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Culture media evaluation on the *Leucoagaricus gongylophorus* and *Escovopsis* sp. fungi development

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ABSTRACT

This study aimed to evaluate the *in vitro* interference of different culture media, Czapek Dox Agar, Nutrient Agar, Starch Casein agar, Malt Extract Broth Base, Nutrient Broth, and the medium containing Peptone, Malt, Agar and crushed peach palm pulp (*Bactris gasipaes*) and tucumã (*Astrocaryum aculeatum*) on the fungus *Leucoagaricus gongylophorus* and the parasitic fungus *Escovopsis* sp. growth. The fungi were inoculated in different culture media and then kept in acclimatized chambers at 28 ºC in the dark for 42 days, being analyzed at weekly intervals, totaling six evaluations. Fungal colonies were evaluated considering the factor "f" (cm), obtained from the product between the width (W) and length (L) of the diameter of the colonies (f = W x L). The results showed that the symbiotic fungus and the leafcutter ant parasitic fungus showed a growth increase compared to the media provided in the plates, and the media added with tucumã peel, tucumã peel+pulp, fresh peach palm, and cooked peach palm had a significant effect in the *L. gongylophorus* fungus development. However, these treatments were not promising for the *Escovopsis* sp. fungus compared to other culture media.

Keywords: *L. gongylophorus, Escovopsis* sp, *culture media.*

Avaliação de meios de cultura sobre o desenvolvimento dos fungos *Leucoagaricus gongylophorus* e *Escovopsis* sp

RESUMO

O objetivo deste trabalho foi avaliar a interferência *in vitro* de diferentes meios de culturas, Czapek Dox Agar, Nutrient Agar, Starch Casein agar, Malt Estract Broth Base, Nutrient Broth e o meio contendo (Peptona, Malte, Agar) e polpa triturada de pupunha **(***Bactris gasipaes) e* tucumã (*Astrocaryum aculeatum*)), *sobre o crescimento* do fungo *Leucoagaricus gongylophorus* e o fungo parasita *Escovopsis* sp. Os fungos foram inoculados em diferentes meios de cultura e posteriormente mantidos em câmaras climatizadas à temperatura de 28 ºC no escuro, por um período de 42 dias totalizando seis avaliações, sendo analisados em intervalos semanais. As colônias dos fungos foram avaliadas considerando o fator "f" (cm), obtido do produto entre a largura (L) e o comprimento (C) do diâmetro das colônias (f = L x C). Os resultados mostraram que o fungo simbionte e o fungo parasita das formigas cortadeiras apresentou um aumento no crescimento em relação aos meios fornecidos nas placas, sendo que o meio acrescido com tucumã casca, tucumã casca+polpa, pupunha *in natura* e pupunha cozida apresentaram efeito significativo no desenvolvimento do fungo *L. gongylophorus.* No entanto, para o fungo *Escovopsis* sp, esses tratamentos não foram promissores em relação aos outros meios de cultivos.

Palavras-chave: *L. gongylophorus, Escovopsis* sp, meios de culturas.

1. Introduction

The leafcutter ants from the Atta genera are severe pests in the neotropical region, known as "saúvas", whose economic importance is highlighted because they cut fresh plant material that they use as fungus substrate, cultivated for their food. (Della Lucia et al., 2014). The leaves and parts of vegetables cut by these insects cause significant financial losses, and the damage can be irreversible, even causing the plants to die (Zanetti et al., 2014). Like saúvas, ants from *Acromyrmex* genera (Quenquéns) also affect the agricultural sector, responsible for building the largest nests, making control very difficult (Mueller et al., 2018). Studies carried out by Cantarelli et al. (2019) demonstrated that *Acromyrmex heyeri* and *Acromyrmex lobicornis* are responsible for 20.8% of the losses in several *Pinus taeda* plantations, being attacked in the first two months after being replanted.

According to Moreno et al. (2017), the relationship between fungi and insects originated more than 50 million years ago. It can be analyzed in antagonistic and symbiotic ways, as several organisms coexist harmoniously in the nest.

Second, Seipke et al. 2011, mutualism was considered rare and of minor importance. However, in recent years, it has come to be seen for its influence on several life forms evolution, such as the symbiosis between microorganisms and leaf-cutting ants. This has improved nutritional aspects and protection against predators, parasites, and pathogens that destroy an entire nest. The chamber where the fungi are kept contains empty spaces allowing the airflow to maintain gas exchange and a temperature of approximately 26 °C.

Knowledge about the life cycle of *Escovopsis* sp. is still very scarce. It is known that this fungus is highly pathogenic for the nest of the leaf-cutting ants, presenting a fast mycelial growth, covering the leaf-cutting ant fungus gardens in 72 hours without the worker ants' presence. The *Escovopsis* sp fungus parasitizes the symbiotic fungus, degrading its cells and feeding on the released nutrients (Currie et al., 1999a; Pagnocca et al., 2011).

The studies already carried out have helped to understand this association, considering that the information about it is, in its majority, outside the Amazon biome and investigations involving the construction and regional understanding of this interaction are scarce. Laboratory growth is complex and somewhat financially costly, occurring more readily in nature. Laboratory procedures in microbiology raised equipment prices, making it difficult to purchase materials such as culture media and culture maintenance, making practical learning of the essential functions of microorganisms in the environment unfeasible (Barbosa, 2010). necessary to the search for alternative and more accessible culture media for use in studies carried out with these microorganisms in laboratories. Other works analyze plant extracts' effects on the development of symbiotic fungi, presenting themselves as a more economical option. A study with leaf macerates of *Citrus* spp., *Ligustrum* spp., *Acalypha* spp., *Eucalyptus* spp., *Alchornea triplinervia,* and *Melia* spp. showed that macerated plants positively influenced symbiotic fungus development (Camargo et al., 2003).

Due to their rich metabolite composition, some fruits from the Amazon region can be investigated as substrates for bioprocesses, including tucumã and peach palm, since fungi need some growth essential nutritional elements, named macro-elements (carbon, nitrogen, hydrogen, oxygen, carbohydrate, etc.). These elements are required in significant quantities by fungi and can be determining factors in growth. The use of part of these fruits, such as peels, can reduce production costs for the laboratory and thus obtain a better performance of these microorganisms and add value to residues that currently present themselves as an environmental challenge.

In this context, this study aimed to observe the mutualistic fungus *L. gongylophorus* and the parasitic fungus *Escovopsis* sp. development to establish the correlation between them to study the organisms and their interaction in the in vitro environment better. Therefore, information about growth, morphology, and interaction between leaf-cutting ant microorganisms in different culture media was obtained.

2. Material and Methods

The garden of fungi and ants of different castes were collected on the farm of the Federal University of Amazonas-UFAM, at 2º38'20" S 2º39'10" S and 60º40' W 60º30' W, at km 38 of the road BR-174. An anthill of *Atta laevigata* (Saúva Cabeça de Vidro) was selected, excavated using a digger and garden shovel, thus reaching the colony of the symbiotic fungus and ants from different castes. Samples were collected in properly sterilized, sealed, and packaged plastic containers and subsequently taken to the Microbiology Laboratory.

In the laboratory, the fungus and ant garden storage boxes were placed in the refrigerator for 2 minutes to temporarily inactive, then handled in a laminar flow cabin, aiming to isolate the symbiont fungus. Fragments of fungal gardens with more evident fungal development were removed. Garden fragments (sponges) were immersed successively in autoclaved distilled water (1 minute), 70% ethanol (1 minute), 2% active chlorine (1 minute), 70% ethanol (30 seconds), and autoclaved distilled water (30 seconds) using fine-tipped tweezers. After this process, smaller fragments of approximately 3 millimeters in diameter were removed and transferred to Petri dishes containing PDA culture media added with

tetracycline (50 mg/L), each with four sponge fragments. The fungus was also isolated directly from the anthill, transferring small fragments from the sponges to Petri dishes.

The plates were incubated in a BOD (Biological Oxygen Demand) incubator at 28 ºC in a dark environment and analyzed daily to measure symbiotic fungus' growth. After the fungus had developed in culture medium (BDA), they were transferred to 25 Petri dishes individually, without antibiotics. The plates were incubated again in a BOD incubator at 28 ºC for fungal development. Then, it was analyzed for its micromorphology using the microculture technique.

The remaining fungus sponge was kept in the same transport box to grow its parasitic fungus *Escovopsis* sp. When observing the parasite growth, it followed all the steps of the process of the first fungus. All microorganisms were purified using serial dilution. These dilutions were carried out by transferring 1 mL of the suspension into a flask containing 9 mL of saline solution (10-1) and vortexed. From this dilution tube (10-1), an aliquot of 1 mL was removed and transferred to a flask containing 9 mL of saline solution (10-2) and so on until the desired dilution was obtained according to the protocol of Azevedo and Costa (1973). All dilutions were seeded in Petri dishes containing PDA plus tetracycline in triplicate to get pure colonies.

The macromorphological identification of the obtained isolates was carried out based on the growth in the PDA medium. Colony characteristics such as colony texture and pigment production were evaluated in the obverse and reverse. For micromorphology analysis, the microculture technique was performed to observe vegetative structures (hyphae) by inoculating the fungus of interest in a cube of culture medium on a glass slide, with a sterile coverslip placed on the agar block. Plates were incubated in a BOD at 28 ºC for seven days or until the fungus sporulation grows on the coverslip. After the incubation period, the coverslip was removed and placed on a slide with a drop of cotton blue lactophenol. The analysis under an optical microscope to visualize microstructures (vegetative and reproductive) was performed using a 40X objective (total magnification of 400X).

Peach palm and tucumã were selected at the Agroufam Fair held monthly at the Federal University of Amazonas - UFAM. A micro company of fruit pulpers from Tucumã, located at 3°05'17.2"S 59°58'44.5"W donated the tucumã peels. Next, they were taken to the Microbiology Laboratory, where they underwent an asepsis process.

The fresh peach palm fruits were washed in running water, cut into small fragments, and then dried in the oven for seven days. After drying, the materials were processed in a blender until they turned into powder and stored in sterilized plastic pots. The culture medium's development was standardized: Agar 15 g, peptone 2 g, Malt 50 g, fresh peach palm 42 g, and distilled water 1000 ml. Subsequently, the material was sterilized at 121°C for 15 minutes and poured into Petri dishes (Victor et al., 2001).

In the cooked peach palm culture medium preparation, 500 g of pulp was used, added to 2 L of distilled water, and then cooked for 20 minutes. After being cooked, it was drained, cut into pieces, and then taken to a stove with air circulation at an average temperature of 55 °C for seven days. After drying, the materials were processed and stored. The culture medium development was standardized: Agar 15 g, peptone 2 g, Malt 50 g, cooked peach palm 42 g, and 1000 mL distilled water.

The pulp, together with the tucumã husk, were washed in running water to eliminate possible microorganisms in the peel and then taken to an oven at 55 °C for seven days to remove moisture, crushed, and stored in sterilized pots. The powder produced was standardized (Agar 15 g, peptone 2 g, Malt 50 g, tucumã peel+pulp 42 g, and 1000 mL distilled water). The tucumã husks underwent the same process described above, and then taken to an electric oven for seven days, crushed, packaged, and standardized: (Agar 15 g, peptone 2 g, Malt 50 g, tucumã+shell 42 g, and distilled water 1000 mL).

Culture media, Czapek Dox (Agar 49 g, agar 15 g, distilled water 1000 mL), Nutrient Agar (28 g, agar 15 g, distilled water 1000 mL), Starch Casein agar (39 g, agar 15 g,1000 ml distilled water), Malt Extract Broth Base (48 g, 15 g agar, 1000 ml distilled water) and Nutrient Broth (13 g, 15 g agar, 1000 ml distilled water) were employed. The routine culture medium of the BDA laboratory was used as standard, being prepared with 200 g of potato, 20 g of dextrose, and 15 g of agar per 1000 mL of distilled water.

With the media ready and deposited in Petri dishes, 20-day culture media discs with a diameter of 5 mm were removed with the aid of a pourer and transferred to the plates. For each test, an agar disc with the parasitic fungus *Escovopsis* sp. and the symbiotic fungus *L. gongylophorus* was added, all performed in five replicates to observe the fungi growth, later used to calculate the means Statistics. Then, they were sealed and incubated in BOD (Biological Oxygen Demand) climatized chambers at a temperature of 28 ºC in the dark.

The observation of fungus growth was performed macroscopically, based on the colony diameter with the aid of a caliper, being defined as width (W) and length (L) as the smallest and largest dimensions of colony growth, respectively, in centimeters (cm) and at weekly intervals, totaling six evaluations (at 7, 14, 21, 28, 35 and 42 days after inoculation) according to Borba et al. (2006). The fungi's diameter average value was calculated.

Two straight lines were drawn, with a crossing point coinciding with the center of the inoculum disc, and measurements were taken from the edge of the inoculum to the end of the fungus growth. Then each plate's diameter average value was calculated. *Escovopsis* sp. vs. *L. gongylophorus* fungus in the peach palm and tucumã culture media

As it has a slow growth, the symbiotic fungus was inoculated 20 days before in Petri dishes containing the experimental medium (Peptone, Malt, Agar, fresh peach palm) and the culture medium containing the tucumã peel. These treatments were mounted in triplicates. The disc was inoculated 0.5 cm from the edge of the plate and kept in a BOD oven at 28°C. On the 20th day, a disc of the *Escovopsis* sp. fungus mycelium was inoculated 0.5 cm from the edge opposite to the culture of the symbiotic fungus in the Petri dish and measured for *L. gongylophorus* after the *Escovopsis* sp. inoculation.

Colony growth was measured on days 3, 5, 7, and 14. A caliper was used to calculate the simple growth average, also observing a possible antibiosis. In negative controls, both the parasitic fungus and the symbiotic fungus were placed individually in Petri dishes. An analysis was carried out to compare the mycelial growth (cm) of the mutualistic fungus in the presence (confrontation) and absence (control) of *Escovopsis* sp. to visualize growth differences in the presence of the mutualistic fungus, using the same culture medium mentioned above.

Some duplicates of the fungi were stored, containing isolates from the symbiotic and the parasitic fungi used in the present work. They were preserved using the method reported by Castellani (1939) and maintained in the collection of the Microbiology Research Laboratory at UFAM (Federal University of Amazonas). Data were subjected to analysis of variance (ANOVA). The means were compared by the Tukey test at a 5% probability level. Statistical analyzes were performed using Sisvar software, version 5.6, according to the Ferreira (2014) recommendations.

3. Results and Discussion

There were significant differences between the fungal mycelial growths in the different culture media the first week after the tests began. Regarding the fungus *L. gongylophorus* (Table 1), the largest mycelial diameter was observed in the treatments of tucumã peel, tucumã peel + pulp, fresh peach palm, and cooked peach palm, being significantly higher than the other treatments in most of the evaluated periods.

For forty-two days, six evaluations were carried out. In the first three observations, the culture medium containing tucumã peel proved to be efficient in the leaf-cutting ant symbiotic fungus development, unlike the medium containing the PDA, a reference medium for the *L. gongylophorus* fungus growth in the laboratory.

The symbiotic fungus of the leaf-cutting ant also grew in the treatment tucumã peel + pulp and the medium containing only the peels. Still, there was a small but significant difference between them. In addition to increasing mycelial growth, macroscopic morphological changes and darkening of the culture medium were also observed, such as a change in the color of the culture medium from light yellow to dark brown where the mutualistic fungus grew. Garcia et al. (2013) demonstrated that pulp and peel of tucumã have important bioactive compound content such as total phenols, flavonoids, and carotenoids.

According to Siqueira et al. (1998), the symbiotic fungus of *A. sexdens* grows easily in components found in plants such as flowers and fruits. When metabolized by the fungus, polysaccharides become important carbon sources, essential for the ants' survival. During the six evaluations of the tests, it was verified that the medium supplemented by fresh peach palm and the cooked one was efficient for the symbiotic fungus of the leafcutter ant, where mycelia grew, following the same pattern of the previous tucumã medium, occurring a significant difference between them.

For the basidiomycete fungus, *Lentinus crinitus* from the Amazon region, whose growth is slow in the laboratory, Nepomucena (2010) proposes that the culture medium added to peach palm was efficient for this fungus development. Indeed, the medium contained macromolecules required for this organism's growth. It also had high N content (59.5–79.9%), one of the essential chemical elements for this specific fungus development.

When comparing the treatments with Czapek Dox Agar, Nutrient Agar, Starch Casein, Malt Extract Broth Base, Nutrient Broth, and BDA, it was observed that these cultures media were not very efficient for the *L. gongylophorus* fungal area growth even with significant differences among them. Considering the results obtained by the analyses, the finding of a diameter growth greater or less than the others is directly linked to the treatment composition it was subjected to in the study. According to Koch (1975), growth differences can occur in two or three dimensions. Furthermore, the constitution of the culture medium used can influence the way the fungus grows.

In the bioassays (Table 2) with the parasitic fungus *Escovopsis sp*., treatments showed differences in development patterns, which is noticeable when comparing the fungus development results in the first and fifth evaluations.

Table 1. Diameter of mycelial growth of the fungus *L. gongylophorus* determined by growth (f = L x C), expressed in (cm), cultivated in different culture media for six periods.

Treatments	7 days	14 days	21 days	28 days	35 days	42 days
PUCI	1.2 b	1.48 c	2.06 b	2.88b	3.74 b	5.32 c
CE	1.0 b	1.06 g	1.16 d	1.28 e	1.38 d	1.64 f
ST	1.0 b	1.0 g	1.0 e	1.0 f	1.0 e	1.0 g
NU	1.0 b	1.0 g	1.0 e	1.0 f	1.0 e	1.0 g
BDA	1.0 b	1.12 f	1.26 d	1.54 e	1.74 c	2.5 e
TU	1.6 a	1.84 b	2.38 a	3.48 a	4.88 a	6.08 b
NB	1.0 b	1.0 g	1.0 e	1.0 f	1.0 e	1.0 g
M	1.12 a	1.26 e	1.42 c	1.74 d	1.88 c	2.0 e
PUCC	1.1 b	1.35 d	2.0 b	2.30 c	3.30 b	4.70 d
TUC	1.9 a	2.1 a	2.50 a	3.78 a	5.4 a	7.3 a

Means followed by the same letter in the columns do not differ by the Tukey test – 95% significance. * PUCI = Fresh peach palm; CE=Czapek; ST = Starch; NU = Nutrient Agar; PDA; TU= tucumã; NB = Nutrient Broth; M =Malt; PUCC = cooked peach palm; TUC = tucumã peel.

Mycelial growths on plates with the application of tucumã and peach palm supplements were slow as a function of time, indicating a scarcity of the culture medium. However, this observation is in the opposite direction regarding treatments with Cezapek, Starch, BDA, and Nutrient Broth, which showed growth as a function of time in the first observation.

The addition of treatments fresh peach palm and cooked peach palm did not differ in the 35 days as their growth in the treatments was slow since they only took up the entire plate extension on the fifth observation. Chemical food analysis allows chemical groups' identification that can interfere with plant food digestion. Such groups can be called secondary metabolic and appear as a plant defense system such as tannins, alkaloids, among others; the nutritional value, including proteins, carbohydrates, and lipid constituents needed by the ant and the fungus; plant defense mechanisms, such as leaf and fruit hardness, hair density and the presence of latex and leaf moisture content are related to the protection of these plants (Demirtas et al., 2018)

The slow mycelial growth of the parasitic fungus, mainly in the culture medium supplemented with peach palm, may have occurred due to the high tannin concentration, which, despite being compounds with various biological activities, can act as enzyme inhibitors in particular fungi, according to Scalbert (1991). In the culture medium with tucumã peel and the medium with tucumã peel + pulp, the fungus *Escovopsis sp*. developed entirely in the Petri dish within 21 days, still observing macroscopic morphological changes and darkening in some plates on this fungus. The differences observed in growth on different substrates probably occurred due to the different chemical compositions of the substrate.

Contrary to expectations, in treatments with tucumã and peach palm, we observed that the Escovopsis sp. did not have significant mycelial development when in cultivation in the presence of the other treatments.

Regarding Czapek Dox Agar, Starch Casein, Nutrient Broth, and BDA media, these treatments had a significant difference that occurred within seven days for the growth of these microorganisms. Perhaps the fungus has a system for recognizing the presence of sugar, so the growth was greater. Man et al. (2016) states that glucose is the most abundant sugar present in the leaf-cutting ant fungus garden, and it is proposed that Escovopsis sp. is capable of catabolizing these carbohydrates.

In the media with fresh peach palm and tucumã the antagonism tests as shown in Table 3 were carried out to evaluate the effects of culture media with tucumã pulp + peel and peach palm on the fungus *Escovopsis sp* and the fungus *L. gongylophorus* since it was observed that *Escovopsis sp*. had better growth in the medium containing peach palm in 14 days. Still, it had a favorable growth for the fungus *L. gongylophorus* also in the peach palm medium.

Although the growth of the symbiotic fungus was efficient in the culture medium with peach palm, it was completely effective against the *Escovopsis sp* fungus since these results prove toxic chemical compounds production involving the parasitic fungus *Escovopsis* sp. according to what is proposed by Currie et al. (1999b) and Reynolds and Currie (2004).

Even though the addition of tucumã and peach palm can benefit the development of the fungus *L. gongylophorus*, it still showed slow growth in the laboratory, as in all treatments, the fungus did not reach the ends of the Petri dish at the end of the experiment. These results corroborate the results obtained by many authors who analyzed the in vitro development of this fungus (Camargo et al., 2003; Loeck et al., 2004; Borba et al., 2006).

Table 2. Diameter of mycelial growth of the parasitic fungus *Escovopsis sp.* determined by growth (f = W x L), expressed in (cm), cultivated in different culture media for six periods.

Treatments	7 days	14 days	21 days	28 days	35 days	42 days
PUCI	4.3 c	6.4 d	7.2 b	7.6 a	8.0 a	_
CE	8.0 a	_	_	_	_	_
ST	8.0 a	_	_	_	_	_
NU	6.6 b	7.8 c	8.0 a	_	_	_
BDA	8.0 a	_	_	_	_	_
TU	6.3 b	7.56 b	8.0 a	_	_	_
NB	8.0 a	_	_	_	_	_
M	6.8 b	8.0 a	_	_	_	_
PUCC	4.5 c	6.6 d	7.5 b	7.9 a	8.0 a	_
TUC	6.4 b	7.58 b	8.0 a	_	_	_

Means followed by the same letter in the columns do not differ by Tukey's test – 95% significance. * PUCI= Peach palm; CE=Cezapek; ST = Starch; NU= Nutrient Agar; PDA; TU= tucumã: N = Nutrient Broth; M=Malt; PUCC= Boiled Peach palm; TUC= tucumã peel.

Table 3. Simple average showing differences in fungal growth in different media

Tucumã medium	3 days	5 days	7 days	14 days
Escovopsis sp	2.32	4.1	5	5.8
L. gongylophorus	2.1	2.5	3.1	3.9
Peach palm medium				
Escovopsis sp	2.35	4.5	5.3	6.1
L. gongylophorus	2.18	2.76	3.06	3.59

*(m/cm) = mean/centimeters, for each observed day.

4. Conclusions

Culture media prepared from components of tucumã peel, peel+pulp, fresh peach palm, and cooked peach palm significantly affected the *L. gongylophorus* fungus colony's development compared to treatments with Czapek Dox Agar, Nutrient Agar, Starch Casein, Malt Extract Broth Base, Nutrient Broth and BDA. It was observed that these culture media did not develop the fungus in any of the periods.

However, for the fungus *Escovopsis sp*, the treatments with peach palm and tucumã decreased the growth rate compared to other culture media. In the media prepared with tucumã and peach palm, during the fungi L. *gongylophorus* and Escovopsis sp. development, the production and release to the culture medium of exudates were observed, suggesting a more detailed analysis since this is a study that reveals promising data.

Thus, it is essential to continue with scientific studies to evaluate the active principles of the fruits, which may in the future be studied as to their biotechnological potential.

Authors' Contribution

Maria Lucidalva Ribeiro de Sousa contributed to the conduct the experiment and writing of the manuscript. Janaína da Costa Nogueira writing the manuscript. Adriana Dantas Gonzaga de Freitas contributed to the statistical analysis and writing of the manuscript.

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