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Isolation and Maintenance of Symbiotic Fungi of Ants in the Tribe Attini (Hymenoptera: Formicidae)

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Isolamento e Manutenção de Fungos Simbiontes de Formigas da Tribo Attini (Hymenoptera: Formicidae)

RESUMO - O isolamento e a manutenção de fungos basidiomicetos simbiontes de formigas da tribo Attini tem sido dificultado pela baixa velocidade de crescimento desses fungos, bem como pela presença de muitos microrganismos que vivem na superfície do material que as formigas mantêm no interior nos ninhos como substrato para o crescimento dos seus fungos simbiontes. No presente trabalho nós descrevemos um método que aumenta em mais de sete vezes a eficiência de isolamento desses fungos, quando comparada àquela obtida por procedimentos tradicionais. Ninhos subterrâneos de formigas atíneas dos gêneros *Atta*, *Acromyrmex*, *Trachymyrmex* e *Mycetarotes* foram localizados e deles foram coletadas amostras contendo fungos simbiontes e formigas, que foram transportadas para o laboratório, onde as formigas foram capazes de limpar a cultura do fungo e estimular o seu crescimento. Em seguida, porções dos micélios foram assepticamente coletadas e transferidas para meio Yeast Nitrogen Base contendo glicose e cloranfenicol. Para facilitar a manutenção dos isolados em culturas de laboratório, diferentes nutrientes foram analisados para a elaboração de um meio de cultivo complexo, que possibilitou aumentar a velocidade de crescimento dos fungos e estocá-los por longos períodos. O método foi aplicado com sucesso para os fungos simbiontes de todos os gêneros de formigas estudados, gerando, assim, um procedimento extremamente útil para a formação e manutenção de uma coleção representativa de diferentes fungos simbiontes de formigas da tribo Attini.

PALAVRAS-CHAVE: *Atta, Acromyrmex, Trachymyrmex, Mycetarotes*, basidiomiceto, simbiose

ABSTRACT - The isolation and maintenance of symbiotic basidiomycete fungi living in association with ants of the tribe Attini has been hindered by the slow growth rate of these fungi and the presence of other microorganisms on the surface of the material which the ants maintain inside their nests to provide a growth substrate for their symbiont. In this paper we describe a method which increases the efficiency of isolation of these fungal symbionts by over seven fold as compared to traditional isolation procedures. Underground nests of attine ants of the genera *Atta*, *Acromyrmex*, *Trachymyrmex* and *Mycetarotes* were located, from which samples containing the fungal symbiont and ants were collected and transported to the laboratory where the ants were able to clean the fungal culture and stimulate its growth. As the symbiotic fungus grew, portions of its mycelium were collected and transferred to solid Yeast Nitrogen Base culture medium containing glucose and chloramphenicol. To facilitate the maintenance of the isolates in laboratory cultures, several nutrients were tested to formulate a complex culture medium for fast fungal growth and long-term storage. We successfully applied this methodology to the fungal symbionts of all the ant genera studied, thus producing a useful tool for the creation and maintenance of a comprehensive collection of fungi symbiotic of ants in the tribe Attini.

KEY WORDS: *Atta, Acromyrmex, Trachymyrmex, Mycetarotes*, basidiomycete, symbiosis

An obligate symbiosis exists between basidiomycete fungi and leaf-cutting ants of the tribe Attini. This association results in the exploitation of a great variety of vegetation by leaf-cutting ants (Cherrett 1968), which are important both as herbivores in natural habitats (Wint 1983) and pests in agriculture settings (Cherrett & Pregrine 1976, Fowler *et al.* 1986).

In their nests, leafcutter ants propagate the fungal symbiont by providing it with cut leaves and inhibiting the growth of competing microorganisms (Cherrett *et al*. 1989), while the fungus produces enzymes, principally polysaccharidases and proteinases (Boyd & Martin 1975, Cherrett *et al.* 1989, Siqueira *et al.* 1998), which digest the cut leaves to produce the nutrients necessary for the survival of the larvae and adult ants (Quinlan & Cherrett 1979, Bass & Cherrett 1995). The fungus growing in the cut leaves inside the nests being is called 'fungus garden'.

The dependence of Attini ants on their fungal symbiont is so great that the ants have never been seen living isolated from their fungi and any disruption to this symbiotic association is highly deleterious for the ants, thus the control of the attine pest ants would greatly benefit from an understanding of the mechanisms by which the fungal symbiont contributes to ant herbivory and survival. However, the study of these symbiotic fungi has been hampered by their slow growth rate in laboratory culture, which makes isolation and storage a very laborious and time-consuming task. To facilitate future work on Attini symbiotic fungi, we have improved the isolation procedure and developed a culture medium which allows fast fungal growth and which can also be used for long-term storage.

Materials and Methods

Ants and Nest Location. Underground nests of Attini ants of the genera *Atta*, *Acromyrmex*, *Trachymyrmex* and *Mycetarotes* were located and samples of the fungus garden and ants were collected and transported to the laboratory where the ants were able to reorganize the fungus garden material and clean the fungal culture and stimulate its growth. The ant species, nest location and date of sampling are listed in (Table 1), all the ant species involved in this study being deposited in the Coleção Entomológica Adolph Hempel at the Instituto Biológico, São Paulo-SP, Brazil.

Table 1. Attini ants from which symbiotic fungi have been isolated.

Ant	Nest location[1]	Isolation date
Atta sexdens (L.)	Rio Claro	Aug, 1997
Atta capiguara Gonçalves	Botucatu	Sep, 1995
Atta laevigata (Fr. Smith)	Rio Claro	Nov, 1995
Atta cephalotes (L.)	Almeirin	Aug, 1997
Acromyrmex crassispinus (Forel)	Rio Claro	Sep, 1995
Acromyrmex hispidus fallax Santschi	Rio Claro	Mar, 1996
Trachymyrmex fuscus Emery	Rio Claro	Dec, 1998
Mycetarotes parallelus (Emery)	Rio Claro	Jul, 1997

[1]All the collection sites, except for the *A. cephalotes* site, were in the state of São Paulo, Brazil. *A. cephalotes* was collected in the state of Pará, Brazil.

Culture Media. For fungal isolation from the fungus garden we used Yeast Nitrogen Base Glucose Chloramphenicol (YNBGC) agar consisting of Yeast Nitrogen Base media (YNB, Difco 100697) supplemented with (gl^{-1}) glucose (Merck, 108342, Darmstadt, Germany), 5; agar-agar (Merck, 1.01614), 17; chloramphenicol (Sigma C-0378), 0.1 and sufficient 2M-NaOH to adjust the pH to 6.0. For maintenance we used YNB-glucose agar (i.e. YNBGC without chloramphenicol).

To assess biomass production as a measure of growth we used unsupplemented Pagnocca's *et al*. (1990) liquid Medium A containing (gl^{-1}) glucose, 10; NaCl (Labsynth C1060.01.AH, São Paulo, Brazil), 5; peptone (Sigma P-0556), 5; malt extract (Difco 0186-01), 10, as well as Medium A agar (containing 17 gl^{-1} agar-agar). We also used liquid Medium A supplemented with (gl^{-1}) casein hydrolysate (Sigma 'Amicase' A-2427), 20; flaked oats (Maisvita, São Paulo, Brazil), 20; flaked soybean (Imalaia Oriente, Santa Gertrudes, Brazil), 20. Both unsupplemented and supplemented liquid Medium A had a final pH of 6.0. We also used YNB media supplemented with 5 gl^{-1} of one of the following: bovine albumin (Sigma A-6003), casein (Quimibrás 10578, São Paulo, Brazil), casein hydrolysate, egg albumin (Riedel de Häen 188 01), gelatin (Sigma G-9382), glucose, flaked oats, flaked soybean, soybean flour (Sigma S-9633) or xylose (Sigma X-1500), the final pH being 6.0.

Based on the experimental evidence presented in this paper and published data we produced a new buffered and supplemented formulation of Pagnocca's Medium A agar that we called Medium B agar and which contained (gl^{-1}) glucose, 10; NaCl, 2; peptone, 2; malt extract, 10, agar-agar, 17; casein hydrolysate, 20; flaked soybean, 20; flaked oats, 20; dibasic sodium phosphate, 3.8; citric acid, 2.5; final pH 5.0. All media were sterilized for 20 minutes at 120°C.

Fungal Isolation and Cultivation. The fungus garden material collected from underground nests of the different ant species was incubated (25°C, 80% humidity) for 10 to 20 days in the dark in petri dishes containing worker ants which cleaned the garden material by removing soil fragments to a different part of the petri dish. When white mycelial spots of the fungal symbiont appeared on the leaf material some of the mycelia was collected using aseptic technique and transferred to YNBGC agar in petri dishes where it was incubated at 25ºC in the dark for 30 days. Alternatively, the fungus garden material from underground nests was collected and immediately plated onto YNBGC agar and incubated at 25ºC in the dark for 30 days. In both cases, after 30 days of cultivation on YNBGC agar the fungal isolates were subcultured to YNB-glucose agar (without chloramphenicol) and cultured for a further 30 days. To evaluate biomass production by the symbiotic fungus of the ant *Atta sexdens* (L.) (formerly *Atta sexdens rubropilosa* Forel) on various carbon substrates we subcultured the fungus on YNB-glucose agar for 30 days to produce large amounts of mycelia which was collected and suspended in sterile water with a Potter homogenizer. A suitable volume (containing 2.1 ± 0.11 mg dry weight) of this suspension was transferred to tubes containing 5 ml of liquid Medium A (supplemented and unsupplemented), supplemented YNB media or supplemented culture media A agar and B agar. After 30 days static incubation at 25°C,

liquid media without insoluble components was filtered through a 0.25 µm Millipore filter to collect the mycelia while for liquid media containing insoluble carbon sources (casein, gelatin, soybean, flaked flour or flaked oats) mycelia was harvested with a loop. In both cases the mycelia was dried at 65°C for 24h and weighed. Fungal growth in solid culture media was estimated based on the mycelial surface and density as previously described in Pagnocca *et al.* (1990).

Statistics. Cultures in liquid media were replicated six to ten times for each of the carbon sources. The determined dry weight values were expressed as means ± standard deviations, the Tukey test being used to test for differences between the weight of mycelia produced in the different media and the Mann-Whitney test for differences between initial and final pH (Zar 1996).

Results and Discussion

The maintenance of fungus garden material from ants' nests in the absence of ants resulted in the development of many filamentous fungi and yeasts which often overgrew the characteristic white spots of the symbiotic fungus (Fig. 1a) initially seen on the fungus garden material, and because of this only one in about 30 attempts resulted in the isolation of the symbiotic fungus.

Isolation efficiency was increased by keeping the fungus garden material in petri dishes at 25°C and 80% humidity in the presence of worker ants which started to clean the fungus garden material by removing soil fragments to a different part of the plate, almost all soil debris being removed from the leaf material within 24h. This cleaning activity stimulated the growth of the fungal symbiont as characterized by the accumulation of white mycelial spots on the surface of the leaf material in the fungus garden (Fig. 1b). These spots were collected over the 20 days of the experiment and plated onto YNBGC agar, a procedure which resulted in one successful isolation out of every four attempts. As explained in the Materials and Methods, after 30 days of cultivation on YNBGC agar the fungal isolates were transferred to YNB-glucose agar (without chloramphenicol) and cultured for 30 days to produce larger amounts of mycelia. Few contaminants developed at this stage allowing the mycelia to be subcultured to YNB-glucose without antibiotics for further cultivation and maintenance.

Among the symbiotic fungi isolated (Table 1), those from *Atta*, *Acromyrmex* or *Trachymyrmex* ants exhibited white mycelium which slowly spread as a thin film over the YNB-glucose agar (Fig. 1c) and had swollen hyphal tips (Fig. 1d) called gongylidium (Chapela *et al.* 1994, Fisher *et al.* 1994), the isolates from *Mycetarotes* ants rarely, if ever, presenting gongylidia. In general, isolates started to become dark brown after 30 to 40 days so that further replication in fresh culture media resulted poor or no fungal growth. This loss of viability

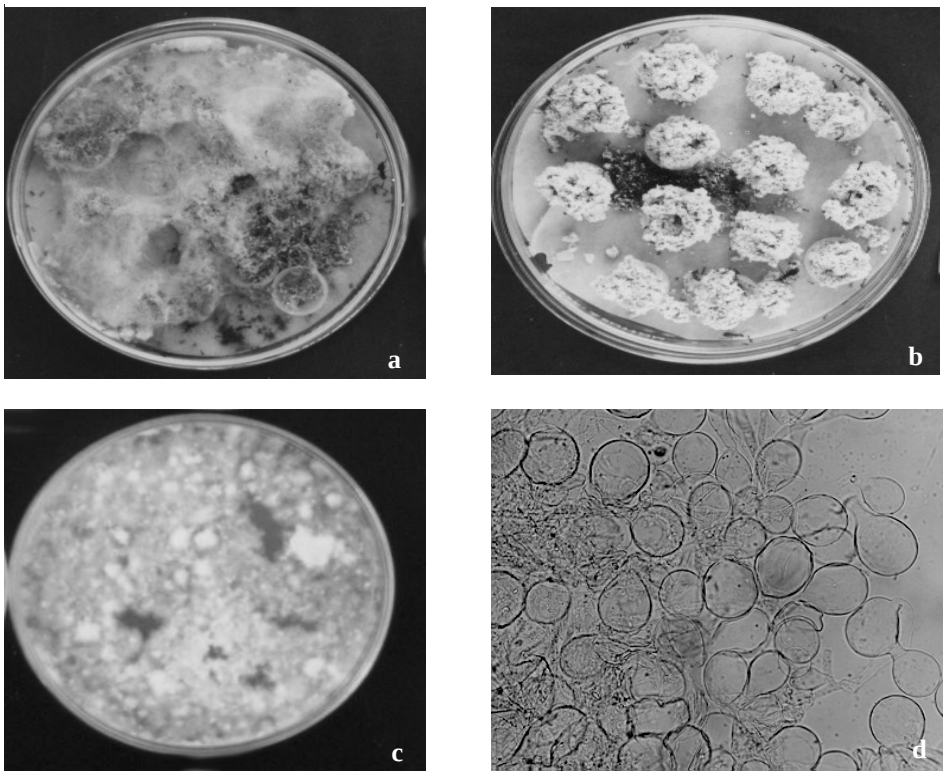

Figure 1. Isolation of the fungal symbiont of the ant *A. crassispinus*. Note the development of contaminants in fungus garden samples after 15 days without ants (a), in contrast to the selective propagation of the fungus in the presence of ants (b). After isolation, the fungus grew as white mycelium on Yeast Nitrogen Base Glucose (YNBG) agar (c) and presented swollen hyphal tips called gongylidium (d) which is a general characteristic of the symbiotic fungi of higher Attini.

means that many replications are needed to maintain the isolates, making laboratory cultivation a continuous and exhaustive task.

To minimize laboratory work, attempts have been made to formulate a culture medium which was more efficient at maintaining laboratory cultures of symbiotic fungi. To accomplish that we investigated the growth rate of the *A. sexdens* fungal symbiont by cultivation in liquid Medium A and YNB containing different nutrients (Table 2), glucose and xylose being used as controls because these simple carbohydrates efficiently support the growth of this fungus in laboratory cultivation and it has been proposed (Siqueira *et al.* 1998) that the production of glucose and xylose by the fungal degradation of starch and xylan is the major process for the generation of nutrients from the vegetal matter which the ants bring into their nests.

Table 2. Weight of mycelia and final pH (mean ± SD) of liquid culture media after growth of the symbiotic fungus of *A. sexdens*.

Medium	Weight of mycelia (mg, dry weight)	pH
Supplemented[1] Medium A (9)[2]	34.4 ± 8.82 a	5.0 ± 0.11*
Medium A (9)	13.2 ± 1.44 b	5.0 ± 0.11*
YNB-xylose (9)	15.5 ± 0.91 b	4.8 ± 0.12*
YNB-glucose (9)	14.6 ± 0.91 b	2.7 ± 0.11*
YNB-flaked oats (7)	12.0 ± 1.33 b, c	6.1 ± 0.11
YNB-gelatin (10)	9.4 ± 0.32 c, d	6.3 ± 0.13*
YNB-flaked soybean (9)	8.3 ± 1.19 d	6.2 ± 0.09*
YNB-bovine albumin (8)	7.9 ± 0.8 d	5.9 ± 0.33
YNB- casein hydrolysate (8)	7.6 ± 0.38 d	6.3 ± 0.23*
YNB-soybean flour (8)	6.4 ± 0.63 d	6.2 ± 0.12*
YNB-egg albumin (6)	5.1 ± 0.58 d	5.9 ± 0.11
YNB-casein (6)	4.7 ± 0.5 d	6.2 ± 0.11*

Values followed by different letters are significantly different by the Tukey test (95% confidence limit)
[1]Supplemented with (gl^{-1}) casein hydrolysate, 20: flaked soybean 20; flaked oats, 20
[2]Number of replicates
*Values significantly different from the initial pH value (6.0) by the Mann-Whitney test (95% confidence limit)

Our results (Table 2) using supplemented YNB confirm that the simple carbohydrates glucose and xylose were the most efficient carbon sources in respect to the production of fungal biomass, while the most efficient complex carbon sources was flaked oats, followed by gelatin, flaked soybean, bovine albumin, casein hydrolysate, soybean flour, egg albumin and casein. It seems, therefore, that the *A. sexdens* fungal symbiont is better adapted to grow on carbohydrates and not on proteinaceous substrates, the slow growth rate on proteins having also been reported for the symbiotic fungus of the ant *Atta colombica tonsipes* Santschi (Martin & Martin 1970).

After 30 days incubation there was a statistically significant (Mann-Whitney test, 95% confidence limit) decrease in the original pH (6.0) of YNB broth supplemented with xylose to pH 4.8 and to pH 2.7 for YNB supplemented with glucose. Supplementation of YNB with bovine or egg albumin or flaked oats resulted in no significant pH change, while supplementation with gelatin, flaked soybean, soybean flour, casein or casein hydrolysate resulted in a small (but statistically significant) increase in pH to 6.2-6.3 (Table 2). It thus seems that metabolism of carbohydrates by fungal symbiont causes a decrease in pH while the metabolism of proteins either does not affect or slightly increases pH.

Medium A agar, described by Pagnocca *et al*. (1990), is normally used for culturing the fungal symbiont of *A. sexdens*. We tested the effect of supplementing liquid Medium A with casein hydrolysate, flaked soybean and flaked oats and found that the quantity of fungal biomass produced in supplemented liquid Media A after 30 days cultivation was over 2.5 times higher than in unsupplemented liquid Medium A (Table 2) and more than twice the biomass achieved in YNB-glucose. Both supplemented and unsupplemented liquid Medium A reached pH 5.0 after 30 days incubation, significantly different from their initial pH of 6.0 (Table 2). That supplemented liquid Medium A attained the same pH value as unsupplemented liquid Medium A may have been due to the fact that both media contained glucose and peptone, the metabolism of which had opposing effects on pH and which may have helped to stabilize the final pH of these media.

Considering the optimum pH values (around pH 5) reported for fungal symbionts of Attini ants (Silva 1999) and the good growth achieved on supplemented liquid Medium A we decided to buffer Medium A agar to pH 5.0 as described by Stoll & Blancard (1990) and to supplement it, as described in Materials and Methods, in order to produce what we called Medium B agar. We found that Medium B agar supports faster growth of the symbiotic fungus of *A. sexdens* than that found in Medium A agar and that although Medium B agar contained suspended particles of the insoluble components (flaked soybean and flaked oat) of the medium and appeared lumpy, removal of these particles by filtration (before agar addition) caused a decrease in fungal growth rate (Table 3). Cultures could be conserved for up to 40 days in Medium B agar instead of starting to become dark brown after 30 to 40 days as on

Table 3. Relative growth of the symbiotic fungus of *A. sexdens* in different solid culture media.

Medium	Relative growth (30 days)
Medium A agar (8)[1]	2 +
Medium B agar (9)	5 +
Filtered[2] Medium B agar (6)	3 +

[1]Number of replicates
[2]Filtration was carried out (before agar addition) to remove suspended particles of the insoluble components (flaked soybean and flaked oat) of the medium, which minimized the lumpy appearance of Medium B agar, but however caused a decrease in fungal growth rate.

YNB-glucose agar or Medium A agar, indicating that the mycelia is still viable, while there also appeared to be less contamination on Medium B agar. These characteristics indicate that Medium B agar is better optimized for the cultivation of Attini ant symbiotic fungi and can facilitate the maintenance of several isolates simultaneously.

The methods for the isolation and maintenance of Attini ant symbiotic fungi described in this paper will facilitate the formation of collections of the fungi associated with these ants, and thus help to provide important information that may be useful for the control of those Attini ant species which are agricultural pests.

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