains (Borba et al., 2006).

On the other hand, *Atta mexicana* (F. Smith, 1858) is abundant across a distribution range from Arizona (USA) to Honduras (Mintzer, 1979), including tropical and subtropical ecosystems (Zavala-Hurtado et al., 2000). Few studies on the mutualistic fungus of this species have been conducted. Romero et al. (1987) showed that *Leucoagaricus gongylophorus* can be grown *in vitro* on solid media, and Vigueras et al. (2017) revealed that it also grows on mineral medium (MM) enriched with grass and sugarcane bagasse. The latter authors additionally tested a semi-solid culture medium based on cellulose and added either peptone, yeast extract, or MM.

In this study, we aimed to make a molecular identification of *Leucoagaricus gongylophorus* from the fungal garden of *Atta mexicana*. Moreover, we compared the myce-

[2]Universidad Tecnológica del Mar de Tamaulipas Bicentenario, Carretera Estatal N° 52 Soto La Marina- La Pesca, km 46+400 S/N, 87678 La Pesca, 87678 Soto la Marina, Tamaulipas, Mexico.
[3]El Colegio de la Frontera Sur, A.C., Av. Centenario, KM 5.5 S/N, 77014 Chetumal, Quintana Roo, Mexico.
[4]Instituto de Ecología, A.C., Red de Estudios Moleculares Avanzados, Carretera antigua a Coatepec 351, Col. El Haya, 91073 Xalapa, Veracruz, Mexico.
[5]Instituto de Ecología, A.C., Red de Biodiversidad y Sistemática, Carretera antigua a Coatepec 351, Col. El Haya, 91073 Xalapa, Veracruz, Mexico.
[6]Instituto de Ecología, A.C., Red de Ecología Funcional, Carretera antigua a Coatepec 351, Col. El Haya, 91073 Xalapa, Veracruz, Mexico.
[7]Authors for correspondence: joseantonio.guerrero@inecol.mx, jorge.valenzuela@inecol.mx
[8]These authors contributed equally to this work.

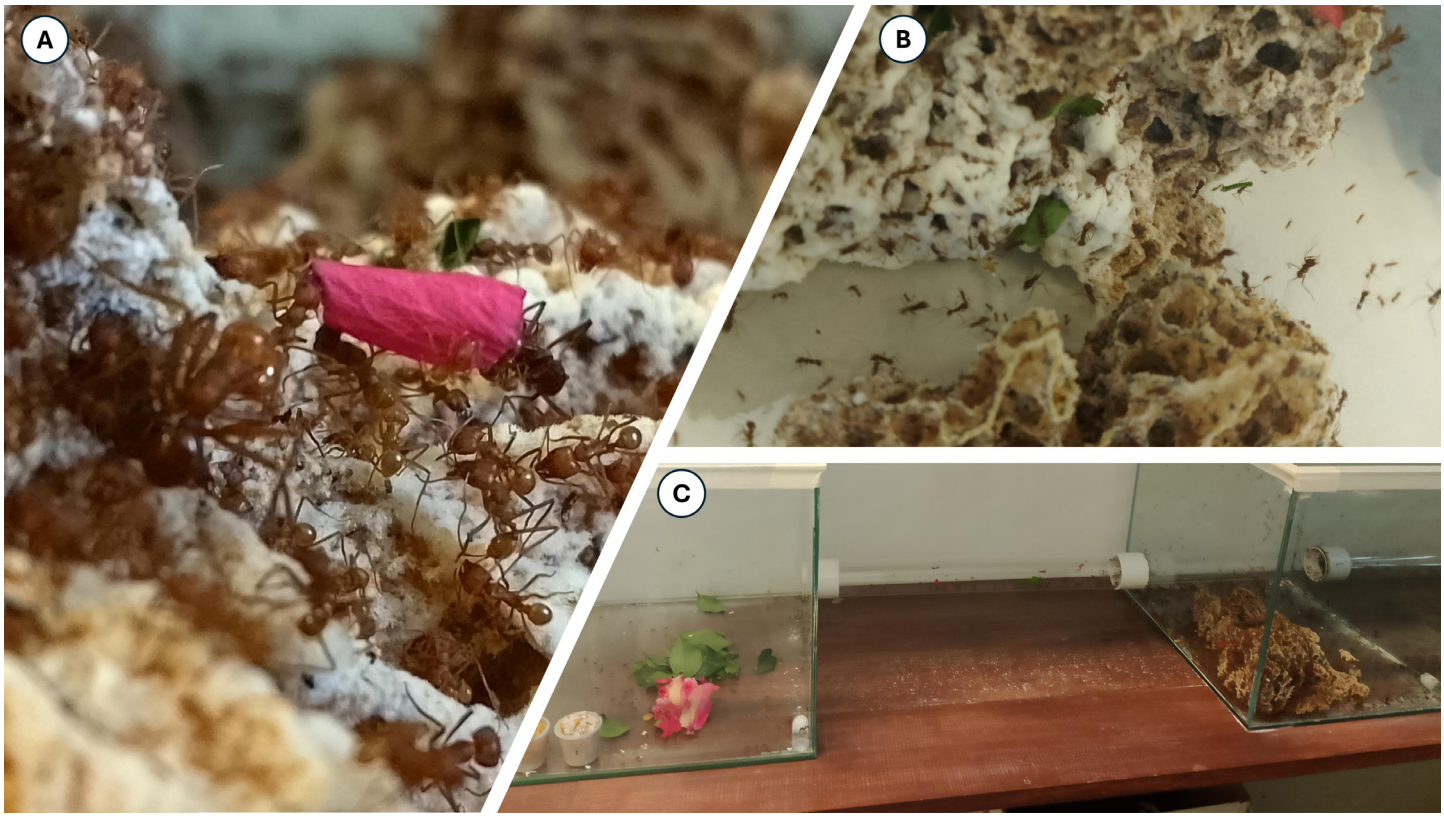

Figure 1: Mutualistic interaction between *Atta mexicana* (F. Smith, 1858) and *Leucoagaricus gongylophorus* (A. Möller) Singer. A. workers processing plant material; B. symbiont fungus alongside the gardener ants; C. nest under laboratory conditions.

lial growth of one strain of *Leucoagaricus gongylophorus*, isolated from an *A. mexicana* nest, on seven solid culture media as well as the mutualistic fungus growth on PDA medium enriched with foliar material obtained from plants known to be well-foraged or avoided by ants (Infante-Rodríguez et al., 2020). Finally, we performed a cost analysis for the *in vitro* maintenance of the symbiont fungus. We expected that the addition of foliar material from preferred plants could improve the growth of the symbiotic fungus, while the addition of foliar material from rejected plants would inhibit it.

Materials and Methods

Isolation and characterization of the *L. gongylophorus* strain

The mutualistic fungus used in this study was obtained from an ant colony that was founded from a fecundated ant queen of *Atta mexicana* placed in captivity after its mating flight and maintained under controlled conditions (25 °C; 16:8 h light/obscurity photoperiod; 80% relative humidity). For the maintenance and growth of the fungal garden, fresh young leaves of *Rosa alba* L. (Rosaceae), *Styrax glabrescens* Benth. (Styracaceae), and *Trema micrantha* (L.) Blume (Cannabaceae) were offered to the ant colonies due to a wide acceptance as foraging material during preliminary observations. Voucher samples of the former three plant species were deposited in the herbarium XAL of the Instituto de Ecología, A.C. (voucher numbers XAL0106252, XAL 0106256 and XAL0106255, respectively; all samples collected by D. Infante-Rodríguez). This foliar material was collected at the Botanical Garden and an adjacent cloud forest fragment to the Instituto de Ecología, A.C. Foliar material from other plant species (e.g. *Psidium guajava* L. (Myrtaceae, XAL0106250), *Citrus reticulata* Blanco (Rutaceae, XAL57987), and *Coffea arabica* L. (Rubiaceae, XAL0106253) was also tested, albeit only occasionally, because it was usually avoided by the ant colony.

To isolate the mutualistic fungal strain, samples (n=50) of 1 mm^3 of mycelium were taken from the laboratory-established nests. Serial reseedings were put into Petri dishes (60 × 15 mm) containing 25 ml of PDA and incubated at 25 °C and 60% relative humidity until purifying the strain. After successful isolation, the mutualistic fungus was maintained in the dark at 25 °C until use.

Genomic DNA was extracted from the isolated white fungus. Universal primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used to amplify the internal transcribed spacer (ITS) region of the fungal rDNA. The ITS regions were reamplified from isolated amplicons, purified, and sent out for sequencing by Macrogen Co. Ltd. (South Korea). Furthermore, three strains of the mutualistic fungus were obtained from three other colonies of *A. mexicana* maintained under laboratory conditions. These were also sequenced, and a BLAST search was conducted to ascertain whether the percentage of coverage and identity corresponded to isolates of *Leucoagaricus gongylophorus*.

Growth of *Leucoagaricus gongylophorus* on culture media

Culture media

Seven different solid media were tested for the culture of the *Leucoagaricus gongylophorus* strain isolated from the *Atta mexicana* nest. Also, local distributors were quoted to compare the costs of PDA, AEM, and Agar-Agar media to determine the cost of 500 g presentations.

The mutualistic fungus was grown in Petri dishes containing 25 ml of each of the seven sterilized solid culture media. The first four media have been reported to be effective in growth experiments of LCA fungal symbionts, and the components are described as follows:

1) Malt Extract Agar (MEA): malt extract (20 g), bacteriological peptone (5 g), yeast extract (2 g), agar (20 g), H_2O (1 l) (Miyashira et al., 2010).

2) Pagnocca A (Pag A): glucose (10 g), sodium chloride (5 g), bacteriological peptone (5 g), malt extract (10 g), agar (15), H_2O (1 l) (Pagnocca et al., 1990).

3) Pagnocca B (Pag B): glucose (10 g), sodium chloride (2 g), bacteriological peptone (2 g), malt extract (10 g), casein hydrolysate (20 g), soybean flakes (20 g), oat flakes (20 g), dibasic sodium phosphate (3.8 g), citric acid (2.5 g), agar-agar (17 g), H_2O (1 l) (Silva-Pinhati et al., 2005).

4) Potato Dextrose Agar (PDA) (Difco, Detroit, MI, USA): dried potato (18.5 g), D-glucose (16.5 g), agar (12 g), H_2O (1 l) (Gadd, 1982).

The three additional culture media have yet to be evaluated for their suitability for the culture of *Leucoagaricus gongylophorus*. Two of them, complete basic medium (CBM) and dry *Agaricus bisporus* (J.E. Lange) Imbach compost extract added with a mineral mixture (CE), have been previously employed for the culture of some edible *Agaricus* L. fungi and the components are described as follows:

5) Complete basic medium (CBM): glucose (10 g), peptone (1 g), yeast extract (1 g), KH_2PO_4 (1 g), $MgSO_4.7H_2O$ (0.5 g), $CaCl_2$ (0.5 g), agar (13 g), H_2O (1 l) (Maia et al., 2012; Velázquez-Narváez et al., 2018).

6) Dry *Agaricus bisporus* compost extract added with a mineral mixture (CE): compost extract (800 ml), bacteriological agar (20 g), glucose anhydride (10 g), H_2O (200 ml) (Straatsma et al., 1993).

Finally,

7) α-cellulose culture medium (αC): α-cellulose (20 g), glucose (10 g), yeast extract (1 g), bacteriological peptone (1 g), $(NH_4)2SO_4$ (1 g), $CaCl_2$ (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), KH_2PO_4 (1 g), agar (15 g), H_2O (1 l).

To conduct growth assays on *Leucoagaricus gongylophorus* within each medium, each culture medium plate was inoculated with 1.5 cm diameter *L. gongylophorus* mycelium discs previously grown on PDA for 30 days and maintained at 25 °C for 28 days. Radial growth of mycelium was measured in mm weekly for each treatment with three replicates. To estimate the total growth diameter of *Leucoagaricus gongylophorus* from radial growth, the following formula was employed:

$$D = 2 \times R$$

where D represents the total diameter of mycelial growth in millimeters, and R denotes a radial growth measured in millimeters.

Growing media with dried leaves

The growth of *Leucoagaricus gongylophorus* mycelium was evaluated on a PDA culture medium with the addition of leaves from six different plant species. The culture media were prepared with PDA medium added with two concentrations of freeze-dried leaves (100 mg ml^{-1} and 10 mg ml^{-1}) from three well-foraged plants (*Styrax glabrescens*, *Rosa alba*, and *Trema micrantha*) and three avoided ones (*Psidium guajava*, *Citrus reticulata*, and *Coffea arabica*). Each treatment consisted of three replicates, following the same procedure as in the previous tests. Foliar material was obtained from young and undamaged leaves from three healthy trees of each plant species collected in a cloud forest patch. Fresh leaves (200 mg) of each plant individual were freeze-dried (FreeZone 1, Labconco, Kansas, MO, USA) for seven days at -56° C and 0.02 mBar of vacuum. Dry leaves were pulverized using a food blender (Nutribullet®) and the plant powder obtained was used to enrich PDA media.

Statistical analyses

To detect differences in the mycelial growth of the symbiont fungus between solid and plant-supplemented culture media treatments, the growth average measurements were analyzed using a Kruskal-Wallis test with a posterior Dunn´s multiple comparison test using R software v. 4.1.2 (R Core Team, 2020).

Results

Isolation and characterization of the symbiont fungus

The mutualistic fungal strain on the PDA medium grew as a white mycelium, producing the characteristic bulbous structures in the apical part, named gongylidia (Fig. 2). Sequencing of the ITS was used to identify the isolated strain; the PCR amplified fragment spanned the ITS region for 608 pb long (GenBank accession number: PQ586137). The comparison of the fungal strain sequence in the NCBI through BLAST showed 99.51% of similarity with *Leucoagaricus gongylophorus* isolates cultivated by *Atta insularis* (Guérin-Méneville, 1845). Three additional strains obtained from our laboratory colonies showed similarities higher than 99.5% with *L. gongylophorus* isolates cultivated by *Atta* sp., *Trachymyrmex* Forel, 1893 and *Acromyrmex octospinosus* Reich, 1793 (Table 1).

Growth of the symbiont fungal on different solid media

The mutualistic fungus grew on all seven tested media (Fig. 3). After 28 days of cultivating *Leucoagaricus gongylophorus* strain, greater mycelial growth was obtained on PDA, CE, and CMB media, but significant growth differences were only detected with the Pag A and Pag B culture media on which the lowest mycelial growths occurred ($H=15.05$, $df=9$, $P<0.05$).

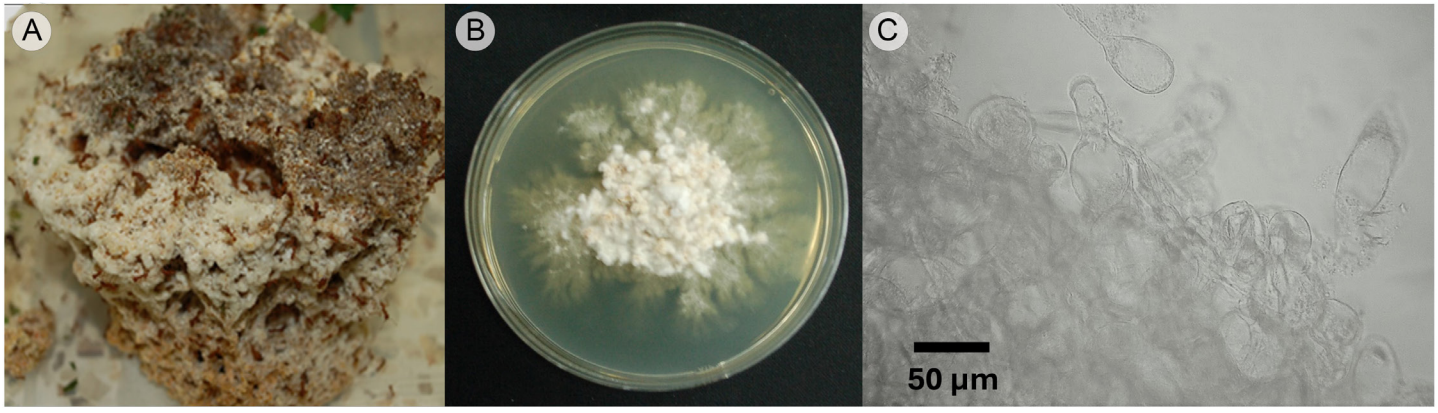

Figure 2: Morphological characterization of the symbiont fungus *Leucoagaricus gongylophorus* (A. Möller) Singer: A. mycelium grown by worker ants in a nest maintained under laboratory conditions; B. isolated strain of the symbiont fungus grown on PDA medium; C. gongylidia from the isolated strain.

Table 1: BLAST results of four isolates of the mutualistic fungus of *Atta mexicana* (F. Smith, 1858) obtained from laboratory nests. ID=number of the laboratory colony from which the sample of the symbiont fungus was obtained, AN / BP=Accession number in the NCBI database and the number of base pairs of the curated sequences herein generated that have been compared by BLAST in the NCBI database, Best Hit NCBI=Type specimen from the NCBI database for comparison of the generated sequences of the symbiont fungus, Accession ID=Identification code of type specimens from the NCBI database.

	ID	AN / BP	Best Hit NCBI	Accession ID	Coverage (%)	Identity (%)	Host
Tested mutualistic fungal strain	Col10	PQ586137 / 608	*Leucoagaricus gongylophorus* (A. Möller) Singer, voucher SIANTDB3174	DQ779958.1	100	99.51	*Atta insularis* (Guérin-Méneville, 1845)
Additional mutualistic fungal strains obtained from laboratory colonies of *A. mexicana* (F. Smith, 1858)	Col5	PQ586135 / 473	*Leucoagaricus gongylophorus* (A. Möller) Singer, voucher JSC041011-20	MK685756.1	100	100	*Atta* (Fabricius, 1805) sp.
	Col6	PQ586136 / 665	*Leucoagaricus gongylophorus* (A. Möller) Singer, voucher SIANTDB3174	MK685742.1	100	99.55	*Trachymyrmex* (Forel, 1893)
	Col12	PQ586138 / 612	*Leucoagaricus gongylophorus* (A. Möller) Singer, voucher SIANTDB3174	MK685742.1	100	99.51	*Acromyrmex octospinosus* (Reich, 1793)

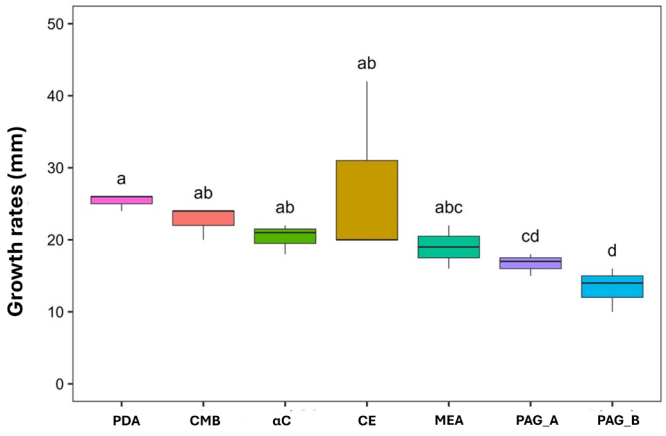

Figure 3: Growth of the *Leucoagaricus gongylophorus* (A. Möller) Singer strain (isolated from the fungus cultivate by *Atta mexicana* (F. Smith, 1858) after 28 days on seven different culture media. αC=α-cellulose medium; CBM=complete basic medium; CE=compost extract; MEA=malt extract agar; PDA=potato dextrose agar; Pag A=Pagnocca A; Pag B=Pagnocca B media. Different letters indicate significant differences (Dunn's multiple comparison test P<0.05).

Growing media supplemented with dried leaves

Significant growth differences of the mutualistic fungus were found in PDA media enriched with 100 mg ml^{-1} of freeze-dried leaves (H=18.69, df=6, P<0.01). Lower mycelial growth percentages were obtained on media enriched with foliar material of *Rosa alba*, which is the preferred plant of *Atta mexicana*, and *Psidium guajava*, *Citrus reticulata* and *Coffea arabica*, which are the three most avoided plant species (Fig. 4), in comparison with the control treatment on PDA medium and PDA medium enriched with foliar material of *Trema micrantha*. *Styrax glabrescens* foliar material also produced some inhibitory effect on the growth of *Leucoagaricus gongylophorus*, but this was significantly lower than in the case of the four former plants.

The inhibitory effects of *Coffea arabica*, *Citrus reticulata*, *Psidium guajava* and *Rosa alba* extracts are much more pronounced at 100 mg ml^{-1} than at 10 mg ml^{-1} (especially for *Coffea arabica*, and *Citrus reticulata*). In PDA media enriched with 10 mg ml^{-1} of freeze-dried leaves, significantly lower mycelial growth percentages were obtained with *Psidium guajava* and *Rosa alba* (H=15.24, df=6, P<0.05), indicating that both plants had inhibitory effects on the growth of *Leucoagaricus gongylophorus* at this concentration (Fig. 5).

Cost analysis on different solid media

In terms of cost analysis, the solid base mediums of PDA (Difco®) and AEM (Condalab®) fall within price ranges of $149 to $248 USD for a 500 g presentation, while the solid base medium of Agar-Agar can be obtained in generic brands at a cost of less than $50 USD for a 500 g presentation (Table 2).

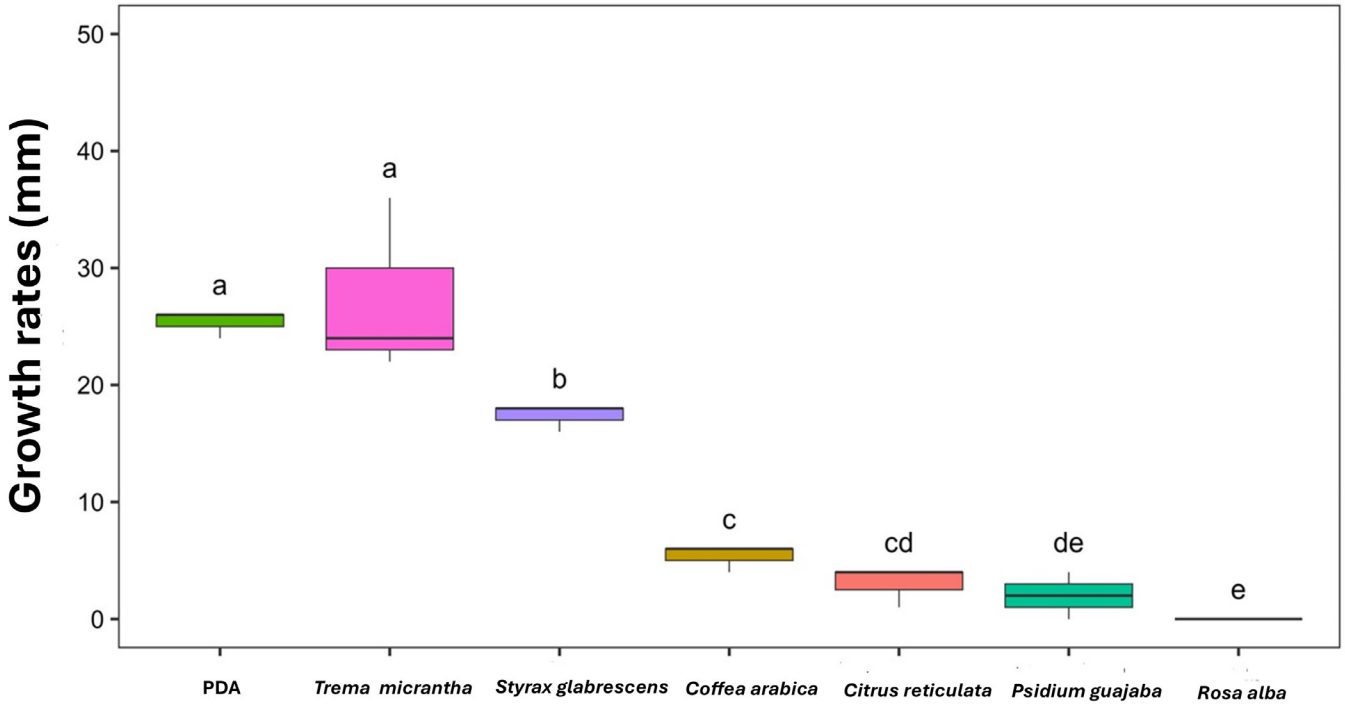

Figure 4: Box plots of growth (mm), after 28 days, of *Leucoagaricus gongylophorus* (A. Möller) Singer on PDA culture medium with the addition of freeze-dried leaves (100 mg ml^{-1}) of six plant species. Different letters indicate significant differences (Dunn's multiple comparison test P<0.05).

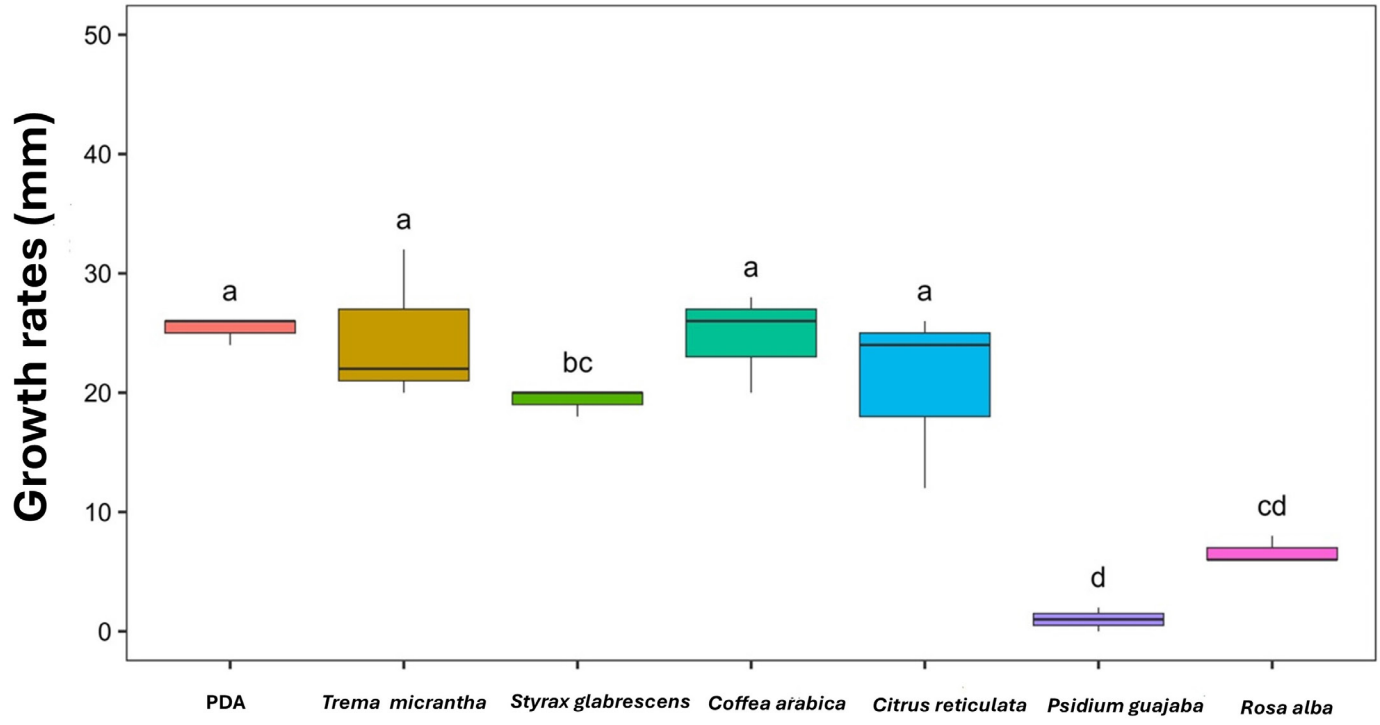

Figure 5: Box plots of growth (mm), after 28 days, of *Leucoagaricus gongylophorus* (A. Möller) Singer on PDA culture media with the addition of freeze-dried leaves (10 mg ml^{-1}) of six plant species. Different letters indicate significant differences (Dunn´s multiple comparison test P<0.05).

Table 2: A comparative analysis of the financial implications of utilizing solid base media for *in vitro* cultivation of *Leucoagaricus gongylophorus* (A. Möller) Singer.

Culture medium	Cost	Presentation	Brand
PDA	$149 USD	500 g	Difco®
Malta Extract Agar	$248 USD	500 g	Condalab®
Agar Agar	$50 USD	500 g	Mi granero®

Discussion

Isolation and characterization of the symbiont fungus

The isolated *Leucoagaricus gongylophorus* strain showed slow growth on the different culture media tested as has already been observed in the *in vitro* growth of mutualistic fungi isolated from other leaf-cutting ant species (Borba et al., 2006; Miyashira et al., 2010; Lugo et al., 2013). Genetic data of ITS sequencing confirmed that the mutualistic fungus isolated from *Atta mexicana* fungal garden belongs to the species *Leucoagaricus gongylophorus*. The comparison in the NCBI database through BLAST showed 99.5% in similarity with *Leucoagaricus gongylophorus* isolates cultivated by other Attini ants.

There is evidence that the higher Attini ants cultivate the same mutualistic fungus, and gongylidia are characteristic structures used to identify the mutualistic fungus of *Atta* and *Acromyrmex* species (Mikheyev et al., 2007). These structures were observed in the isolated fungal strain cultivated in this study (Fig. 2C). Gongylidia exhibits high concentrations of nutrients that are vital for ant colony development, including lipids, free sugars, and polysaccharides. They serve as the primary food source for the colony (De Fine Licht et al., 2014). Furthermore, gongylidia contain enzymes, including pectinases, proteinases, and laccases, that can be utilized to degrade plant substrates (Schiøtt et al., 2010; De Fine Licht et al., 2013; Kooij et al., 2014). This may confer a selective advantage to the ant foragers in utilizing diverse plant substrates.

Growth of *Leucoagaricus gongylophorus* on culture media

This is the first study reporting the growth of the mutualistic fungus of *Atta mexicana* on CE, CBM, Pag A, and Pag B media. The growth of the mutualistic fungus was observed to be similar on CE, CBM, PDA, αC, and MEA media, as compared to that observed on PDA medium. However, Pag A and Pag B media were found to be less effective for the development of the fungus. The results indicate that the culture of *Leucoagaricus gongylophorus* under laboratory conditions is a viable option with the use of various media, even in the absence of ants.

Pag A and Pag B media have been demonstrated to be effective for the isolation and growth of the *Leucoagaricus gongylophorus* strain from nests of *Acromyrmex ambiguus* (Emery, 1888), *Acromyrmex crassispinus* (Forel, 1909), and *Acromyrmex lundii* (Guérin-Méneville, 1838) (Borba et al., 2006). However, these media are less favorable for strains isolated from *Acromyrmex heyeri* (Forel, 1899). These findings, along with our own, indicate the potential for differing requirements between mutualistic fungal strains isolated from different species of LCA.

Two studies have reported the isolation of the mutualistic fungus of *Atta mexicana in vitro* using PDA and MEA as culture media (Espinoza et al., 2017) or semi-solid culture media based on cellulose with peptone, yeast extract, or mineral medium (Vigueras et al., 2017). The latter authors utilized grass and sugarcane bagasse as a carbon source, demonstrating that plant waste material can be employed for the *in vitro* culture of this mutualistic fungus. In the first study, no data was presented regarding the growth diameters of the fungus, or the biomass obtained. In the second study, only biomass between 1 and 5 mg of dry weight was reported. After 20 days, the fungal biomass was found to be twice as high in the cultures that had been supplemented with peptone and yeast extract in addition to α-cellulose or microcrystalline cellulose, as compared to those cultures that had been supplemented with α-cellulose or microcrystalline cellulose in the mineral medium. However, the present study obtained a higher mycelial growth on CE (27.3±12.7 mm) and PDA media (25.3±1.15 mm) at 28 days of growth. The CE and CBM media have been employed for the cultivation of *Agaricus subrufescens* Peck (Agaricaceae) (Maia et al., 2012; Velázquez-Narváez et al., 2018), a species that is phylogenetically closely related to *Leucoagaricus gongylophorus* (Schulzová et al., 2009).

We propose that the CE medium may represent a cost-effective alternative to PDA, MEA, or protein-based media for the *in vitro* culture and maintenance of the symbiont fungus. For example, compost is the conventional substrate utilized for the cultivation of common edible mushroom (*Agaricus* spp.). However, compost is a substrate rich in nitrogen and other elements that, when incorporated into a solid medium, presented some diffusion problems in the Petri dishes. It is a complex mixture that has yet to be fully characterized, so it is unclear whether some of its components can be incorporated more effectively or homogenously into the agar base. It is proposed that the use of fine sieving of the extract may assist in addressing the issue of diffusion within the medium. In terms of cost analysis, it can be observed that the solid base medium of PDA (Difco®) and AEM (Condalab®) fall within price ranges of $149 to $248 USD for a 500 g presentation, while the solid base medium of Agar-Agar can be obtained in generic brands at a cost of less than $50 USD. This would result in a reduction in the price of the solid base medium used in the compost extract medium (Table 2).

It has been demonstrated that *Leucoagaricus gongylophorus* can metabolize plant polysaccharides, including xylan, starch, pectin, and cellulose. Nevertheless, the degradation and assimilation of cellulose by the fungus appears to be relatively inefficient. It can thus be assumed that cellulose is not the primary carbon source for the symbiosis between leaf-cutting ants and their mutualistic fungus (Vigueras et al., 2017). The usefulness of cellulose for the nutrition of *Leucoagaricus gongylophorus* remains under controversy, and some reports have failed to demonstrate a significant cellulose-degrading activity by this fungus (De Siqueira et al., 1998). Abril and Bucher (2004) suggested that the fungus garden does not degrade cellulose, and that fungal nutrition is based on cytoplasm-soluble compounds of the plant cells as amino acids and glucose. Nevertheless, the mutualistic fungus could grow on semi-solid and solid cultures using cellulose and lignocellulosic-based media, showing that *Leucoagaricus gongylophorus* could metabolize polysaccharides as cellulose found in plant tissues (Vigueras et al., 2017).

The mutualistic fungus of *Acromyrmex echinatior* (Forel, 1899) expresses a functional xylanase gene, which indicates that it can degrade plant cell wall material and corroborates that the Attini ant mutualistic fungus has a saprotrophic origin (Schiøtt et al., 2008). The fungal growing inside *Atta* nests is a very complex microbiome. Using metagenomic and metaproteomic approaches, Aylward et al. (2012) revealed that abundant populations of Enterobacteriaceae are found inside the fungal crop, including the genera *Enterobacter* (Hormaeche and Edwars, 1960), *Pantoea* (Gavini, 1989), *Klebsiella* (Trevisan, 1885), *Citrobacter* (Werkman and Gillen, 1932), and *Escherichia* (Castellani and Chalmers, 1919). These bacterial communities possess genes associated with lignocellulose degradation and diverse biosynthetic pathways and play a role in nutrient cycling by converting the nitrogen-poor forage of the ants into vitamins, amino acids, and other cellular components. *Leucoagaricus gongylophorus* isolated in culture media lacks this microbiome, which may explain the low cellulose-degrading activity previously reported for this fungus under laboratory conditions (Vigueras et al., 2017).

Growing media with dried leaves

Contrary to our expectations, the addition of leaf material from various plant species that are foraged by ants did not improve the growth of the fungus. Some contrasting results were observed depending on the species and concentration of foliar material used. *Leucoagaricus gongylophorus* cultivated on PDA medium supplemented with both concentrations (100 mg ml^{-1} and 10 mg ml^{-1}) of *Trema micrantha*, one of the plants well-accepted by foragers ants, had a mycelial growth response similar to the one obtained in PDA medium. Mycelial growth on PDA media enriched with 10 mg ml^{-1} of foliar material of *Coffea arabica*, *Citrus reticulata*, and *Styrax glabrescens* was analogous to controls. However, at 100 mg ml^{-1} there was a significant inhibitory effect, being particularly high in the case of *Coffea arabica* and *Citrus reticulata*, which are avoided plants by foragers ants. PDA medium supplemented with foliar material of *Psidium guajava* (an avoided plant) and *Rosa alba* (the best foraged plant in the case of *Atta mexicana* (Infante-Rodríguez et al. 2020)) had inhibitory effects at both concentrations tested. Some authors have suggested that the ability of the leaf-cutting ants to exploit a plethora of plants (inedible or toxic for the fungus) is based on their ability to handle

enzymes produced by *Leucoagaricus gongylophorus* (Nichols-Orians, 1991; Rønhede et al., 2004). Leaf-cutting ants are well-known to concentrate these enzymes in fecal droplets that are deposited on the fresh foliar material brought by the ants for the culture of the symbiont fungus (De Fine Licht et al., 2013). The case of *Rosa alba*, which is the plant that is the most foraged by *Atta mexicana*, but which also exhibited the strongest inhibitory effect on the *in vitro* growth of *Leucoagaricus gongylophorus*, strongly suggests the existence of a detoxification mechanism through the use of ant-produced enzymes, as is known for several LCA species (De Fine Licht et al., 2014).

This puts forward that the fungal garden employs ants to transport fungal enzymes from the most productive central sections of the garden to the upper peripheral layers, where new substrate is abundant but mycelial growth is rare (De Fine Licht et al., 2014). The enzymes produced can be classified into three main groups: pectinases, proteinases, and laccases (Schiøtt et al., 2010). Laccases have been demonstrated to play a role in the degradation of secondary plant defense metabolites, including phenolic compounds (De Fine Licht et al., 2013; Kooij et al., 2014). Additionally, some authors have proposed that plant material is degraded through bacteria associated with the fungal symbiont (Powell and Stradling, 1991; Francoeur et al., 2023).

Conclusions

Our results reveal that the addition of foliar material to culture media does not improve the growth of the symbiont fungus (PDA in this case). These results suggest that the preparation of foliar material by ants is necessary to make it available for the mutualistic fungus. Moreover, they highlight the importance of investigating furthermore the roles played by ants, the symbiotic fungus, and the microbiome found inside the fungus garden in the processing and detoxification of the plant material collected by foragers for the culture of *Leucoagaricus gongylophorus*. Also, PDA is a medium that is rich in sugars and other nutrients, which may explain why the fungus does not utilize the supplemented leaf material, as it has other nutrients available. It is recommended that future experiments utilize agar without sugar to assess the growth of mutualistic fungi on plant-supplemented media.

Author contributions

DAIR, ACVN, and JEVG contributed to the conceptualization, realization of the experiments, and formal analysis and writing of the original manuscript. JAGA, JLMV, KM, and JPL contributed to the drafting, revision, and editing of the manuscript; GLCV contributed to the realization of the analysis and interpretation of the DNA sequencing. All authors contributed to the discussion and revision of the final version of the manuscript.

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Dennis Adrián Infante-Rodríguez,
Alberto Carlos Velázquez-Narváez,
Juan Luis Monribot-Villanueva, Gloria Carrión,
Klaus Mehltreter, Jean-Paul Lachaud,
José Antonio Guerrero-Analco, Jorge E. Valenzuela González

In vitro culture of Leucoagaricus gongylophorus (Agaricaceae), symbiont fungus of the leaf-cutting ant Atta mexicana (Hymenoptera, Formicidae), using solid and plant-supplemented culture media
Cultivo in vitro de Leucoagaricus gongylophorus (Agaricaceae), hongo simbionte de la hormiga cortadora de hojas Atta mexicana (Hymenoptera, Formicidae), utilizando medios de cultivo sólidos y suplementados con material vegetal

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