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COMPARISON OF RADIAL GROWTH RATE OF THE MUTUALISTIC FUNGUS OF *ATTA SEXDENS RUBROPILOSA* FOREL IN TWO CULTURE MEDIA

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ABSTRACT

In vitro culture of the mutualistic fungus of leaf-cutting ants is troublesome due to its low growth rate, which leads to storage problems and contaminants accumulation. This paper aims at comparing the radial growth rate of the mutualistic fungus of *Atta sexdens rubropilosa* Forel in two different culture media (Pagnocca B and MEA LP). Although total MEA LP radial growth was greater all along the bioassay, no significant difference was detected between growth efficiencies of the two media. Previous evidences of low growth rate for this fungus were confirmed. Since these data cannot point greater efficiency of one culture medium over the other, MEA LP medium is indicated for *in vitro* studies with this mutualistic fungus due its simpler composition and translucent color, making the analysis easier.

Key words: leaf-cutting ants, mutualistic fungus growth, mycelial growth, *Leucoagaricus*, *Atta sexdens*

INTRODUCTION

Fungus-growing ants are distributed only in the New World and belong to the monophyletic tribe Attini (Hymenoptera-Formicidae-Myrmicinae), which is composed of 12 genera and approximately 210 species.

Among all attine, usually referred to as fungus-growers, the two most phylogenetically derived genera, *Acromyrmex* and *Atta*, are more commonly known as leaf-cutting ants (8). The leaf-cutting ants are an important forest herbivore, exploring a great variety of plants which are used to cultivate a specific basidiomycete fungus (6). Several authors have identified this microorganism as *Leucoagaricus gongylophorus* (=*Leucocoprinus gongylophorus*) based on the morphology of fruit-bodies which grow inside *Atta sexdens rubropilosa* (2) and *Atta cephalotes* (10) nests, or using molecular sequences (25). This fungus produces a special mycelia structure called gongylidia, rich in glycogen and used as food for the leaf-cutting larvae (1).

Leaf-cutting ants and the basidiomycete fungus possess an intrinsic mutualistic relationship, strongly integrated with antibiotic, nutritional and physiological co-dependence. Leaf-cutting ants protect the mutualistic fungus from parasites and potentials competitors (7, 8), while the fungus is an essential food source for the larvae and queen (17, 20). Other ants in the nests have plant sap as an important food source (23).

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Leaf-cutting ants have been considered a dangerous herbivore to some important crop fields. Several methods have already been proposed for the control of these insects. Since all of them present some environmental disadvantage, continuous search for an effective and less pollutant control method acting directly on ants or on fungal development is the focus of much research (14, 21, 22).

Studies using mutualistic fungi are difficult due to the very slow fungal growth in culture media (15, 16, 21, 22). For example, the mutualistic fungus of *Atta sexdens piriventris* reached 57 mm in diameter after 63 days of experiment with an organic medium called Pagnocca A; 34,4 mm with the medium called V8 juice agar; 46,2 mm with an organic medium called Celulose-asparigine; and 16,8 mm with the mineral medium called Murashige & Scoog (15). Such slow growth rates turn the storage very difficult, and the fungus culture is often impregnated with contaminants.

In this paper, two solid culture media, MEA LP and Pagnocca B, were evaluated for the efficiency of *in vitro* fungal growth promotion. MEA LP culture medium was prepared by combination of two traditional culture media (MEA with yeast addition and MEA peptone). Pagnocca B was first described by Silva-Pinhati *et al.* (25) for bioassays with the mutualistic fungus of *Atta sexdens* and consists of a new buffered supplemented formulation of Paggnoca Medium A.

MATERIAL AND METHODS

Eight leaf-cutting ant nests maintained in laboratory were used as fungi source. Fungi fragments and some ants were removed from each nest and transferred to sterile pots, previously autoclaved at 120ºC and 1.1 atm (eight fungi matrix). The transport of some ants was crucial for successfully fungi culture, because they are able to clean fungi fragments and stimulate its growth. Small isolated mycelium portions of each matrix were inoculated on Petri dishes containing MEA LP, using a sterile laminar flux chamber, for development of the initials cultures.

Two culture media compositions were tested. The first is a common fungi culture medium containing malt extract combined with yeast and peptone (MEA LP – 20 g malt extract, 5 g bacteriological peptone, 2 g yeast extract, 20 g agar, distilled water up to 1 L). The other culture medium, called Pagnocca B by Silva-Pinhati *et al.* (26), is composed of 10 g glucose, 2 g sodium chloride, 2 g bacteriological peptone, 10 g malt extract, 17 g agar, 20 g casein hydrolysate, 20 g soybean flakes, 20 g oat flakes, 3.8 g sodium phosphate, 2.5 g citric acid, distillated water up to 1 L, and pH adjusted to 5.0. Both MEA LP and Pagnocca B culture media were autoclaved at 120 °C and 1.1 atm for 30 min.

Sterile Petri dishes were prepared with 15 mL of culture medium, and kept in a sterile laminar flow chamber under UV light until culture medium solidification. Five millimeter discs containing the mutualistic fungus from initial cultures were transferred to test Petri dishes and placed in the center. Eight Petri dishes, each corresponding to one fungi matrix, were prepared for both MEA LP and Pagnocca B media. The dishes were incubated in a B.O.D. chamber in the dark at 25 ºC (±1ºC).

Two perpendicular straight lines were drawn on the bottom of each Petri dish. The crossing point coincided with the center of the 5 mm initial fungi disc. Radial growth measurements were recorded weekly from the edge of the initial inoculum until the extreme area of fungi mycelia development, following the four segments formed by the two perpendicular lines (Figure 1). Data for each week correspond to means of four measurements, each one carried out with one segment.

Bioassays were ended when the fungi mycelia reached the Petri dish wall in any dish. Daily fungal growth rate was calculated for each fungi matrix, and expressed as $mm.day^{-1}$. Student's T test was used to evaluate differences significance of fungal growth rates between the two culture media.

RESULT AND DISCUSSION

Previous bioassays with traditional *in vitro* culture medium suggested that MEA LP is more effective regarding the growth promotion of the mutualistic fungus of *Atta sexdens* development, in comparison with MEA+yeast (MEA LP

without peptone) or BDA+yeast (10 g dextrose, 20 g agar, juice from 200 g potate cooked in 500 mL water, distilled water up to 1 L) (data not shown). Pagnocca *et al*. (21, 22) and Godoy *et al*. (11) used a growth medium very similar to MEA LP used at the present research, containing glucose, sodium chloride, bacto-peptona, malt extract and agar. Silva-Pinhat *et al*. (26) suggested a new growth medium, called Pagnocca B, as the best option for this mutualistic fungus culture. In the present paper, both media MEA LP and Pagnocca B are compared for growth efficiency of in vitro culture of this fungus species. The observation of gongylidia on mycelia fragments by optical microscopy supports the identity of the mutualistic fungus (*Leucoagaricus* sp) in the *in vitro* cultures (Figure 2) (6, 13, 27).

Mean values of total radial growth of mutualistic fungus related to time are presented in Figure 3. Bioassays completion took seven weeks. All MEA LP measurements showed higher values than those of Pagnocca B medium, which were more evident after the 28th day. However, no significant differences ($p \leq 0.05$) were observed of *in vitro* radial growth of this fungus species. Although the MEA LP values were always higher than those obtained with Pagnocca B, the available data failed to support the hypothesis of better growth promotion of one medium over the other.

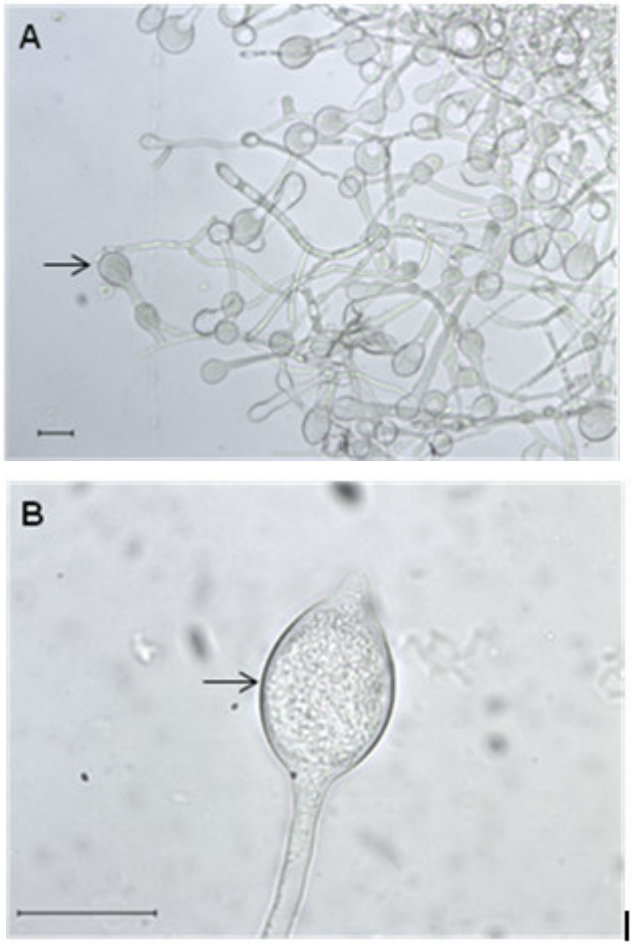

Figure 2. Mycelia detail from mutualistic fungi of *Atta sexdens rubropilosa*. Enlarged apical structures correspond to gongylidia (arrows). Bars = 40 µm. (Photo: A. M. Gugliotta).

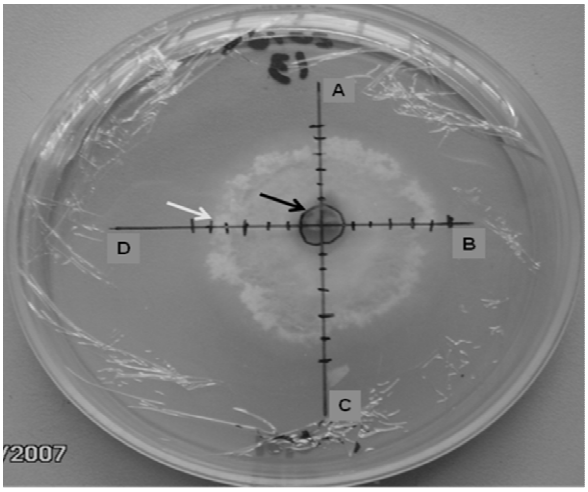

Figure 1. Petri dish used for fungus growth bioassay. Black arrow indicates the edge of initial inoculum. White arrow indicates the edge of fungi radial growth six weeks after bioassay start. Letters A, B, C, D correspond to the four segments used for growth measurements.

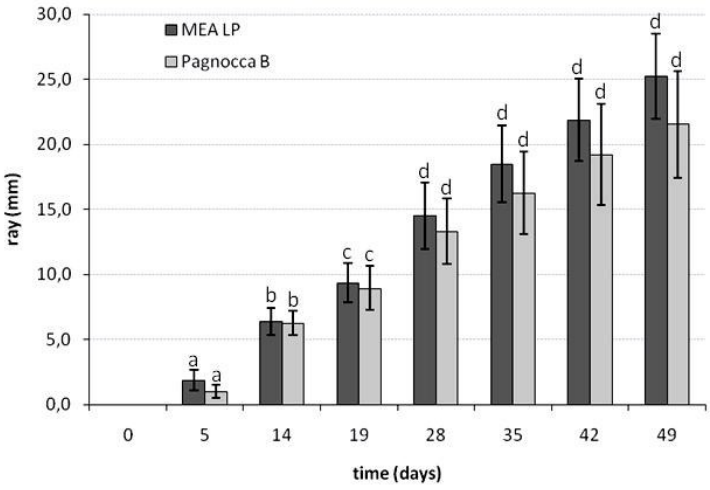

Figura 3. Means and confidence intervals of in vitro radial growth of mutualistic fungus of *Atta sexdens rubropilosa* in MEA LP medium and Pagnocca B medium (n = 8). Same letter over bars indicates that there is no significant difference by using Student T test (P<0.05).

Based on total radial growth, daily growth rate was calculated for each one of the eight fungus matrices (Table 1). Daily growth rates among MEA LP samples were less variable, in comparison with Pagnocca samples. The standard deviation calculated using the eight MEA LP dish plates was very low. Only one sample presented daily growth rate lower than 0.4 mm.day^{-1} with MEA LP medium (matrix 6 = 0.380 mm.day^{-1}). All other samples presented values between 0.426 mm.day^{-1} and 0.684 mm.day^{-1}. Mean daily growth rate for samples cultivated with Pagnocca B medium was lower than the value obtained for MEA LP, 0.440 mm.day^{-1} and 0.515 mm.day^{-1}, respectively. Four of the eight sample matrices presented growth rate lower than 0.4 mm.day^{-1} with Pagnocca B medium. Comparing MEA LP and Pagnocca B values for the same matrix, four of them presented higher MEA LP values (matrices 4, 5, 7 and 8), while the others (matrices 1, 2, 3 and 6) grew better with Pagnocca B. Matrix 8 presented, at the same time, the highest growth rate with MEA LP (0.684 mm.day^{-1}) and the lowest one with Pagnocca medium (0.316mm.day^{-1}).

Table 1. Radial growth rate (mm.day^{-1}) for each fungi matrix cultivated in MEA LP medium and Pagnocca B medium.

	Culture medium	
Fungi matrix	**MEA LP**	**Pagnocca B**
1	0.528	0.543
2	0.520	0.617
3	0.584	0.541
4	0.543	0.342
5	0.454	0.332
6	0.380	0.490
7	0.426	0.337
8	0.684	0.316
mean ± sd	0.515 ± 0.0957	0.440 ± 0.1208

These large differences among matrices could be explained taking into account that the mutualist is clonally propagated by queens that carry a pellet of the fungus in their mouth during their nuptial flight to establish new colonies (1). In addition to vertical propagation from one generation to another, recent research has suggested that horizontal fungi transmission may also happen among ant nests or among close related ant species (12, 19).

Students's T test was applied to verify whether there is a significant difference among daily fungal growth rates on petri dishes filled with MEA LP or Pagnocca B media. Since the observed t-value (T= 1.381) is below the absolute t-value (T = 1.7613), no significant difference has been found between daily growth rates using both MEA LP or Pagnocca B culture media.

The growth rate of *Leucoagaricus* species, mainly those with mutualistic relationship with leaf-cutting ants, has already been pointed out as very slow. The low growth rate has been considered a limiting factor regarding several experimental analyses (15; 21; 22). Loeck et al. (16) reported radial growth rate for the mutualistic fungus associated with another leaf-cutting ant species (*Atta sexdens piriventris*) with several culture media. The highest value obtained was 56.7 mm after 49 weeks or 0.165 mm.day^{-1}. This value is even lower than those obtained in the present work. Studies carried out with other non-mutualistic basidyomicetes species show values of growth rate at least 2,000 times higher in comparison with the mutualistic fungus used in this study. For example, Matheus (18) detected 0.90 ± 0.13 cm.day^{-1} for *Agrocybe perfecta* (Rick) Sing., 1.14 ± 0.33 cm.day^{-1} for *Coprinus jamaicensis* Murr., 2.53 ± 0.53 cm.day^{-1} for *Pycnoporus sanguineus* (L:Fr.) Murr., and 3.77 ± 0.42 cm.day^{-1} for *Phanerochaete chrysosporium* Burds. The intrinsic relationship inside higher attine nests could partially account for the low growth rate of the mutualistic fungus. The dynamics of the association between the ants and their fungi are complex, including other organisms such as the filamentous fungus *Pseudonocardia* (Actinomycetes) which produces antibiotics that inhibits the growth of *Escovopsis* (a virulent pathogen fungus), aiding in the garden maintenance (8).

Besides culture medium composition, other parameters have already been investigated concerning fungal growth rate improvement. Cazin *et al*. (5) tested three temperatures for *in vitro* culture, and observed higher growth rate at 24°C than at 30°C or 37°C. Our experiments have been conducted at 25°C ±

1°C, very close to the ideal temperature previously established.

Another problem regarding leaf-cutting ant mutualistic fungus culture is contaminants accumulation. This problem is closely correlated with the low growth rate of *Leucoagaricus* species in *in vitro* culture. Although reduced time for bioassays was the focus of some research (11, 21, 22), no ideal assay time can be defined based on the available data. Even though no contaminants have developed at most dish plates, in some bioassays lasting more than seven weeks changes were noted in the fungus color and in the culture medium, which were determinant for the experiment failure.

Distinct measurement ways have been used for determination of *in vitro* fungi growth. Although estimative evaluation based on visual observation (15, 22) and determination of relative percentage increase in comparison to the initial condition (9, 11, 21, 24) has been successful measurements, such criteria were dependent on the researcher interpretation. The measurement strategy used in the present work achieved more accurate results, since all values were obtained using a measure instrument. Loeck *et al*. (16) and Borba *et al*. (3, 4) have already employed the same radial-growth measures to evaluate the growth rate of the mutualist fungus of another leaf-cutting ant species.

Radial-growth rate has been shown to be a good measurement approach, although it does not take into account the fungal vertical growth or the increase of density in the Petri dish. Only radial growth (e.g. horizontal growth) is considered in this method. Notwithstanding the growth rate measurements presented have probably been underestimated, radial-growth use turns the obtained values easier to compare with other results and improve data accuracy.

Finally, since no significant difference was found between both tested culture media (MEA LP and Pagnocca B), none of the media can be considered more efficient than the other for fungal growth promotion. However, MEA LP is pointed out as more convenient, due its simpler composition and visual transparency, turning the radial measurements easier and more precise.

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