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Isolation, Growth Characteristics, and Long-Term Storage of Fungi Cultivated by Attine Ants

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Seven pure-culture strains of fungi cultivated by attine ants (ant-garden fungi) were isolated from locally maintained leaf-cutting ant colonies. An ant-garden fungus strain obtained from an *Atta cephalotes* colony, when offered to ants of the colony from which the fungus was isolated, was accepted as their own. Young fungus cultures were harvested and incorporated into the fungus garden, and cultures of intermediate age were used to begin a new fungus garden; old cultures were simply harvested. To facilitate further research on this fungus, growth characteristics of the different isolates were studied under a variety of conditions. They grew better at 24°C than at 30°C, and growth did not occur at an incubation temperature of 37°C. In a broth culture medium, growth was enhanced by aeration of the culture and by addition of yeast extract, olive oil, sesame oil, peanut oil, soybean oil, corn oil, sunflower oil, cottonseed oil, walnut oil, safflower oil, or mineral oil. Glycerol did not noticeably affect growth, but Tween 80 inhibited growth. These fungi were extremely sensitive to cycloheximide, growth being totally inhibited at cycloheximide concentrations ranging from 0.4 to 4.0 μg/ml. To date, the ant-garden fungus isolates have remained viable in long-term mineral oil-overlay storage cultures for up to 4 years.

For some time, we have been involved in studies that use leaf-cutting ants and the mutualistic fungus cultivated by these ants. Up to now, our work has centered around the chemical ecology of host plant selection (5) and the antifungal activities of crude plant extracts and of purified natural compounds that were found to deter leaf-cutting ant attack (4). In this report, we describe certain microbiological characteristics of the fungus cultivated by attine ants.

The mutualistic fungus of the attine ants is an extremely slow-growing fungus when cultivated on common laboratory media. Consequently, assays for antifungal activity, or studies designed to obtain fundamental physiological information about the organisms, are quite arduous and must be run over time periods of from several weeks to several months in order to yield meaningful results. Since relatively little has been published in the way of microbiological studies on the mutualistic fungus itself, a series of studies was undertaken to more specifically characterize optimal conditions for its growth.

The specific identity of the fungus cultivated by attine ants has been a subject of some controversy; moreover, it has not been demonstrated that all attine ant colonies use the same organism for their fungus gardens. The term ant-fungus, sometimes used (3, 10, 11) to describe pure-culture isolates of the symbiotic fungus cultivated by attine ants, is ambiguous and inadequate from the standpoint of proper microbiological nomenclature. In order to minimize ambiguity, we will refer to the fungus, or group of fungi, cultivated by these ants as the ant-garden fungus.

MATERIALS AND METHODS

Isolation of the ant-garden fungus. Most of our own ant colonies were collected from a tropical deciduous forest in Santa Rosa National Park, Guanacaste Province, Costa Rica; each colony contained one queen. The colonies were maintained in Plexiglas boxes in an insectarium at the Department of Biology, University of Iowa. The room was maintained at 23°C and 40 to 60% relative humidity on a 12 h-12 h light-darkness cycle. Colonies were fed fresh lilac leaves in season and frozen leaves during winter months.

Specimens of fungus garden material ranging in size from 1 to 2 cm^3 were removed from individual colonies of fungus gardens cultivated by the ants and were stored temporarily in sterile test tubes. The fungus garden material was placed in a sterile petri dish and separated into small pieces with sterile dissecting needles, and the ants remaining on the material were removed with sterile forceps.

In order to minimize the number of extraneous bacteria and airborne-fungus spores present on specimens, the fungus garden material was washed with successive changes of distilled water as follows. The small pieces of fungus garden material were suspended in 10 ml of sterile distilled water, vigorously vortexed, and centrifuged briefly to sediment the particles. The supernatant fluid was carefully decanted and replaced with 10 ml of fresh sterile distilled water, and the process described above was repeated through a total of five successive washes.

Following the last wash, the fungus garden particles were resuspended in 1 ml of sterile distilled water and samples were removed with a bacteriological loop for inoculation onto culture medium (Sabouraud dextrose agar, Mycosel agar [BBL Microbiology Systems, Cockeysville, Md.], potato dextrose agar, corn meal dextrose agar, corn meal agar with 0.5% Tween 80, and Czapek Dox agar) contained in petri dishes. The cultures were incubated at room temperature (24°C) under fluorescent light.

Ant-garden fungus stock culture storage. Pure-culture isolates of the fungus strains were inoculated onto potato dextrose agar slants prepared in screw-cap tubes. The cultures were incubated at room temperature with the caps loosened for a period of 4 to 6 weeks to allow growth to cover most of the slant. A mineral oil overlay was used to

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TABLE 1. Origins of ant-garden fungus strains

Fungus strain	Accession number[a]	Ant colony strain	Geographic source of ant colony[b]
AF-1	NRRL 13773	*Atta cephalotes*	Santa Rosa National Park, Costa Rica
AF-2	NRRL 13774	*Acromyrmex octospinosus*	Santa Rosa National Park, Costa Rica
AF-3	NRRL 13775	*Atta cephalotes*	Santa Rosa National Park, Costa Rica
AF-4	NRRL 13776	*Atta cephalotes*	Santa Rosa National Park, Costa Rica
AF-5	NRRL 13777	*Atta columbica*	Barro Colorado Island, Panama
AF-6	NRRL 13778	*Atta texana*[c]	College Station, Tex.
AF-7	NRRL 13779	*Atta mexicana*[d]	Organ Pipe Cactus National Monument, Ariz.
AF-8	NRRL 13780	*Acromyrmex octospinosus*	Santa Rosa National Park, Costa Rica

[a] Cultures have been deposited at the Northern Regional Research Center, Peoria, Ill.
[b] Santa Rosa National Park has now been expanded to Guanacaste National Park, Costa Rica.
[c] Ant colony provided by Alex Mintzer.
[d] Pure culture isolate provided by Alex Mintzer.

prepare individual cultures for long-term storage. Sterile mineral oil was pipetted into each slant culture in sufficient volume to allow for an excess of the oil to extend about 1 cm above the upper edge of the slant. The screw caps were tightened, and the cultures were stored upright at room temperature.

Viability tests were performed on the mineral oil-overlay storage cultures by making subcultures onto fresh potato dextrose agar slants incubated at room temperature. A sterile stiff-wire inoculating needle was inserted through the mineral oil layer, and a piece of fungus mycelium was removed from the surface of the agar slant. The inoculum was then introduced into the surface of a fresh potato dextrose agar slant by deliberately slashing the medium with the needle to ensure that the oil-covered mycelium would make direct contact with the culture medium.

Recognition of the ant-garden fungus by leaf-cutting ants. A pure-culture isolate of the fungus was grown on 50 ml of Sabouraud dextrose agar contained in a 500-ml Erlenmeyer flask, and individual cultures of five different saprophytic fungi isolated from the same ant colony material were prepared in a similar manner. Six flasks, each containing one of the test cultures, were connected one at a time to the ant colony with a Tygon tube (Sani-Tech), and the behavior of the ants toward the cultures was observed.

In order to determine whether there might be a difference in response to young or old cultures of the ant-garden fungus, culture medium in Erlenmeyer flasks, as described above, was inoculated with fungus at 2-week intervals over a period of 3 months. The flasks, containing cultures of different ages, were attached to the ant colony and observed in order to determine preferences exhibited by the ants for individual cultures.

Effect of incubation temperature on growth of the ant-garden fungus. Cultures of ant-garden fungus strain AF-1 were prepared in Sabouraud dextrose agar or broth medium contained in Erlenmeyer flasks to compare growth at 24°C and at 30°C. Potato dextrose agar slants were inoculated with fungus to test for growth of all eight ant-garden fungus strains at 37°C.

Effect of aeration on growth of the ant-garden fungus. Sabouraud dextrose broth cultures were prepared in 100-ml volumes in 500-ml Erlenmeyer flasks. Duplicate sets of medium were prepared with and without supplements of 1% yeast extract, 1.0 ml of olive oil, or 1.0 ml of oleic acid. After inoculation of the media, one set of cultures was incubated as stationary cultures and the other set was incubated on a Gyrotory shaker (New Brunswick Scientific Co., Inc., Edison, N.J.) at 190 to 200 rpm at room temperature for periods of up to 5 weeks.

Effects of yeast extract, oils, and Tween 80 on growth of the ant-garden fungus. Sabouraud dextrose broth and Sabouraud dextrose broth enriched with 1% yeast extract were prepared in 100-ml volumes contained in 500-ml Erlenmeyer flasks. Individual flasks of medium were supplemented with 1 ml of one of the following vegetable oils: olive, sesame, peanut, soybean, corn, sunflower, cottonseed, walnut, and safflower. Other supplements were mineral oil, glycerol, and 0.1 to 2.0% concentrations of Tween 80. The culture media were inoculated with individual ant-garden fungus strains and incubated as stationary cultures at room temperature for 10 weeks.

RESULTS

After small pieces of washed fungus garden material were inoculated onto plates of culture medium, colonies suspected of being the slow-growing ant-garden fungus became noticeable within 3 to 4 weeks. At that time, subcultures were made from isolated colonies onto potato dextrose agar slants and incubated at 24°C until an amount of growth sufficient to allow for microscopic examination was present. Identification of an isolate as an ant-garden fungus was based on the following criteria: (i) a lack of spore production and the presence of gonglydia when examined by microscopy, (ii) macroscopically evident staphylae in the aerial mycelium of a growing culture, and (iii) extremely slow growth.

At present, our culture collection of ant-garden fungus strains consists of eight individual isolates. Using the method described above, we have isolated seven strains from individual colonies of attine ants maintained at the University of Iowa. Through the generosity of Alex Mintzer (Department of Entomology, Texas A&M University, College Station, Tex.), we have been supplied with one additional pure-culture isolate. Our eight ant-garden fungus strains and their sources are listed in Table 1.

With the exceptions of Mycosel agar and Czapek Dox agar, ant-garden fungi from washed fungus garden material grew on all media inoculated. When Czapek Dox agar slants (1.5% agar; Agar Products Co., Van Nuys, Calif.) were inoculated with each of the pure-culture isolates, growth occurred, but the rate of growth was much slower than that on media containing an organic source of nitrogen. Czapek Dox broth culture medium yielded no growth over a period of 12 weeks of incubation at 24°C.

Mycosel agar is a peptone-dextrose medium that contains cycloheximide at a concentration of 400 μg/ml. After preliminary tests showed that no ant-garden fungus strain would grow in the presence of Actidione (85 to 100% cycloheximide; The Upjohn Co., Kalamazoo, Mich.) at either 400 or

TABLE 2. Growth of ant-garden fungi in the presence of Actidione

Inoculum	Actidione concn (μg/ml)	Growth[a] at week: 1	2	3
AF-1	4.0	–	–	–
	0.4	–	+	2+
	0.04	+	2+	4+
	0.004	+	2+	4+
AF-2	4.0	–	–	–
	0.4	+	+	1+
	0.04	+	4+	4+
	0.004	+	4+	4+
AF-3	4.0	–	–	–
	0.4	+	+	1+
	0.04	+	2+	4+
	0.004	+	2+	4+
AF-4	4.0	–	–	–
	0.4	+	+	1+
	0.04	+	2+	3+
	0.004	+	2+	4+
AF-5	4.0	–	–	–
	0.4	+	+	1+
	0.04	+	2+	4+
	0.004	+	2+	4+
AF-6	4.0	–	–	–
	0.4	–	+	1+
	0.04	+	3+	4+
	0.004	+	3+	4+
AF-7	4.0	–	–	–
	0.4	–	+	1+
	0.04	+	3+	3+
	0.004	+	4+	4+
AF-8	4.0	–	–	–
	0.4	–	–	–
	0.04	+	2+	2+
	0.004	+	3+	4+

[a] Symbols: –, no growth; +, barely detectable growth; 1+, 25% of control growth; 2+, 50% of control growth; 3+, 75% of control growth; 4+, no inhibition.

40 μg/ml, a neopeptone-dextrose agar medium was prepared that contained serial dilutions of Actidione with a concentration ranging from 4.0 to 0.004 μg/ml. The media were inoculated with each of the eight strains, incubated at room temperature, and observed over a period of 3 weeks. Results of tests done with serial dilutions of 4.0 to 0.004 μg/ml are listed in Table 2.

Individual cultures of each of our ant-garden fungus isolates were prepared on potato dextrose agar slants contained in screw-cap tubes for the purpose of preparing long-term-storage mineral oil-overlay cultures. Our first isolate, AF-1, has remained viable over a period of 48 months. Our other seven isolates, stored for shorter periods of time, also have remained viable. AF-2, AF-3, AF-4, and AF-5 have remained viable over a period of 19 months, AF-6 has remained viable over 18 months, AF-7 has remained viable over 13 months, and AF-8 has remained viable over 9 months.

Pure cultures of isolate AF-1 and cultures of five saprophytic fungus strains (*Penicillium* sp., *Aspergillus* sp., *Cladosporium* sp., *Scopulariopsis* sp., and *Cephalosporium* sp.) isolated from the fungus garden material were connected via a Tygon tube to the leaf-cutting ant colony (*Atta cephalotes*) from which the fungus strains had been isolated. The ants readily accepted the AF-1 culture and incorporated it into their fungus garden. They also cut and removed the agar from the culture flask. None of the five saprophytic fungi was accepted by the ants; rather, the flasks containing these fungi were used as trash piles.

When AF-1 cultures of various ages were attached to the *Atta cephalotes* colony, the ants again recognized the AF-1 culture as their own fungus. Their behavior included careful exploration (with their antennae) and test bites of the cultures before any action was taken. The ants expressed different responses to the culture isolates, depending upon the age of the individual culture. In the case of relatively young cultures (2 weeks of age), the ants carried mycelium back to the colony for incorporation into their fungus garden. With cultures of intermediate age (1 month old), they brought cut leaves into the culture flasks and attempted to start a new fungus garden. With old cultures (greater than 8 weeks of age), the ants simply harvested the fungus mycelium.

When duplicate cultures of AF-1 were prepared on Sabouraud dextrose broth or on Sabouraud dextrose agar and incubated at 24°C and at 30°C, the elevated temperature did not increase the rate of growth on either of the two media. On the contrary, fungus growth appeared to be somewhat better in cultures incubated at the lower temperature. Cultures of each of the eight ant-garden fungus isolates, prepared on potato dextrose agar slants and incubated at 37°C, exhibited no evidence of growth after a period of 3 weeks.

AF-1 was found to grow better in shake cultures than it did in stationary cultures. When incubated at room temperature in stationary or shake culture, or with broth or agar, growth was markedly stimulated by the addition of yeast extract or olive oil but less so by oleic acid. Raising the yeast extract concentration in culture medium from 1% to 2% did not increase the rate of growth of AF-1. Oleic acid appeared to be inhibitory early in the incubation period, but it later became stimulatory, and growth eventually became about equal to that in unsupplemented medium. At 30°C in stationary or shake culture, or with broth or agar, there was very little stimulation by any of these supplements.

After it had been shown that olive oil would stimulate growth of AF-1, other vegetable oils and related compounds were tested. Sesame, peanut, soybean, corn, sunflower, cottonseed, walnut, safflower, and paraffin oils, when incorporated into Sabouraud dextrose broth with or without 1% yeast extract, were found to stimulate growth of the fungus. Glycerol had no obvious effect on AF-1 growth.

Tween 80, when added to Sabouraud dextrose broth containing 1% yeast extract, inhibited growth of AF-1 at concentrations of 0.5, 1.0, and 2.0%. Inhibition was approximately 25% with 0.5% Tween 80, increasing to about 50% with a 2.0% Tween 80 concentration. In culture media containing Tween 80, the fungus did not grow on the surface; rather, it grew only deep within the medium. Moreover, addition of Tween 80 to culture medium containing olive oil did not improve the growth of AF-1. On the contrary, Tween 80 appeared to inhibit growth of the fungus at any of the four concentrations (0.1, 0.5, 1.0, and 2.0%) tested.

DISCUSSION

Initial attempts to isolate the ant-garden fungus were hampered by large numbers of bacteria and fungus contam-

inants on the surface of fungus garden material. This complicating factor was mentioned by Weber (10), but it is not clear from his report whether an ant-garden fungus isolate was obtained free from contaminating organisms or whether the fungus grew on culture medium along with a mixture of other organisms. After eliminating many surface contaminants by washing the fungus garden material with sterile distilled water, we were able to obtain individual, well-separated colonies of the ant-garden fungus.

Although isolated colonies of the fungus appeared on most media used, it grew best on medium containing appreciable amounts of free carbohydrate and an organic nitrogen source, a finding also noted by Weber (10) and by Martin et al. (9). Martin (7, 8) and Boyd and Martin (1) reported that the ant-garden fungi grow better on hydrolysates of polypeptides than when nitrogen is supplied as a peptone or polypeptide, presumably because the fungus does not secrete significant amounts of proteolytic enzymes when growing under pure-culture conditions.

The fungus grew best on Sabouraud dextrose agar, grew less well on potato dextrose agar, and grew only sparsely on corn meal dextrose agar or on corn meal agar with 0.5% Tween 80. Although initial isolations were not achieved on Czapek Dox agar, we subsequently found that pure-culture isolates of the ant-garden fungi would grow, albeit very slowly, on this medium. The ant-garden fungi did not grow, however, on Czapek Dox broth. It appears that growth on Czapek Dox agar medium can be attributed to trace nutrients in the agar that is used to solidify the medium.

The lack of growth of the fungus strains on Mycosel agar was found to be due to the presence of cycloheximide, a known antifungal antibiotic to which the ant-garden fungi appear to be exquisitely sensitive. We have not yet determined whether cycloheximide might be repellent to leaf-cutting ants, because we do not want to risk losing a captive ant colony. Whether or not cycloheximide is repellent, it would appear that because of its extreme toxicity for the ant-garden fungus, this chemical might serve some practical purpose in geographical areas where leaf-cutting ants present a serious problem. Cycloheximide has been used as a commercial fungicide, and it probably could be applied to commercially valuable plants at risk of leaf-cutting ant attack. If leaves that had been sprayed with cycloheximide were harvested by the ants, the chemical might prevent or retard ant-garden fungus growth on the leaf substrate, thereby eventually eliminating the fungus garden, which is needed for ant colony survival.

The mineral oil overlay technique appears to be a suitable method for preserving viable ant-garden fungus isolates for periods of at least 4 years. When making subcultures from mineral oil-overly stock cultures, it is important to introduce the inoculum into the fresh medium by slashing the medium. Unless this is done, the mineral oil may prevent the inoculum from making contact with the nutrient medium, and the subculture may not be able to grow. Although specific assays have not yet been done to compare freshly recovered cultures with those that have been subcultured at regular intervals over the same period of time, obvious morphological changes have not occurred in the storage cultures. Being a simple and relatively inexpensive method for long-term storage of cultures, the mineral oil overlay method provides a means for maintaining an ant-garden fungus culture collection without the necessity of periodically making fresh subcultures.

Even though the ants responded differently to ant-garden fungus cultures of different ages, they always recognized and accepted their own fungus. In contrast, five strains of saprophytic fungi isolated from the same colony were not accepted; the ants treated these organisms as part of their colony trash heap. This type of recognition test, done on the isolate obtained from our first attempt to grow the fungus, established its identity as the leaf-cutting ant ant-garden fungus (11). Once we became familiar with the appearance and growth characteristics of the organism, it was no longer necessary to repeat the test with each new isolate.

Although leaf-cutting ants make good use of the fungus that they cultivate, growth of the organism is distressingly slow for microbiological work. On media such as Sabouraud dextrose broth, Sabouraud dextrose agar, or potato dextrose agar, some of our assays and growth studies must be incubated for as long as 12 to 16 weeks to obtain enough growth to make meaningful measurements. To try to reduce such extremely long incubation periods, we began exploring ways to enhance the growth rate of the fungus.

When cultures were incubated at an elevated temperature of 30°C, the growth rate was somewhat less than that at room temperature, a finding also noted by Weber (11). Since our leaf-cutting ant ant-garden fungus cultures grow very poorly at 30°C, and since no growth occurs at 37°C, they appear to present little or no infectious hazard for humans.

Broth cultures of the organisms, aerated by incubation on a Gyrotory shaker, grow as small ball-like clumps that float in the broth. When growth is light, the ball-like clumps are larger than they are in cultures in which growth is very heavy.

Addition of Tween 80 to a broth culture medium or to the medium supplemented with olive oil inhibited growth of the ant-garden fungus. We have not determined whether the Tween 80 has a direct toxic effect on the organism or whether its activity is simply a surface-active effect, such as preventing the fungus inoculum from floating on the surface of the broth. In stationary broth cultures that do not contain Tween 80, growth of the fungus is optimal only when the inoculum floats on the surface of the broth. If the inoculum sinks to the bottom of the flask, growth is very slow until peripheral mycelial growth reaches the surface of the broth medium.

We have reason to believe that stimulation of AF-1 by yeast extract is due to its water-soluble-vitamin content. Hervey et al. (3) reported that several vitamins stimulated the growth of ant-garden fungus isolates in a synthetic culture medium, and when vitamins were tested individually, the most marked response was to thiamine. Vitamin-requirement and -supplement studies on our ant-garden fungus isolates currently are in progress.

Finding a marked growth-stimulating effect by olive oil, and a lesser stimulatory effect by oleic acid, was somewhat surprising. Preliminary studies with other vegetable oils and hydrocarbons suggest that our ant-garden fungus isolates are able to metabolize a variety of oils. These fungi may have become adapted to utilizing such substances as a result of their exposure to oils and waxes in leaf materials brought into the colony by the ants.

The ability of ant-garden fungi to utilize hydrocarbon substrates is not unique among filamentous fungi since it was shown some time ago (6) that many fungi can utilize such substances. Moreover, a fungus found on skin surfaces, and which occasionally causes an infectious process (tinea versicolor) in humans, has been shown to have an obligate requirement for fatty acid supplementation of culture media (2). Quantitative studies on various oils and other nutrient supplements for the leaf-cutting ant ant-garden fungus cur-

rently are in progress and will be reported in a subsequent paper.

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