JULIANA DE OLIVEIRA AUGUSTIN

NOVAS ESPÉCIES DE *ESCOVOPSIS*, SUA DISPERSÃO E VIRULÊNCIA NA SIMBIOSE FORMIGAS ATTINI – *LEUCOAGARICUS GONGYLOPHORUS*

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para obtenção do título de *Doctor Scientiae*.

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APROVADA: 30 de novembro de 2011.

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(Coorientadora

Prof. Gustavo Ferreira Martins

Pesq. Christina Cléo Vinson

Prof. Luiz Antônio Maffia

Prof. Simon Luke Elliot
(Orientador)

Dos sóis da imensidão às ultimas gotas d'àgua no centro da Terra,

tudo o que há de bom e belo nasce e vive do trabalho constante.

Emmanuel

Às mulheres que buscam se superar através das bênçãos da maternidade

e do grato trabalho no ambiente profissional,

bem como à Myriam de Oliveira Fernandes (*in memoriam*),

dedico humildemente esta tese.

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Em minha pesquisa, esforcei-me para conciliar trabalho de campo, experimentação e biologia molecular. Esta abordagem interativa não teria sido possível sem o generoso apoio de colaboradores em cada uma destas etapas:

- Por auxiliar a minha exploração por fragmentos de mata secundária na região da Zona da Mata Mineira, gostaria de agradecer a Manuel Ferreira, cujo conhecimento e experiência de campo, além da disposição e boa vontade de sair para as coletas, foram absolutamente essenciais ao trabalho.

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O primeiro capítulo desta tese, que está no formato ABNT, surgiu a partir do voto de confiança do meu orientador, que devotou em mim credibilidade para fazê-lo. Encontra-se publicado na 1ª edição do livro "Formigas Cortadeiras: da bioecologia ao manejo", editado por Terezinha Maria de Castro Della Lucia. Meus co-autores deste trabalho de revisão são: Elena Diehl, da Universidade do Vale do Rio dos Sinos; Richard Ian Samuels, da Universidade Estadual do Norte Fluminense Darcy Ribeiro e Simon Luke Elliot, da Universidade Federal de Viçosa.

O segundo capítulo desta tese apresenta a descrição de quatro novas espécies do micoparasita *Escovopsis* (Ascomycota: Hypocreales) e encontra-se no formato da PlosOne. Meus co-autores neste trabalho são Harry Charles Evans, pesquisador da CABI, Simon Luke Elliot, Eduardo Seiti Gomide Mizubuti e Robert Weingart Barreto, da Universidade Federal de Viçosa.

O ponta-pé inicial que resultou no trabalho apresentado no terceiro capítulo foi dado por Harry C. Evans, que, providencialmente, tropeçou – literalmente -, em um montículo de lixo de uma colônia attine. Qual não foi sua surpresa ao perceber que nos fragmentos deste lixo havia *Escovopsis* esporulando! A partir desta descoberta o trabalho foi delineado e os resultados são apresentados no capítulo 3. No entanto, somente foi possível executá-lo com a valiosa colaboração das seguintes pessoas: Luiz Gustavo Zingoni Neiva, Marcela Cristina Silva Caixeta, Lucimar Aparecida de Oliveira Cardoso e Renan Batista Queiroz, às quais expresso sinceros agradecimentos.

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Gostaria de registrar um agradecimento especial aos meus maiores incentivadores. Minha família sempre me inspirou com o amor e o apoio necessários à minha formação. Meus pais propiciaram-me com o ambiente favorável à valorização do que constitui importante conquista na vida, o desejo pela busca de conhecimento. Não é possível agradecer-lhes o suficiente por esta dádiva.

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BIOGRAFIA

Juliana de Oliveira Augustin nasceu em Curitiba-PR e cresceu em São João del-Rei-MG. É filha do gaúcho Sérgio Walter Augustin e da mineira Maria Lúcia de Oliveira Augustin.

Ingressou na faculdade de Ciências da Fundação de Ensino Superior de São João del-Rei, atual UFSJ, onde despertou o interesse pela matemática e física, e fez bons amigos ao longo de três anos de curso.

Tendo sempre tido grande interesse por biologia, formou-se em Ciências Biológicas pela Universidade Federal de Juiz de Fora em 2005. Durante a graduação amadureceu o gosto pela Etologia. Em 2007, concluiu o mestrado pelo Programa de Pós-Graduação em Biologia e Comportamento Animal da UFJF.

Em 2008 ingressou o Programa de Pós-Graduação em Entomologia pela Universidade Federal de Viçosa, submetendo-se à defesa de tese em 30 de novembro de 2011.

SUMÁRIO

RESUMO

AUGUSTIN, Juliana de Oliveira, D.Sc., Universidade Federal de Viçosa, novembro de 2011. **Novas espécies de *Escovopsis*, sua dispersão e virulência na simbiose formigas Attini – *Leucoagaricus gongylophorus*.** Orientador: Simon Luke Elliot. Coorientadores: Robert Weingart Barreto e Terezinha Maria Castro Della Lucia.

Um exemplo notável de mutualismo é a relação simbiótica entre as formigas attine (Formicidae: Attini) e seus jardins de fungos (Leucocoprineae e Pterulaceae) cultivados como fonte de alimento. Muito embora exemplo clássico de mutualismo, evidências crescentes mostram que esta simbiose representa uma complexa associação de parasitas e hospedeiros, como por exemplo, *Escovopsis*, que é um micoparasita específico dos jardins de fungos das formigas attine. Do ponto de vista evolutivo, há grande interesse neste micoparasita, embora pouco se saiba sobre sua biologia e diversidade. Esta tese marca o início de um esforço que almeja investigar o modo de transmissão e a diversidade de *Escovopsis* isolados de colônias de *Acromyrmex*. As principais conclusões desta tese são: i) Utilizando dados morfológicos e moleculares, quatro novas espécies de *Escovopsis* são descritas e ilustradas - *E. niveo-chlamydosporiformans*, *E. lentecrescens*, *E. microsporum* e *E. moelleri*. Uma chave dicotômica prontamente os separa uns dos outros e das duas espécies já descritas no gênero. Como essa pesquisa se concentra em apenas uma pequena região geográfica, este achado indica que pode haver muito mais espécies de *Escovopsis* dentro da faixa geográfica das Attini. ii) O trabalho de campo revela que *Escovopsis* é provavelmente transmitido horizontalmente. Porem, não se pode afirmar que este micoparasita seja exclusivamente transmitido horizontalmente e atualmente estamos estudando a possibilidade de transmissão vertical do mesmo.

iii) As espécies de *Escovopsis* estudadas não se mostraram virulentas, ao contrário do que aponta a literatura. Esta informação se baseia não somente em nosso experimento como também no fato de colônias saudáveis de laboratório naturalmente abrigarem pelo menos dois morfotipos do micoparasita (J.O. Augustin, H.C. Evans & S.L. Elliot, unpublished data) e em evidências da literatura para sua presença consistente em colônias hospedeiras. A simbiose formigas attine - microrganismos é um sistema com potencial para ser usado em futuras pesquisas sobre o estudo das interações parasita-hospedeiro, uma vez que a taxa de infecção colonial pode ser acessada ao longo do tempo e do espaço. Isto se deve ao fato dos simbiontes poderem ser isolados e mantidos em condições de laboratório por longos períodos de tempo e reisolados. Além disso, se considerarmos a grande diversidade de outros microrganismos que atualmente são conhecidos da simbiose formigas cultivadoras de fungo, parece possível a existência de outros microrganismos parasitas que não somente *Escovopsis*. Se isso for verdade, então podemos prever que eles possam representar caminhos promissores para futuros estudos.

ABSTRACT

AUGUSTIN, Juliana de Oliveira, D.Sc., Universidade Federal de Viçosa, November, 2011. **New species of Escovopsis, their dispersal and virulence in the Attine ant symbiosis - Leucoagaricus gongylophorus.** Adviser: Simon Luke Elliot. Co-Advisers: Robert Weingart Barreto and Terezinha Maria Castro Della Lucia.

A notable example of mutualism is the symbiotic relationship between attine ants (Formicidae: Attini) and their fungal gardens (Leucocoprineae and Pterulaceae) cultivated for food. Although a classic example of mutualism, empirical data shows that this symbiosis represents a complex association of hosts and parasites. *Escovopsis* spp. is a prevalent specific mycoparasite that attacks the ant's cultivars. From an evolutionary point of view, there is great interest in this mycoparasite although little is known about its biology and diversity. This thesis makes a start at assessing the diversity of *Escovopsis* morphotypes isolated from *Acromyrmex* colonies and its mode of transmission. The major findings are: i) Using both morphological and molecular data, four new species of *Escovopsis* are described and illustrated: *E. niveo-chlamydosporiformans*, *E. lentecrescens*, *E. microsporum* and *E. moelleri*. A dichotomous key separates them from one another and from the two previously described species in the genus. As this survey concentrates on only a small region of the Zona da Mata Mineira in Brazil, this finding indicates there may be many more species of *Escovopsis* throughout the geographic range of the Attini. ii) Field work reveals that *Escovopsis* is probably horizontally transmitted. Whether this mycoparasite relies solely on this mode of transmission we currently do not know and we are presently studying the possibility of vertical transmission. iii) *Escovopsis* is not a virulent parasite, contrary to its portrayal in the literature. This

information is based not only on our experimental procedures but also on the fact that healthy laboratory colonies can naturally inhabit at least two *Escovopsis* morphotypes (J.O. Augustin, H.C. Evans & S.L. Elliot, unpublished data) and the abundant evidence in the literature for its consistent presence throughout the geographic range of the Attini. The fungus-growing ant symbiosis is a fruitful system for future research on the study of host-parasite interactions, because infection in attine gardens can be accessed over time and space and since the symbionts can be isolated and maintained in laboratory conditions for long periods of time. Moreover, if one considers the great diversity of other micro-organisms currently known from the attine symbioses, it seems possible there could be parasites other than *Escovopsis* inhabiting attine colonies. If this is true then we can predict they may represent promising avenues for future studies.

INTRODUCTION

"Design is not just what it looks like and feels like.
Design is how it works."

Steve jobs
American businessman, co-founder of Apple Inc.
1955 - 2011

The symbiosis between fungus-growing ants (Formicidae: Attini) and their cultivated fungi (Leucocoprineae and Pterulaceae) has emerged in recent years as a model system for studying parasitism in social insect colonies. This is because fungus-growing ant colonies are host to complex interactions between mutualists and parasites. Here I briefly focus on three partners of this symbiosis and outline key features of the fungus-growing ant microbe symbiosis that make them a suitable model system for research on host-parasite interactions.

The mutualistic interaction found in this system is represented by the insect and the fungal cultivar that they cultivate as their primary food source. Fungus-growing ants have been practising fungiculture for approximately 50 million years and rely almost exclusively on their fungal gardens as an essential source of nutrients. While larvae are almost entirely dependent on the cultivated fungus for food (Weber 1972; Quinlan and Cherrett 1978), the adult worker's diet can be more varied and includes, in addition to the fungus, nectar from flowers and extrafloral nectaries (Murakami and Higashi 1997) or leaf sap that workers ingest when cutting and processing fresh vegetal fragments (Bass and Cherrett 1995). In the particular case of the leaf-cutters, workers of *Atta* and *Acromyrmex* ants cultivate a leucocoprineaceous fungus, the anamorph of which is *Leucoagaricus gongylophorus*

(Möller) Singer (Agaricales: Basidiomycota) (Kreisel 1972; Chapela et al 1994), in underground nest chambers and provide it with plant fragments as a substrate and a stable habitat with few competitors.

Leaf-cutting ant colonies are model system for the study of host-parasite interactions, because, among other aspects, small *Acromyrmex* and *Atta* colonies are relatively easy to collect and maintain in laboratory conditions, compared with other ant genera within the Attini. They can even be collected during their mating flights (in Minas Gerais this occurs between October and December) when one can collect the reproductive females. Once they are provided with appropriate temperature and humidity, each reproductive female will claustral or semi-claustrally, *Atta* and *Acromyrmex*, respectively, start up their respective colony (Fernandéz-Marín et al, 2007; Augustin et al. 2011). This is particularly interesting because then one can keep track of the history of each colony, right from the very beginning, i.e., the colony founding stage.

Attine agriculture is susceptible to invasion by a mycoparasite of the genus *Escovopsis* (Ascomycota: Hypocreales). This fungus is only known from the attine ant-microbe symbiosis, implying that *Escovopsis* is a specific mycoparasite. We currently do not know if *Escovopsis* can sustain an independent life style away from the fungus gardens of attine ants.

The parasitic nature of *Escovopsis* has previously been demonstrated by Reynolds and Currie (2004), who showed that *Escovopsis weberi* is a non-invasive necrotrophic parasite, i.e., it kills its host and then digests the dead biomass without penetrating the host hyphae. According to the authors, it secretes digestive compounds that break down host mycelium before contact occurs.

Apart from being a mycoparasite, evidences are that infection rates by *Escovopsis* vary not only across host species, but also across the geographic range of the Attini. It is a prevalent mycoparasite throughout sympatric Attini genera in Panama (Currie et al. 1999a; Gerardo et al. 2004) and parts of Southeastern Brazil (Rodrigues et al. 2005a, b; J.O. Augustin, unpublish data). Moreover, *Escovopsis* can be found in healthy laboratory (J.O. Augustin, unpublish data) and natural leaf-cutting ant colonies (Rodrigues et al. 2005b; Chapter 2, this thesis). What is more, however, is that naturally *Escovopsis*-infected colonies still forage well and grow ergonomically (J. O. Augustin, personal observation). This is an important observation because it indicates that the ants manage to overcome the potential negative effects of *Escovopsis*, suggesting that it is not such virulent parasite. There are currently no empirical data that support the general belief that *Escovopsis* is virulent to its host. Thus, potential avenues of research include investigating the nature of virulence of *Escovopsis lato sensu*, which I am currently investigating (preliminary results are in Chapter 4), as well as the parasitic nature of other *Escovopsis* species. New species of *Escovopsis* are described in Chapter 2, based on morphological and molecular data.

While the attine ant-microbe system comprises microorganisms of more than 40 different genera, among bacteria and fungi, as shown in a revision presented in Chapter 1, this thesis focuses on the antagonistic partner of the symbiosis, *Escovopsis*. The aim of this work was to describe four new species of *Escovopsis*, isolated from three sympatric *Acromyrmex* species. Chapter 3, on the other hand, presents data in support of horizontal transmission of *Escovopsis*. I also

aimed at investigating potential negative effects of *Escovopsis* on healthy *Atta* colonies; preliminary results are in Chapter 4.

Another striking aspect of the model is that attine ant colonies allow for feasible sampling, long-term laboratory maintenance and genotyping of both host and parasite, as well as other microsymbionts present in this symbiosis. Fungal and bacteria samples can be cultured and stored under axenic conditions for long periods of time until subsequent revival. All these characteristics are crucial for facilitating constant and thorough investigation.

Due to the relative ease of both molecular characterization and experimental manipulation of the symbionts the fungus-growing ant symbiosis offers a promising system model in which to test hypothesis on host-parasite interactions in social insect societies.

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CAPÍTULO 1

FUNGOS PARASITAS DE FORMIGAS CORTADEIRAS E DOS SEUS FUNGOS MUTUALÍSTICOS

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1 Introdução

As relações simbióticas são imprescindíveis na vida de todos os seres vivos no planeta e são consideradas hoje em dia responsáveis pela evolução de organismos multicelulares, entretanto, as associações não são necessariamente benéficas para todos os membros. As relações simbióticas são classificadas em quatro formas: amensalismo, comensalismo, mutualismo, e parasitismo. Parasitismo por definição é uma forma de simbiose entre dois ou mais organismos de espécies diferentes, vivendo em associação/proximidade, em que um dos membros depende do outro para adquirir nutrientes, proteção e/ou outro fator ligado a vida funcional. O membro dependente (parasita) ganha benefícios do relacionamento enquanto o outro (o hospedeiro) é prejudicado pela associação.

Nesse capítulo será descrita a ocorrência de fungos parasitos das formigas cortadeiras e dos jardins do fungo mutualístico, considerando a colônia como um superorganismo, ao invés de tratar cada um de seus elementos individualmente. O modo como as formigas e seus fungos simbiontes se protegem contra possíveis ataques de parasitas tem despertado interesse e polêmica desde a descoberta, em 1999, de um então novo parceiro neste mutualismo, uma bactéria filamentosa, subsequentemente identificada como *Pseudonocardia*. Dados empíricos mostraram que esse actinomiceto protege as colônias especificamente contra o ataque do fungo *Escovopsis* (Ascomycota: Hypocreales), parasito dos jardins das Attini. Entretanto, a classificação de *Pseudonocardia* como um organismo mutualístico tem sido questionada recentemente. Antes de entrar em maiores detalhes sobre os parasitas das colônias, é importante revisar a literatura sobre o mutualismo clássico das formigas e seus fungos simbiontes.

2 Mutualismo entre formigas cortadeiras e fungos

As relações interespecíficas de simbiose favorecidas pela seleção natural moldam o processo evolutivo dos seres vivos em todos os níveis de organização biológica (BOUCHER et al., 1982; PRICE et al., 1986; CALDERA et al., 2009). Os organismos não vivem isolados; ao contrário, eles são frequentemente encontrados

vivendo em estreita relação com organismos muitas vezes filogeneticamente distantes. O mutualismo, por exemplo, se considerado como um tipo de relação simbiótica em que os membros envolvidos se beneficiam mutuamente (RICKLEFS, 2003) é reconhecidamente uma força evolutiva importante, que ao longo do tempo, favoreceu o surgimento das células eucarióticas. Isso por sua vez permitiu um aumento na diversidade de vida no planeta. Alguns exemplos que ilustram a importância do mutualismo são as micorrizas, das quais muitas plantas terrestres dependem para a fixação de íons de fósforo, e os microrganismos que auxiliam diversos animais na digestão de alimentos.

Evidências crescentes apontam em favor da inclusão de simbiontes nas teias alimentares (THOMAS et al., 2005; THRALL et al., 2007). Estudos recentes têm demonstrado que teias alimentares possuem mais ligações parasito-hospedeiro do que presa-predador (LAFFERTY et al., 2006); e que parasitas podem alterar a fisiologia e consequentemente o comportamento de hospedeiros que possuem funções chave no ecossistema, alterando a composição, o fluxo energético e a estrutura das comunidades (WOOD et al*.,* 2007; HERNANDEZ; SUKHDEO, 2008). Portanto, a tendência atual e crescente é a de que um organismo vivo não pode mais ser considerado como um organismo isolado, e sim, como um organismo e seus simbiontes, sejam eles mutualistas ou parasitas.

Uma das mais notáveis relações mutualísticas do reino animal ocorre entre as formigas cultivadoras de fungo (Myrmicinae, Attini) e seu fungo simbionte (Basidiomycota: Lepiotaceae e Pterulaceae). O início das muitas adaptações que permitiram o desenvolvimento e a manutenção desta estreita relação deve-se ao surgimento do hábito micófago pelas formigas. Corroborando com antigas especulações, análises filogenéticas e moleculares recentes confirmaram a origem única do hábito micófago há aproximadamente 50 milhões de anos atrás, na América do Sul (SCHULTZ; BRADY 2008).

De fato, a especialização da fungicultura para alimentação atinge seu ápice evolutivo com as formigas cortadeiras, representadas pelos gêneros *Acromyrmex* e *Atta*, únicas capazes de cortar e processar material vegetal fresco para o cultivo do simbionte, tornando-se herbívoros considerados dominantes nos neotrópicos

(HÖLLDOBLER; WILSON, 1990; FARJI-BRENER; ILLES, 2000; FARJI-BRENER; GHERMANDI, 2004).

Tão especializado tornou-se a micofagia entre as Attini que ela é hoje o resultado de uma relação simbiótica envolvendo não apenas dois, mas cinco participantes. Os três mutualistas são hoje representados pelas formigas, o fungo que elas cultivam, e a bactéria filamentosa do gênero *Pseudonocardia* (Actinomycetes) que cresce em locais específicos do exoesqueleto de *Myrmicocrypta*, *Apterostigma*, *Mycocepurus*, *Cyphomyrmex*, *Trachymyrmex* e *Acromyrmex* (CURRIE et al., 1999). Os dois parasitas são o fungo do gênero *Escovopsis* (Ascomycetes), o qual é conhecido somente do jardim de fungo das Attini (SEIFERT et al., 1995) e a levedura preta (*black yeast*) *Phialophora* (Ascomycota) a qual cresce na mesma região da cutícula das formigas onde também se encontra a bactéria *Pseudonocardia* (LITTLE; CURRIE, 2007). Posteriormente, esses dois parasitas serão descritos em maiores detalhes.

3 Parasitismo de formigas cortadeiras e do seu fungo simbionte

Os jardins das formigas Attini abrigam uma diversidade enorme de microrganismos como fungos filamentosos (DIEHL-FLEIG; LABRES, 1993; DIEHL-FLEIG; LUCIANO 1995; CURRIE et al., 1999), leveduras (CARREIRO et al., 1997, 2004) e bactérias (VAN BORM et al., 2002; SANTOS et al., 2004), que exploram com maior ou menor intensidade os recursos energéticos e nutritivos deste mutualismo.

A fim de compreender como as complexas sociedades de insetos se defendem do ataque de parasitas, é necessário estudá-las sob o conceito de superorganismo (HÖLLDOBLER; WILSON, 1990; CREMER; SIXT, 2009), em que cada casta e cada parte da estrutura física do ninho – que possuem funções específicas na colônia - equivalem a um órgão dos organismos multicelulares – também com função específica naquele organismo. No caso particular de colônias maduras de saúvas (*Atta*), que chegam a produzir o maior número de castas físicas de operárias já determinado para uma colônia de formigas (WILSON, 1980), podem-se considerar as diferentes castas e seu fungo simbionte como sendo diferentes tipos de tecido deste organismo. Seria possível, portanto, um parasita utilizar preferencialmente um ou outro tecido do hospedeiro como porta de entrada. Um parasita poderia

também se especializar em utilizar um ou outro tecido como recurso para se manter e se reproduzir.

A princípio, colônias inteiras de formigas podem ser devastadas por um parasita que seja suficientemente virulento, ou mesmo quando a colônia esteja particularmente vulnerável em determinado momento – colônias em processo de fundação, por exemplo. Entretanto, as evidências de uma alta virulência inerente a qualquer parasita são escassas. Poucos experimentos foram realizados com o intuito de avaliar a virulência de um parasita de colônias de formigas cortadeiras. Um dos poucos exemplos de estudos deste tipo foi realizado por Currie et al. (1999) com uma abordagem evolutiva da interação parasita-hospedeiro. Outro trabalho foi desenvolvido por Jaccoud et al. (1999), no entanto, com um enfoque mais aplicado. A fim de verificar a patogenicidade (isto é, a capacidade de causar doença) e a virulência (isto é, o nível de prejuízo causado pelo parasita) de *Escovopsis*, Currie et al. (1999) retiraram as formigas de pequenas porções do jardim simbionte, deixando-os livres de operárias, e inocularam aproximadamente 300–500.000 esporos do microfungo diretamente em porções de 60-75 mL do jardim de *Trachymyrmex*, *Acromyrmex* e *Atta*. Como era de se esperar, todas as porções de jardim de fungo sucumbiram. Já nas colônias onde *Escovopsis* foi endêmico (sem ter sido inoculado experimentalmente) e cujas operárias foram retiradas a fim de se observar o desenvolvimento do jardim de fungo, esperava-se encontrar vários tipos de patógenos, mas foi encontrado somente *Escovopsis*. A partir somente destas observações, os autores concluíram que *Escovopsis* é um microfungo parasita especializado com alta virulência contra jardins de fungo de Attini, o que pode ser apenas uma falácia experimental.

Já o trabalho de Jaccoud et al. (1999) simula os efeitos da infecção de mini-colônias de *Atta sexdens rubropilosa* pelo fungo entomopatogênico *Metarhizium anisopliae* (Ascomycota: Hypocreales) com a finalidade de usá-lo no controle biológico desta subespécie de formiga. As mini-colônias consistiam de porções de 75 mL de fungo simbionte, juntamente com eventuais operárias e formas imaturas do inseto ali presentes, mas sem a rainha. Todas as 19 mini-colônias procederam de uma única colônia-mãe coletada do campo seis anos antes do experimento. Este consistiu em avaliar os efeitos do entomopatógeno nas mini-colônias, inoculando-o

sob a forma de esporos secos unicamente, ou de esporos secos misturados a pó de citrus, ambos nas quantidades de 0,5 g (considerado alta dose) e de 0,05 g (baixa dose) por mini-colônia. De modo geral, as operárias foram capazes de sobreviver à baixa dosagem do patógeno, removendo os esporos da cutícula através de comportamentos de limpeza e, em seguida, depositando-os nas pilhas de lixo. Entretanto, a alta dosagem utilizada no experimento de Jaccoud et al. (1999) foi capaz de levar à morte 100% das mini-colônias até o final do experimento. Resultados similares já haviam sido observados por Diehl-Fleig e Lucchese (1991) em testes realizados com colônias naturais de *Acromyrmex striatus* diante do fungo *Beauveria bassiana* (Ascomycota: Hypocreales) cultivado em grãos de arroz e também de esporos secos formulados com farinha de casca de laranja. Para colônias de *Acromyrmex heyeri* e de *Ac. striatus* mantidas em laboratório também foram descritos comportamentos de reconhecimento de fungos filamentosos, acompanhados por comportamentos de auto-higiene e limpeza mútua das operárias quando em contato com esporos secos de fungos entomopatogênicos (FRITSCH; DIEHL-FLEIG, 1996).

A questão que surge ao se examinar os estudos acima referidos é: basta se aplicar altas doses de patógeno em amostras de colônias para se inferir sobre sua virulência? Amostras do jardim simbionte sem operárias ou mini-colônias sem rainhas consistem em amostras realmente representativas de uma situação natural? Esta questão será abordada em maiores detalhes mais adiante.

4 Fungos parasitas associados às formigas cortadeiras e seu simbionte

Uma gama de fungos filamentosos e leveduras tem sido encontrada associada ao jardim de fungo simbionte ou às próprias formigas cortadeiras – operárias e rainhas fundadoras (Apêndice Tabelas 1 (pag. 27) e 2 (pag. 36)). Dentre estes fungos, a maioria é de solo (*Mucor*, *Absidia*, *Cunninghamella*, *Chaetomium*, *Phoma*, *Chrysosporium*, *Acremonium*, *Gliomastix*, *Trichoderma*, *Volutella* e *Arthrobotrys*) enquanto somente *Beauveria* e *Metarhizium* são formalmente caracterizados como entomopatógenos facultativos, ou seja, podem ou não utilizar insetos como hospedeiros em alguma fase da vida. Fungos entomopatogênicos são inimigos naturais de muitas espécies de insetos, agindo como importantes

reguladores das suas populações (HAJEK; ST. LEGER, 1994). De fato, fungos entomopatogênicos foram os primeiros agentes utilizados no controle microbiano de insetos (ALVES, 1998).

4.1 Patógenos de Formigas Cortadeiras – *Beauveria, Metarhizium* e *Ophiocordyceps*

Espécies dos gêneros de fungos ascomicetos *Metarhizium* e *Beauveria* são entomopatógenos generalistas capazes de infectar uma ampla variedade de insetos. Existem relatos desses gêneros infectando insetos sociais, mas para estes hospedeiros os dados são bastante limitados (SCHMID-HEMPEL, 1998). Tanto *Metarhizium* quanto *Beauveria* obrigatoriamente levam seus hospedeiros à morte ao se reproduzirem, e têm sido amplamente estudados por seu inerente potencial como agentes no controle biológico de insetos-praga. Em regiões temperadas, ambos os entomopatógenos estão associados aos hospedeiros principalmente terrestres, de forma que os insetos que constroem ninhos no solo estão mais susceptíveis à contaminação (HÖLLDOBLER; WILSON, 1990). Embora amplamente estudados para uso em controle biológico (MACHADO et al*.,* 1988; SILVA; DIEHL-FLEIG, 1988; DIEHL-FLEIG et al., 1993; SPECHT et al., 1994) relativamente pouco se conhece a respeito de sua diversidade, e os dados são escassos nas regiões tropicais, ironicamente onde a diversidade de muitos outros organismos vivos é sabidamente maior.

Apesar de raros, há relatos de ocorrência natural de fungos entomopatogênicos em formigas do gênero *Atta.* Assim, foi isolada uma linhagem de *B. bassiana* a partir de uma operária de *Atta sexdens piriventris* naturalmente infectada (DIEHL-FLEIG et al., 1992). Esse fungo e *Metarhizium anisopliae* var. *anisopliae* foram isolados de rainhas de saúva (ALVES, 1998) e do jardim de fungo simbionte de ninhos de campo de *A. sexdens rubropilosa* (RODRIGUES et al., 2005b).

O ascomiceto *Beauveria* pertence à ordem Hypocreales e engloba espécies com ampla gama de hospedeiros além da morfoespécie *B. bassiana sensu lato*, capaz de infectar mais de 200 espécies de insetos, ácaros e carrapatos (ALVES, 1998). Além disto, um estudo recente mostra que *B. bassiana* não possui especificidade quanto ao hospedeiro, mas atua como entomopatógeno generalista de insetos (MEYLING et al., 2009). O fungo pode ocorrer como epibionte e endófito

de plantas (CHERRY et al., 1999) e seus conídios são capazes de sobreviver no solo de ambiente natural ou agrícola (MEYLING et al., 2009). Os hospedeiros atacados mostram-se cobertos por uma massa pulverulenta branca (Figura 1A, pag. 29), o que confere o nome muscardine branca à doença provocada por *B. bassiana s.l.*, em contraste com o muscardine verde, provocado por *M. anisopliae*.

O ascomiceto *Metarhizium* pertence à família Clavicipitaceae Clado A dos Hypocreales (SUNG et al., 2007) que caracteristicamente infecta uma grande diversidade de espécies de insetos, porém, é considerado entomopatógeno facultativo, uma vez que é facilmente isolado dos solos, onde é capaz de sobreviver por longos períodos (ST. LEGER, 2008). Contudo, *M. anisopliae* é considerado um dos mais importantes parasitas de insetos, possivelmente ocorrendo naturalmente em mais de 300 espécies de hospedeiros de diferentes ordens, principalmente Coleoptera (DOMSCH; GAMS, 1980). Amostras de solo nas proximidades de ninhos de *Acromyrmex echinatior*, *Acromyrmex octospinosus*, *Atta cephalotes* e *Atta colombica*, revelaram abundância (18 dos 20 ninhos amostrados) de *M. anisopliae* var. *anisopliae*, particularmente nos 5cm de terra ao redor de cada ninho (HUGHES et al., 2004). Apesar desta alta prevalência do patógeno ao redor dos ninhos, poucas operárias estavam de fato infectadas (apenas três operárias de *A. colombica*, as quais inclusive já estavam no depósito de lixo da colônia). Isto indica que as estratégias de defesa individuais e de grupo empregadas mostram-se eficientes contra esse entomopatógeno. *Metarhizium anisopliae* pode ser reconhecido pelos conídios cilíndricos, uninucleados, hialinos ou fracamente coloridos, e por suas colônias em tons de verde, que variam do claro ao escuro ou esbranquiçadas com pontos verdes, formando uma camada pulverulenta cobrindo o inseto morto (Figura 1B, pag. 29).

O solo é um ambiente muito rico em microrganismos, os quais exercem influências cruciais à sua diversidade biológica (INGHAM et al., 2000). Assim, considerando que as fêmeas fundadoras de formigas cortadeiras podem facilmente se contaminar com patógenos durante a fundação de novas colônias no solo, seria possível a existência de isolados de fungos especializados em infectar particularmente esta casta temporária. Isso coincide com a fase mais vulnerável do ciclo de vida de uma colônia. Espécies monogínicas de formigas geralmente são

capazes de fundar uma colônia sem a ajuda de outras companheiras de ninho, exibindo o que se conhece por modo independente de fundação de colônia. Durante este processo, a fêmea fundadora perde peso a cada dia, travando uma corrida entre se manter viva sem alimento e produzir operárias rapidamente e em número suficiente capazes de sustentar a colônia. Este investimento impõe grande pressão sobre a sobrevivência dessas fêmeas, tornando muito vulnerável esta fase do seu ciclo de vida (HÖLLDOBLER; WILSON, 1990, BAER et al., 2006). De fato, Augustin (2007) verificou que das 107 fundadoras de *Atta sexdens* em início de processo de fundação colonial em laboratório, 12 (11,21%) morreram durante este período, exibindo sobre sua cutícula uma massa pulverulenta de conídios esbranquiçados, típica de infecção por *Beauveria*.

Um evento recentemente relatado e aparentemente raro entre as formigas cortadeiras é a infecção seguida de morte provocada pelo fungo entomopatogênico *Ophiocordyceps* (Ascomycota: Hypocreales), que é a fase sexual de *Hirsutella* (HUGHES et al., 2009). Uma fêmea fundadora de *Ac. Octospinosus* e outra de *A. colombica* em processo de fundação colonial foram coletadas vivas no campo, e após sete dias desde suas mortes em laboratório, ambas exibiam o estroma do fungo em sua forma anamórfica (fase assexual), ou seja, *Hirsutella*. Os autores também realizaram um experimento que comprovou a habilidade de *Ophiocordyceps* de alternar entre hospedeiros de diferentes subfamílias e gêneros (no caso, entre os gêneros *Atta*, *Acromyrmex*, *Sericomyrmex* e *Apterostigma* (Myrmicinae, Attini) e o gênero *Camponotus* (Formicinae). Esta alternância entre hospedeiros, e a relativa baixa especificidade por eles, indica se tratar de uma relação parasita-hospedeiro recente na evolução.

4.2 Possíveis Patógenos das Formigas – *Aspergillus, Clonostachys* e *Fusarium*

Espécies dos gêneros *Aspergillus* e *Fusarium* são comumente encontradas em solos tropicais ou subtropicais, onde atuam principalmente como decompositores (DOMSCH; GAMS, 1980). No entanto, algumas espécies, como *A. flavus* e *F. oxysporum*, têm sido também isoladas de operárias vivas (HUGHES; BOOMSMA, 2004) ou moribundas (RODRIGUES et al., 2005a), do depósito de lixo (HUGHES et al., 2004) e mesmo do jardim de fungo de formigas cortadeiras

(RODRIGUES et al., 2005b). O ascomicota do gênero *Clonostachys* também atua como saprófita de solo, tendo sido isolado (em baixa frequência) do jardim simbionte de colônias de laboratório de *A. sexdens rubropilosa* (RODRIGUES et al., 2005a).

Para os gêneros de fungos citados acima, sempre existe a dúvida se são de fato entomopatógenos ou se sua presença no inseto representa saprofitismo. Quando encontrados no jardim de fungo ou no lixo, existe também a possibilidade de estarem colonizando esse material como oportunistas, ou de serem fitopatógenos ou endofíticos trazidos para a colônia pelas formigas. Nesses casos, tais fungos podem ser considerados agentes secundários e não agentes causadores da morte do inseto.

4.3 Parasitas Especializados do Jardim do Fungo – *Escovopsis*

Atualmente, existe grande interesse em pesquisas envolvendo espécies do fungo parasita *Escovopsis*. Uma das principais razões em se conhecer melhor esta simbiose é que *Escovopsis* é somente encontrado parasitando o fungo simbionte das Attini (SEIFERT et al., 1995; SCHULTZ; BRADY, 2008). Tem sido demonstrado que certas linhagens deste parasita associam-se exclusivamente com linhagens específicas de fungo simbionte do "sistema agricultural" Leucocoprineae e Pterulaceae (SCHULTZ; BRADY, 2008) das Attini basais do grupo das *Apterostigm*(GERARDO et al., 2006) e das espécies *Cyphomyrmex longiscapus*, *Cyphomyrmex muelleri* e *Cyphomyrmex costatus* (GERARDO et al., 2004), conforme revelam dados experimentais e de análises filogenéticas. Esta especificidade é interpretada como tendo as linhagens de *Escovopsis* uma história coevolutiva comum com seus fungos hospedeiros, pontuada por trocas apenas ocasionais de hospedeiros. Os estudos filogenéticos, contudo, não mostraram uma congruência completa entre as filogenias do parasita e do hospedeiro, indicando que a gama de hospedeiros passíveis de infecção por *Escovopsis* foi e provavelmente poderia continuar mudando (GERARDO et al., 2006). Outro motivo pelo qual essa interação merece ser melhor estudada é sua possível aplicabilidade no controle das formigas cortadeiras utilizando *Escovopsis*.

Contrariamente ao que foi demonstrado para as Attini basais, análises moleculares recentes e evidências experimentais revelaram pouca especificidade parasita-hospedeiro dentro do clado mais derivado das Attini constituídos pelos gêneros *Atta* e *Acromyrmex* (TAERUM et al., 2007). Neste estudo, as análises filogenéticas indicaram que a história evolutiva entre *Escovopsis* e o fungo simbionte das cortadeiras não acompanhou a história evolutiva das próprias formigas. Além disso, ficou demonstrado que oito linhagens filogeneticamente distantes de *Escovopsis*, divididas em dois clados distintos, foram capazes de infectar todas as sete linhagens de fungos hospedeiros utilizadas no experimento, indicando que elas não são especializadas em linhagens específicas do fungo simbionte das cortadeiras. Entretanto, a metodologia de inocular pequenos pedaços de jardim com *Escovopsis* significa que é difícil avaliar diferenças sutis visualmente. Os resultados de Taerum et al. (2007) concordam com os resultados de Gerardo et al. (2004) que mostraram a falta de congruência filogenética entre a Attini basal *Cyphomyrmex* e *Escovopsis*. Portanto, os isolados deste parasita não seriam especializados em linhagens específicas de jardins de fungos e a especificidade de *Escovopsis* não seria mediante as possíveis defesas dos cultivares dos fungos na ausência das formigas.

Tornou-se um dogma que *Escovopsis* é altamente virulento às colônias Attini, com base nos resultados de Currie et al. (1999). Os autores relataram que *Escovopsis* pode dominar os jardins mesmo na presença de formigas, em colônias observadas em campo e laboratório. Uma vez retiradas todas as formigas de uma colônia, *Escovopsis* rapidamente (um a dois dias) cresceu, matando o fungo mutualístico de três espécies de Attini derivadas: *Trachymyrmex* cf. *zeteki, Ac. octospinosus* e *A. colombica*. Ainda mais, a remoção das formigas não resultou em crescimento de outros contaminantes, aparecendo somente *Escovopsis* nos jardins simbiontes. Em um experimento em que colônias de *A. colombica* foram contaminadas artificialmente com esporos de *Escovopsis*, ficou demonstrada a completa destruição de 37% das colônias em 72 horas. Quando o fungo micoparasítico *Trichoderma* (Ascomycota: Hypocreales) foi inoculado, nenhum sintoma de perda ou estresse foi observado no jardim. Finalmente, Currie et al.

(1999) conseguiram reisolar *Escovopsis* das colônias infectadas mas não foi possível reisolar *Trichoderma*.

Se uma colônia de formigas fungicultoras for considerada como um superorganismo, com vários níveis de interação e organização social (HÖLLDOBLER; WILSON, 1990), os testes de virulência corresponderiam melhor à realidade se abrangessem o organismo como um todo, e não somente partes dele. Amostrar porções do fungo simbionte ou mesmo mini-colônias seria o mesmo que amostrar apenas parte do organismo, limitando a compreensão dos mecanismos empregados pelo parasita para aumentar seu valor adaptativo. Contudo, estudos deste tipo são difíceis de realizar.

Quais são os mecanismos de interação de *Escovopsis* com seus hospedeiros? Foi verificado que *Escovopsis weberi* atua como parasita necrotrófico de contato, ou seja, primeiramente mata seu hospedeiro para posteriormente digerir a biomassa recém-morta, não sendo necessária a penetração das hifas para o parasitismo ocorrer (REYNOLDS; CURRIE, 2004). Este mecanismo diverge do mecanismo biotrófico empregado pelos micoparasitas, os quais se alimentam do hospedeiro ainda vivo (JEFFRIES; YOUNG, 1994 *apud* REYNOLDS; CURRIE, 2004).

Um estudo realizado por Currie et al. (1999) buscou *Escovopsis* em 105 jovens colônias naturais e de laboratório de *A. colombica* iniciadas a partir de fêmeas fundadoras provenientes de um único vôo nupcial em Gamboa, Panamá. Os autores analisaram essas colônias antes e depois das primeiras operárias saírem do ninho para forragear. Como os inóculos do fungo simbionte trazidos da colônia de origem pelas fêmeas – bem como seus jardins iniciados durante a fundação claustral – estavam livres de *Escovopsis*, concluíram que este parasita não é transmitido verticalmente entre colônias. Contudo, o fato de não ter sido encontrado *Escovopsis* em colônias iniciadas por fêmeas de um único voo nupcial não implica ausência de transmissão vertical deste parasita. Para se chegar a uma resposta mais conclusiva, o ideal provavelmente seria investigar diversas novas fundações, considerando colônias com vários padrões de horários de revoada, talvez até em áreas geográficas distintas.

4.4 Micoparasitas Não-Especializados Encontrados no Jardim do Fungo – *Trichoderma*

Espécies de *Trichoderma* são comumente encontradas em diversos tipos de solo, especialmente naqueles ricos em matéria orgânica, onde outras espécies de fungo também estão presentes (DOMSCH; GAMS, 1980; SAMUELS, 1996). Há algumas décadas, isolados de *Trichoderma* começaram a ser um dos agentes naturais mais utilizados no controle de fitopatógenos (BÉLAGER et al., 1995; ZHANG et al., 1996), devido as suas propriedades antagonísticas, que envolvem principalmente a produção de antibióticos (GHISALBERTI; SIVASITHAMPARAM, 1991) e/ou enzimas proteolíticas (HARAN et al., 1996). Mais recentemente, alguns isolados de *Trichoderma* também começaram a ser vistos como possíveis candidatos ao controle das formigas cortadeiras, devido às suas propriedades antagonísticas em relação ao fungo simbionte por elas cultivado (ORTIZ; ORDUZ, 2000; LOPEZ; ORDUZ, 2003). Entretanto, Currie et al. (1999) não acharam *Trichoderma* patogênico aos jardins. Além disto, algumas espécies deste gênero têm sido encontradas tanto no jardim simbionte (RODRIGUES et al., 2005b, 2008a) quanto no depósito de lixo (RODRIGUES et al., 2005b) de colônias naturais e de laboratório de *Atta* e *Acromyrmex*, bem como no exoesqueleto de fêmeas fundadoras de *Atta capiguara* e *Atta laevigata* (PAGNOCCA et al., 2008). Estas observações indicam se tratar de um parasita generalista, sem preferência pelo fungo simbionte das formigas como hospedeiro. Mas esses resultados não descartam a existência de isolados específicos em atacar o simbionte.

Acredita-se que o mecanismo de interação entre *Trichoderma* e o fungo hospedeiro seja através da produção de substâncias antifúngicas, ou mesmo tóxicas, como enzimas, metabólitos, antibióticos, substâncias voláteis e não-voláteis, as quais impediriam o crescimento micelial do simbionte das Attini sem a necessidade de contato do parasita com seu hospedeiro (ORTIZ; ORDUZ, 2000).

4.5. Outros Fungos

Grande parte da diversidade de fungos encontrada de alguma forma em associação com as formigas cortadeiras é composta por fungos de solo que atuam principalmente como saprófitas ou mutualistas (DOMSCH; GAMS, 1980; INGHAM et

al., 2000). Dados que comprovem sua ação antagônica frente ao fungo simbionte das Attini ainda não existem. Assim, é possível especular que a grande frequência com que eles vêm sendo isolados de colônias de formigas cortadeiras possivelmente indique que: 1) sejam simbiontes destas formigas, agindo como parasitas ou mutualistas ainda desconhecidos; 2) sejam simplesmente comensais ou inquilinos da colônia, sem causar-lhe prejuízos ou benefícios, e/ou 3) que a colônia se contamine acidentalmente a partir do contato direto com o solo e o material vegetal que as operárias trazem para dentro do ninho.

4.6. Leveduras pretas ("*Black yeasts*")

Recentemente foi descoberto mais um simbionte envolvido na interação entre as formigas, seus fungos mutualísticos, as bactérias *Pseudonocardia* e o fungo parasita *Escovopsis*. O novo simbionte é uma levedura preta (Ascomycota; *Phialophora*) encontrada crescendo nos mesmos locais onde está a bactéria *Pseudonocardia* (LITTLE; CURRIE, 2007). Foi demonstrado que a levedura ataca a bactéria filamentosa, reduzindo os nutrientes disponíveis e diminuindo, portanto, a sua capacidade de proteger a colônia contra o ataque de *Escovopsis* (LITTLE; CURRIE, 2008). É provavel que existam ainda mais microrganismos simbiontes a serem descobertos nas colônias das cortadeiras.

5 Considerações Finais

Considerando as estratégias de defesa das formigas cortadeiras, poder-se-ia questionar se o próprio fungo simbionte é ou não capaz de se defender de alguma forma. De fato, conforme mencionado anteriormente, estudos têm demonstrado que o jardim das cortadeiras está longe de ser constituído apenas pelo fungo simbionte, mas o interessante é que algumas evidências sugerem que ele seja incapaz de reconhecer outros fungos "intrusos" ao mutualismo fungo-formiga. Isto indica que o simbionte é um fraco competidor frente a outras espécies de fungo, e, na ausência das formigas, sucumbe rapidamente (STRADLING; POWELL, 1986; FISHER et al., 1996; ORTIZ; ORDUZ, 2000). Aparentemente, portanto, o simbionte

das formigas cortadeiras especializou-se em crescer e produzir o alimento destes insetos.

O fato de haver poucos relatos de patógenos atacando colônias de cortadeiras indica que elas possuem defesas bem desenvolvidas, as quais ainda não são completamente conhecidas e merecem ser elucidadas. Neste capítulo descreveu-se em detalhe uma gama de mecanismos e os possíveis simbiontes envolvidos na defesa contra entomopatógenos e contra os patógenos específicos do jardim de fungo. De certa forma, poder-se-ia esperar que as formigas fossem altamente susceptíveis ao ataque de patógenos, tendo em vista o alto nível de similaridade genética entre as operárias irmãs da colônia, a frequência das interações sociais (trofalaxia, *allogrooming*) e as condições ambientais – alta umidade e temperatura estável – do ninho (normalmente favoráveis aos fungos entomopatogênicos). Um melhor entendimento dos sistemas de defesa empregados pelas formigas cortadeiras poderia levar ao desenvolvimento de uma estratégia de controle destes insetos-praga. Além disto, estudos de como essas formigas protegem seus cultivos contra microrganismos indesejáveis poderiam auxiliar o homem no planejamento de proteção de lavouras ou mesmo no desenvolvimento de novos antibióticos com base nos estudos dos simbiontes das Attini.

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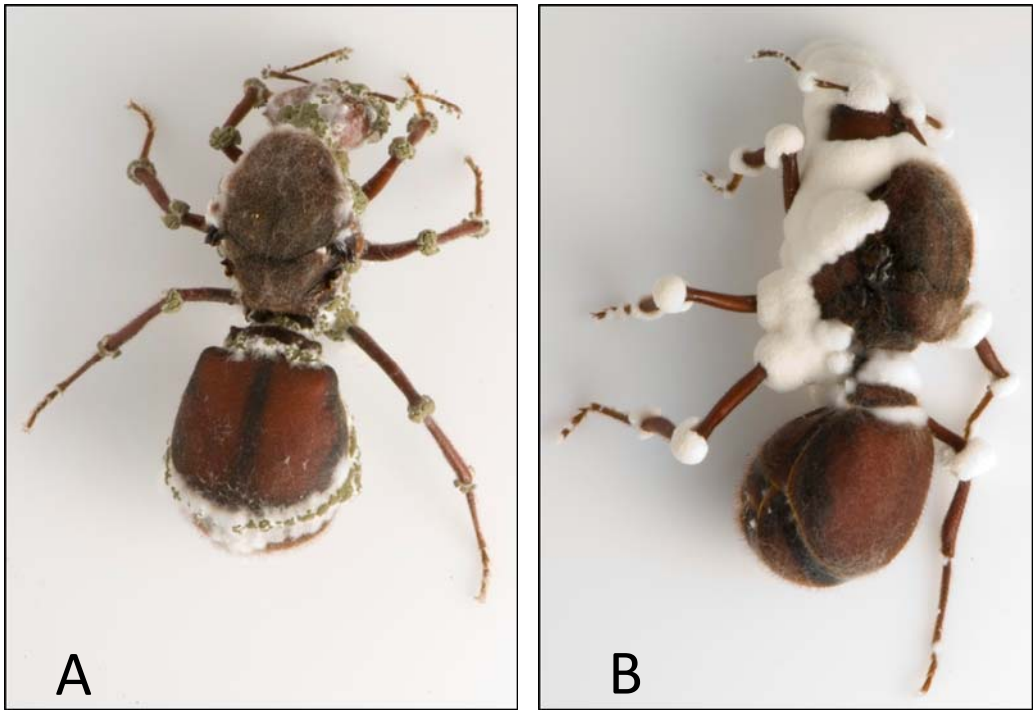

Figura 1: Rainhas de *Atta sexdens rubropilosa* naturalmente infectadas por *Beauveria bassiana* **(A)** e *Metarhizium anisopliae* **(B)**. Fotos: J. L. Neto e J. O. Augustin.

Tabela 1: Fungos patógenos e leveduras isolados de colônias de *Atta* (+: presença; -: ausência).

	Atta spp.							
	Cortadeiras de mono e dicotiledôneas							
	A. sexdens		*A. sexdens rubropilosa*		*A. capiguara*	*A. laevigata*	*A. colombica*	*A. cephalotes*
	Fêmeas fundadoras (exoesqueleto)	Colônias de laboratório (Jardim de fungo simbionte/Lixo/Superfície foliar de *Eucalyptus alba* incorporada ao jardim pelas operárias)	Colônias de laboratório (Jardim de fungo simbionte/Lixo/Superfície foliar de *Eucalyptus alba* incorporada ao jardim pelas operárias/exoesqueleto de operárias)	Colônias de campo (Jardim de fungo simbionte)	Fêmeas fundadoras (exoesqueleto/esponja inicial de fungo simbionte)		Colônias de campo (lixo) / Fêmeas fundadoras	Colônias de laboratório (Jardim de fungo simbionte/Lixo)
Fungo filamentoso não-mutualístico								
Deuteromycota								
Chrysosporium sulphureum[1]	-	-	(+/-/-/-)	-	(-/-)	(-/-)	-	-
Gliomastix murorum[9]	-	-	-	-	-	-	-	(+/-)
Metarhizium anisopliae var. *anisopliae*[3,11]	-	-	(-/-/-/-)	+	-	-	(+/-)	-
Zygomycota								
Absidia corymbifera[8]	-	-	(-/-/-/-)	-	(+/-)	(-/-)	-	-
Mucor hiemalis[3]	-	-	(-/-/-/-)	+	(-/-)	(-/-)	-	-
Mucor microsporus[3]	-	-	(-/-/-/-)	+	(-/-)	(-/-)	-	-
Mucor sp3[8]	-	-	(-/-/-/-)	-	(-/-)	(+/-)	-	-
Piptocephalis sp[3]	-	-	(+/-/-/-)	-	-	-	-	-
Syncephalastrum racemosum[3]	-	-	(+/-/-/-)	-	-	-	-	-
Rhizopus stolonifer var. *stolonifer*[1]	-	-	(+/-/-/-)	-	(-/-)	(-/-)	-	-

Continua

Tabela 1: cont.

	Atta spp.							
	Cortadeiras de mono e dicotiledôneas							
	A. sexdens		*A. sexdens rubropilosa*		*A. capiguara*	*A. laevigata*	*A. colombica*	*A. cephalotes*
	Fêmeas fundadoras (exoesqueleto)	Colônias de laboratório (Jardim de fungo simbionte/Lixo/Superfície foliar de *Eucalyptus alba* incorporada ao jardim pelas operárias)	Colônias de laboratório (Jardim de fungo simbionte/Lixo/Superfície foliar de *Eucalyptus alba* incorporada ao jardim pelas operárias/exoesqueleto de operárias)	Colônias de campo (Jardim de fungo simbionte)	Fêmeas fundadoras (exoesqueleto/esponja inicial de fungo simbionte)		Colônias de campo (lixo) / Fêmeas fundadoras	Colônias de laboratório (Jardim de fungo simbionte/Lixo)
Fungo filamentoso não-mutualístico								
Ascomycota								
Acremonium killiense[1,3]	-	-	(+/+/-/-)	+	(-/-)	(-/-)	-	-
Acremonium strictum[1,3]	-	-	(+/-/-/-)	+	(-/-)	(-/-)	-	-
Arthrobotrys cladodes[3]	-	-	(+/-/-/-)	-	(-/-)	(-/-)	-	-
Aspergillus flavus[3,11]	-	-	(+/+/-/-)	-	(-/-)	(-/-)	-	-
Aspergillus niger[9]	-	-	-	-	-	-	-	(+/+)
Aspergillus niger var. *niger*[3]	-	-	(-/-/-/-)	+	(-/-)	(-/-)	-	-
Aspergillus sp.[8,10]	-	-	(-/-/-/-)	-	(-/-)	(+/-)	-	-
Beauveria bassiana[10]	+	-	-	-	-	-	-	-
Chaetomium funicola[8]	-	-	(-/-/-/-)	-	(-/-)	(-/+)	-	-
Chrysosporium sulphureum[1]	-	-	(+/-/-/-)	-	(-/-)	(-/-)	-	-
Cladophialophora sp.[8]	-	-	(-/-/-/-)	-	(-/-)	(+/-)	-	-

Continua

Tabela 1: cont.

	Atta spp.							
	Cortadeiras de mono e dicotiledôneas							
	A. sexdens		*A. sexdens rubropilosa*		*A. capiguara*	*A. laevigata*	*A. colombica*	*A. cephalotes*
	Fêmeas fundadoras (exoesqueleto)	Colônias de laboratório (Jardim de fungo simbionte/Lixo/Superfície foliar de *Eucalyptus alba* incorporada ao jardim pelas operárias)	Colônias de laboratório (Jardim de fungo simbionte/Lixo/Superfície foliar de *Eucalyptus alba* incorporada ao jardim pelas operárias/exoesqueleto de operárias)	Colônias de campo (Jardim de fungo simbionte)	Fêmeas fundadoras (exoesqueleto/esponja inicial de fungo simbionte)		Colônias de campo (lixo) / Fêmeas fundadoras	Colônias de laboratório (Jardim de fungo simbionte/Lixo)
Fungo filamentoso não-mutualístico								
Ascomycota								
Cladosporium cladosporioides[1,3,8]	-	-	(+/-/-/-)	-	(-/+)	(+/+)	-	-
Cladosporium herbarum[9]	-	-	-	-	-	-	-	(+/+)
Cladosporium oxysporum[9]	-	-	-	-	-	-	-	(+/+)
Cladosporium subtilissimum[8]	-	-	(-/-/-/-)	-	(+/-)	(-/-)	-	-
Cladosporium sp.[8]	-	-	(-/-/-/-)	-	(+/-)	(+/-)	-	-
Clonostachys rosea[1]	-	-	(+/-/-/-)	-	(-/-)	(-/-)	-	-
Cunninghamella elegans[3]	-	-	(+/+/-/-)	-	(-/-)	(-/-)	-	-
Cunninghamella echinulata[3,8]	-	-	(-/-/-/-)	+	(+/-)	(-/-)	-	-
Epicoccum nigrum[9]	-	-	-	-	-	-	-	(+/+)
Escovopsis weberi[1,3]	-	-	(+/-/-/-)	+	(-/-)	(-/-)	-	-
Fusarium culmorum[9]	-	-	-	-	-	-	-	(+/+)

Continua

Tabela 1: cont.

	Atta spp.							
	Cortadeiras de mono e dicotiledôneas							
	A. sexdens		*A. sexdens rubropilosa*		*A. capiguara*	*A. laevigata*	*A. colombica*	*A. cephalotes*
	Fêmeas fundadoras (exoesqueleto)	Colônias de laboratório (Jardim de fungo simbionte/Lixo/Superfície foliar de *Eucalyptus alba* incorporada ao jardim pelas operárias)	Colônias de laboratório (Jardim de fungo simbionte/Lixo/Superfície foliar de *Eucalyptus alba* incorporada ao jardim pelas operárias/exoesqueleto de operárias)	Colônias de campo (Jardim de fungo simbionte)	Fêmeas fundadoras (exoesqueleto/esponja inicial de fungo simbionte)		Colônias de campo (lixo) / Fêmeas fundadoras	Colônias de laboratório (Jardim de fungo simbionte/Lixo)
Fungo filamentoso não-mutualístico								
Ascomycota								
Fusarium equiseti[3]	-	-	(+/-/-/-)	-	(-/-)	(-/-)	-	-
Fusarium solani[3,9]	-	-	(+/-/-/-)	-	(-/-)	(-/-)	-	-
Fusarium verticillioides[3]	-	-	(-/+/-/-)	-	(-/-)	(-/-)	-	-
Fusarium sp.[8,9]	-	-	(-/-/-/-)	-	(-/-)	(+/-)	-	-
Glomerella cingulata[9]	-	-	-	-	-	-	-	(+/-)
Mariannaea elegans var. *elegans*[3]	-	-	(-/-/-/-)	+	(-/-)	(-/-)	-	-
Monilia sp.[8]	-	-	(-/-/-/-)	-	(-/-)	(+/-)	-	-
Moniliella suaveolens[3]	-	-	(+/-/-/-)	+	(-/-)	(-/-)	-	-
Ophiocordyceps[12]	-	-	-	-	-	-	(-/+)	-
Penicillium citrinum[3]	-	-	(+/+/-/-)	-	(-/-)	(-/-)	-	-
Penicillium janthinellum[3]	-	-	(+/+/-/-)	-	(-/-)	(-/-)	-	-

Continua

Tabela 1: cont.

	Atta spp.							
	Cortadeiras de mono e dicotiledôneas							
	A. sexdens		*A. sexdens rubropilosa*		*A. capiguara*	*A. laevigata*	*A. colombica*	*A. cephalotes*
	Fêmeas fundadoras (exoesqueleto)	Colônias de laboratório (Jardim de fungo simbionte/Lixo/Superfície foliar de *Eucalyptus alba* incorporada ao jardim pelas operárias)	Colônias de laboratório (Jardim de fungo simbionte/Lixo/Superfície foliar de *Eucalyptus alba* incorporada ao jardim pelas operárias/exoesqueleto de operárias)	Colônias de campo (Jardim de fungo simbionte)	Fêmeas fundadoras (exoesqueleto/esponja inicial de fungo simbionte)		Colônias de campo (lixo) / Fêmeas fundadoras	Colônias de laboratório (Jardim de fungo simbionte/Lixo)
Fungo filamentoso não-mutualístico								
Ascomycota								
Penicillium sp. subgen. *Furcatum*[3]	-	-	(-/+/-/-)	-	(-/-)	(-/-)	-	-
Penicillium sp.[8]	-	-	(-/-/-/-)	-	(+/-)	(+/-)	-	-
Phoma nebulosa[9]	-	-	-	-	-	-	-	(+/+)
Phomopsis glandicola[9]	-	-	-	-	-	-	-	(+/-)
Phomopsis ilicina[9]	-	-	-	-	-	-	-	(+/-)
Phomopsis quercella[9]	-	-	-	-	-	-	-	(+/-)
Phyllosticta ghaesembillae[9]	-	-	-	-	-	-	-	(+/-)
Trichoderma hamatum[9]	-	-	-	-	-	-	-	(+/+)
Trichoderma harzianum[1,3]	-	-	(+/+/-/-)	+	-	-	-	-
Trichoderma longibrachiatum[9]	-	-	-	-	-	-	-	(+/-)
Trichoderma sp.[1,3,8]	-	-	(+/-/-/-)	+	-	-	-	-

Continua

Tabela 1: cont.

	Atta spp.							
	Cortadeiras de mono e dicotiledôneas							
	A. sexdens		*A. sexdens rubropilosa*		*A. capigu ara*	*A. laevigata*	*A. colombica*	*A. cephalotes*
	Fêmeas fundadoras (exoesqueleto)	Colônias de laboratório (Jardim de fungo simbionte/Lixo/ Superfície foliar de *Eucalyptus alba* incorporada ao jardim pelas operárias)	Colônias de laboratório (Jardim de fungo simbionte/Lixo/Superfície foliar de *Eucalyptus alba* incorporada ao jardim pelas operárias/exoesqueleto de operárias)	Colônias de campo (Jardim de fungo simbionte)	Fêmeas fundadoras (exoesqueleto/esponja inicial de fungo simbionte)		Colônias de campo (lixo) / Fêmeas fundadoras	Colônias de laboratório (Jardim de fungo simbionte/ Lixo)
Fungo filamentoso não-mutualístico								
Levedura								
Aureobasidium pullulans[8,9]	-	-	-	-	(+/+)	-	-	(+/+)
Aureobasidium spp.[5]	-	(-/-/+)	-	-	-	-	-	-
Black yeasts[4]	-	-	(-/-/+/-)	-	-	-	-	-
Candida colliculosa[4]	-	-	(+/+/-/-)	-	-	-	-	-
Candida famata[4]	-	-	(+/+/-/-)	-	-	-	-	-
Candida guilliermondii[4]	-	-	(+/+/-/-)	-	-	-	-	-
Candida homilentoma[4,5]	-	(+/-/-)	(+/+/-/+)	-	-	-	-	-
Candida parapsilosis[8]	-	-	-	-	-	(+/-)	-	-
Candida robusta[4]	-	-	(+/-/-/+)	-	-	-	-	-
Candida sake[4]	-	-	(-/-/-/+)	-	-	-	-	-
Candida valida- similar[4]	-	-	(-/-/-/+)	-	-	-	-	-

Continua

Tabela 1: cont.

	Atta spp.							
	Cortadeiras de mono e dicotiledôneas							
	A. sexdens		*A. sexdens rubropilosa*		*A. capiguara*	*A. laevigata*	*A. colombica*	*A. cephalotes*
	Fêmeas fundadoras (exoesqueleto)	Colônias de laboratório (Jardim de fungo simbionte/Lixo/Superfície foliar de *Eucalyptus alba* incorporada ao jardim pelas operárias)	Colônias de laboratório (Jardim de fungo simbionte/Lixo/Superfície foliar de *Eucalyptus alba* incorporada ao jardim pelas operárias/exoesqueleto de operárias)	Colônias de campo (Jardim de fungo simbionte)	Fêmeas fundadoras (exoesqueleto/esponja inicial de fungo simbionte)		Colônias de campo (lixo) / Fêmeas fundadoras	Colônias de laboratório (Jardim de fungo simbionte/Lixo)
Fungo filamentoso não-mutualístico								
Levedura								
Candida sp1[4]	-	-	(+/-/-/-)	-	-	-	-	-
Candida sp2[4]	-	-	(-/-/+/-)	-	-	-	-	-
Candida sp3[4]	-	-	(-/-/-/+)	-	-	-	-	-
Cryptococcus aerius[5]	-	(+/-/+)	(+/+/+/-)	-	-	-	-	-
Cryptococcus albidus var. *albidus*[4,5]	-	(+/+/-)	(+/-/-/-)	-	-	-	-	-
Cryptococcus albidus var. *aerius*[4]	-	-	(+/-/+/-)	-	-	-	-	-
Cryptococcus curvatus[4,5]	-	-	(-/-/+/-)	-	-	-	-	-
Cryptococcus haglerorum[6]	-	(+/-/-)	-	-	-	-	-	-
Cryptococcus laurentii[4,5,8]	-	(+/-/+)	(+/-/+/-)	-	(+/-)	(+/+)	-	-
Debaryomyces hansenii[5]	-	(+/+/-)	-	-	-	-	-	-
Pichia anomala[4,5]	-	(+/-/-)	(-/-/-/+)	-	-	-	-	-

Continua

Tabela 1: cont.

	Atta spp.							
	Cortadeiras de mono e dicotiledôneas							
	A. sexdens		*A. sexdens rubropilosa*		*A. capiguara*	*A. laevigata*	*A. colombica*	*A. cephalotes*
	Fêmeas fundadoras (exoesqueleto)	Colônias de laboratório (Jardim de fungo simbionte/Lixo/Superfície foliar de *Eucalyptus alba* incorporada ao jardim pelas operárias)	Colônias de laboratório (Jardim de fungo simbionte/Lixo/Superfície foliar de *Eucalyptus alba* incorporada ao jardim pelas operárias/exoesqueleto de operárias)	Colônias de campo (Jardim de fungo simbionte)	Fêmeas fundadoras (exoesqueleto/esponja inicial de fungo simbionte)		Colônias de campo (lixo) / Fêmeas fundadoras	Colônias de laboratório (Jardim de fungo simbionte/Lixo)
Fungo filamentoso não-mutualístico								
Levedura								
Pichia guilliermondii[5]	-	(+/+/-)	-	-	-	-	-	-
Pichia ohmeri[5]	-	(-/+/-)	-	-	-	-	-	-
Pichia mexicana – similar[5]	-	(+/-/-)	-	-	-	-	-	-
Rhodotorula glutinis[4,5,8]	-	(+/+/-)	(+/+/-/-)	-	-	(-/+)	-	-
Rhodotorula mucilaginosa[5]	-	(+/-/-)	-	-	-	-	-	-
Saccharomyces cerevisiae[5]	-	(+/-/-)	-	-	-	-	-	-
Sporobolomyces roseus[4]	-	-	(+/-/-/-)	-	-	-	-	-
Sympodiomyces attinorum[7]	-	(+/+/-)	-	-	-	-	-	-
Torulaspora delbrueckii[5]	-	(+/+/-)	-	-	-	-	-	-
Torulaspora delbrueckii – similar[5]	-	(+/+/-)	-	-	-	-	-	-
Tremella foliacea[4,5]	-	(+/-/-)	(+/-/-/-)	-	-	-	-	-

Continua

Tabela 1: cont.

	Atta spp.							
	Cortadeiras de mono e dicotiledôneas							
	A. sexdens		*A. sexdens rubropilosa*		*A. capiguara*	*A. laevigata*	*A. colombica*	*A. cephalotes*
	Fêmeas fundadoras (exoesqueleto)	Colônias de laboratório (Jardim de fungo simbionte/Lixo/Superfície foliar de *Eucalyptus alba* incorporada ao jardim pelas operárias)	Colônias de laboratório (Jardim de fungo simbionte/Lixo/Superfície foliar de *Eucalyptus alba* incorporada ao jardim pelas operárias/exoesqueleto de operárias)	Colônias de campo (Jardim de fungo simbionte)	Fêmeas fundadoras (exoesqueleto/ esponja inicial de fungo simbionte)		Colônias de campo (lixo) / Fêmeas fundadoras	Colônias de laboratório (Jardim de fungo simbionte/Lixo)
Fungo filamentoso não-mutualístico								
Levedura								
Trichosporon beigelii[4]	-	-	(+/+/-/+)	-	-	-	-	-
Trichosporon firovecii[5]	-	(+/+/-)	-	-	-	-	-	-

Nota: [(1)]Rodrigues et al., 2005a [(2)]Rodrigues et al., 2008a [(3)]Rodrigues et al., 2005b [(4)]Carreiro et al., 1997 [(5)]Carreiro et al., 2002 [(6)]Middelhoven et al., 2003 [(7)]Carreiro et al., 2004 [(8)] Pagnocca et al., 2008 [(9)]Fisher et al., 1996 [(10)]Alves, 1998 [(11)]Hughes et al., 2004 [(12)]Hughes et al., 2009.

Tabela 2: Fungos patógenos isolados de colônias de *Acromyrmex* (+: presença; -: ausência)

	Acromyrmex spp.										
	Cortadeiras de dicotiledôneas									Cortadeiras de monocotiledôneas	
	A. ambiguus	*A. aspersus*	*A. coronatus*	*A. crassispinus*	*A. disciger*	*A. hispidus*	*A. laticeps*	*A. lundi*	*A. octospinosus*	*A. heyeri*	*A. landolti*
	Colônias de campo (Jardim de fungo simbionte)								Fêmeas fundadoras	Colônias de campo (Jardim de fungo simbionte)	
Fungo filamentoso não-mutualístico											
Zygomycota											
Cunninghamella binariae[2]	-	+	+	-	-	-	-	-	-	+	-
Cunninghamella blakesleana[2]	-	-	+	-	-	-	-	-	-	-	-
Cunninghamella echinulata var. *antartica*[2]	-	-	-	+	-	-	+	+	-	-	-
Mucor circinelloides[2]	-	-	-	-	-	-	+	-	-	-	-
Mucor racemosus[2]	+	+	-	-	+	-	+	+	-	-	-
Mucor sp1[2]	-	-	+	-	-	-	-	-	-	-	-
Mucor sp2[2]	-	-	+	-	-	-	-	-	-	-	-
Ascomycota											
Aspergillus flavus[2,3,6]	+	-	-	-	-	+	-	-	-	-	-
Aspergillus versicolor[2]	-	-	-	-	-	-	-	-	-	+	-
Chaetomium sp.[2]	-	-	-	-	-	-	-	-	-	+	-
Cladosporium cladosporioides[1,2,3,4]	-	-	-	-	-	+	-	-	-	-	-
Escovopsis sp.[2]	+	-	+	-	+	-	+	+	-	+	-
Eupenicillium javanicum[2]	-	-	-	-	-	+	-	-	-	-	-
Fusarium equiseti[2,3]	+	-	-	-	-	-	-	-	-	-	-
Fusarium oxysporum[2,3]	+	-	+	-	+	+	+	+	-	+	-
Fusarium solani[2,3,5]	-	-	-	-	-	-	-	-	-	+	-

Continua

Tabela 2: Cont.

	Acromyrmex spp.										
	Cortadeiras de dicotiledôneas									Cortadeiras de monocotiledoneas	
	A. ambiguus	*A. aspersus*	*A. coronatus*	*A. crassispinus*	*A. disciger*	*A. hispidus*	*A. laticeps*	*A. lundi*	*A. octospinosus*	*A. heyeri*	*A. landolti*
	Colônias de campo (Jardim de fungo simbionte)								Fêmeas fundadoras	Colônias de campo (Jardim de fungo simbionte)	
Fungo filamentoso não-mutualístico											
Ascomycota											
Lecythophora sp.[2]	-	-	-	-	-	-	-	-	-	+	-
Moniliella-like fungus[2]	-	+	-	-	-	-	-	+	-	+	-
Ophiocordyceps[7]	-	-	-	-	-	-	-	-	+	-	-
Paecilomyces lilacinus[2]	-	-	+	-	-	-	-	-	-	-	-
Penicillium citrinum[2,3]	-	-	-	-	-	+	+	-	-	-	-
Penicillium waksmanii[2]	-	-	+	-	-	-	-	-	-	-	-
Penicillium sp1[2]	-	-	-	-	-	-	-	+	-	-	-
Penicillium sp2[2]	-	-	-	-	-	-	-	-	-	+	-
Penicillium sp3[2]	-	-	-	+	-	-	-	-	-	-	-
Trichoderma hamatum[2,5]	-	-	-	-	-	-	+	-	-	-	-
Trichoderma harzianum[1,2,3]	-	-	+	-	-	-	-	-	-	+	+
Trichoderma spirale[2]	-	-	-	-	-	-	-	-	-	+	-
Trichoderma virens[2]	-	-	+	-	-	-	-	-	-	-	-
Trichoderma sp.[1,2,3,4]	-	+	+	-	-	-	+	-	-	+	-
Volutella sp.[2]	+	-	-	-	-	-	+	-	-	-	-
Xylaria sp1.[2]	-	-	-	+	-	-	-	-	-	+	-
Xylaria sp2.[2]	-	-	-	-	-	-	-	-	-	+	-

Nota: [(1)]Rodrigues et al., 2005a [(2)]Rodrigues et al., 2008a [(3)]Rodrigues et al., 2005b [(4)] Pagnocca et al., 2008 [(5)]Fisher et al., 1996 [(6)]Hughes et al., 2004 [(7)]Hughes et al., 2009

CHAPTER 2

NEW SPECIES OF *ESCOVOPSIS* PARASITIC ON THE FUNGAL GARDENS OF *ACROMYRMEX* LEAF-CUTTING ANTS IN MINAS GERAIS, BRAZIL

Abstract: A notable example of mutualism is that exhibited by fungus-growing ants and their symbiont cultivar. In recent years, this symbiosis has emerged as a model system for studying coevolution, cooperation and conflict between hosts and ymbionts. The mycoparasite fungus *Escovopsis* (Ascomycota, Hypocreales) is very commonly found in attine fungus gardens and is known only from this habitat. Despite great interest in its biology, ecology and molecular phylogeny, which has led to a steady flow of high-profile publications over the last decade, only two species of *Escovopsis*, *E. weberi* and *E. aspergilloides*, are currently formally described. Here we describe four new morphologically distinct *Escovopsis* species from leaf-cutting ant nests in the region of Zona da Mata, Minas Gerais, Brazil. Using morphological characters as well as molecular data, we provide taxonomic data as well as biological data of the fungi. Our survey indicates there could be many more species throughout the geographic range of the Attini and the present study makes a start at assigning names and formal descriptions to at least a small number of these taxa.

Key words: Ascomycota, Attini, Hypocreales, ITS, morphotyping, phylogeny, taxonomy, leaf-cutting ants

INTRODUCTION

Leaf-cutting ants (Hymenoptera: Formicidae: Attini) are the only known ants (Hölldobler & Wilson 1990) that have evolved the ability to cut and process fresh plant material that is later incorporated into gardens of their symbiont fungal partner, *Leucoagaricus gongylophorus* (A. Mueller) Singer (Basidiomycota: Agaricales) (Fisher 1994). Adult workers and queens of the genus *Acromyrmex* and *Atta* rely entirely on their fungal gongylidia for food, while their symbiont fungus relies on the ants for optimal conditions to grow. The highly-derived domesticated leucocoprineaceous fungi of the leaf-cutters are the only leucocoprineaceous fungi that produce gongylidia, hyphal swellings tips that are rich in lipids and carbohydrates (Martin & Martin 1970; Quinlan & Cherrett 1979), in contrast to the leucocoprineaceous fungi cultivated by the basal attine ants which are capable of free-living and do not produce gongylidia (Schultz & Brady 2008; Vo et al 2009).

Mutualisms, however, are not free from usurpation by parasites (Bronstein 2001; Thomas et al 2005; Thrall et al 2007), and the ant-fungus mutualism is also a target for usurpation. The fungus gardens of the attine ants are hosts of the specialized parasite *Escovopsis* (Ascomycota: Hypocreales), which is only known at the present time from the attine fungus gardens (Seifert et al 1995). It has been demonstrated that *E. weberi* is a necrotrophic parasite, just as its mycoparasitic relatives in the Hypocreales order, with the peculiarity that it is a contact necrotroph, i. e., able to degrade host hyphae from a distance, not necessarily by penetrating the hyphae of its host (Reynolds & Currie 2004).

The fungus genus *Escovopsis* was first isolated by Moeller from a survey he made from leaf-cutting ants in Blumenau, Brazil, in 1890 and 1891 (Seifert et al 1995). Although he carefully described and illustrated the two anamorphic species (Figures 1 and 2 from (Seifert et al 1995)), they remained unnamed until Kreisel (1972) recognized one of these anamorphs among his fungal isolates from *Atta insularis* nests in Kuba. He then described a new genus and species, *Phialocladus zsoltii*, but as he did not designated a holotype, Muchovej and Della Lucia (1990) renamed the genus *Escovopsis* after isolating the fungus that they assumed to be identical to *Phialocladus zsoltii* Kreisel from an unspecified leaf-cutting ant nest in

Brazil. They named the type species as *E. weberii*, in honor of Neil Weber, a contemporary entomologist and myrmecologist, well-known for his studies on leaf-cutting ants (Weber 1966, 1972). The second anamorph described and illustrated by Moeller in 1893 has been rediscovered by Seifert et al (1995) from a nest of a non-leaf-cutting attine ant, *Trachymyrmex ruthae*, from Trinidad. It was named *E. aspergilloides*, due to its similarity to *Aspergillus* in general appearance (Seifert et al 1995). So, the two *Escovopsis* species formally described so far are *E. weberi* and *E. aspergilloides*, despite the fact that a more recent study revealed the possible existence of eight more undescribed species of *Escovopsis* inhabiting attine fungus gardens (Currie et al 1999).

There has been increasing awareness of the impact of mycoparasites on nests of the Attini - and thus of their ecological and evolutionary significance - and, in particular, of the discovery that *Escovopsis* is an obligate pathogen of the attine fungus gardens (Currie et al 1999). This has resulted in a steady flow of high-profile publications on the biology, ecology and molecular phylogeny of *Escovopsis* (Currie 2001a, b; Currie et al 2003; Gerardo et al 2004; Reynolds & Currie 2004; Gerardo et al 2006a; Gerardo et al 2006b; Gerardo & Caldera 2007; Kost et al 2007; Taerum et al 2007; Mueller et al 2008; Schultz & Brady 2008; Caldera et al 2009; Taerum et al 2010). However, the taxonomy of the genus has not been addressed despite the fact that many of these studies repeatedly emphasize that there is considerable morphological and genetic variation within the isolates – most distinguish them simply by colony colour (Gerardo et al 2006b). Clearly, there could be many more species throughout the geographic range of the Attini. The present study makes a start at assigning names and formal descriptions to at least a small number of these taxa.

MATERIALS AND METHODS

Fungal sampling

Attine subcolonies consist of the symbiont fungal garden and worker ants, different from whole colonies, in which one or more functional queens are invariably found.

We collected subcolonies (approx.. 200 mL of fungus garden) from three species of leaf-cutting ants in the genus *Acromyrmex: A. subterraneus molestans* Santschi, *A. subterraneus subterraneus* Forel and *A. niger* F. Smith, between April and May 2010 at sites in the hosts' sympatric range in the Atlantic Rainforest of Minas Gerais (Zona da Mata Mineira): all located within the campus of the Universidade Federal de Viçosa or in a nearby forest reserve (Mata do Paraíso), 650-700 m a.s.l. *Acromyrmex* nests were chosen because they are abundant and easily accessible in the study area*,* being located relatively close to the soil surface, or buried beneath leaf litter in the case of *A. subterraneus molestans*. Samples were taken from both the top of the fungal garden, where fresh vegetation is constantly incorporated, as well as from the oldest part, at the base. Eight garden pieces (~5mm^3) from each subcolony were transferred to Potato Dextrose Agar (PDA) with antibiotics (50mg/L of chloramphenicol) (Riker and Riker 1936) and incubated at 25^{o}C. If *Escovopsis* emerged from a garden piece, which typically occurred within 4 days of initial isolation, the colony was scored as infected. *Escovopsis* mycelium was then subcultured on potato carrot agar (PCA), for hyphal-tip isolation for molecular characterization. All samples have been deposited in the Herbarium VIC, Department of Vegetal Biology, UFV, Viçosa-MG, (accession numbers DOA626-VIC31753; DOA627-VIC31754; DOA628-VIC31755; DOA629-VIC31756).

Morphological data

Colony morphology and radial growth rates of isolates were compared on both Potato Dextrose Agar (PDA) culture medium (Riker and Riker 1936) and Potato Carrot Agar (PCA) (Tuite 1969), as well as on malt extract agar 2% (MEA) (Riker and Riker 1936), at 25^{o}C, in the dark or 12-hour light/12-hour dark. For microscopic analysis, material was mounted in 1% acid fuchsin and examined using an Olympus RX51 microscope with MicroPublisher 3.3 RTV Q imaging camera.

Molecular characterization

DNA EXTRACTION – Fungi were grown in Erlenmeyer flasks containing 100 mL of liquid medium (10 g of sucrose, 2 g L-asparagin, 2 g yeast extract, 1 g KH_2PO_4, 0.1 g $MgSO_4.7H_2O$, 0.44 mg $ZnSO_4.7H_2O$, 0.48 mg $FeCl_3.6H_2O$ and 0.36 mg $MnCl_2.H_2O$) for 5 days at 26^oC on a shaker (170 r.p.m.). The resulting mycelium was washed out with distilled water and placed on sterile filter paper to dry. DNA extraction followed a CTAB (cetyl trimethyl ammonium bromide) extraction protocol modified from Doyle & Doyle (1990), as follows. Using pestle and mortar, the fungal biomass of each isolate was ground in liquid nitrogen and transferred to 1.5 mL microtubes containing 750 µL of CTAB 2X buffer and 15µL of 2-β-mercaptoethanol. Microtubes were placed in water bath at 65^oC/30min. In each tube, 500 µL of phenol chloroform-isoamyl alcohol (25:24:1 v/v) was added followed by centrifugation at 14000 g/5 min. The supernatant was transferred to a new microtube and 500 µL chloroform-isoamyl alcohol (24:1 v/v) was added; this was then centrifuged at 14000 g/5 min. An aliquot of 360 µL of the supernatant was transferred to a new tube, in which 324 µL of cold isopropyl alcohol was added. The suspension was held at -20°C for 10 min and then centrifuged at 14000 g/7 min. The supernatant was discarded and the pellet was washed twice with 500 µL ethanol 70%, followed by centrifugation at 14,000 g/5 min. After the ethanol was discarded, tubes were allowed to dry at room temperature overnight. The dried pellet was then resuspended in 50 µL TE buffer containing RNAse (10 µL/ml), homogenized and held at 37^oC/2 h. The quality and quantity of DNA samples were determined in agarose gels (0.8 %) stained with ethidium bromide (0.15 µg/mL). A DNA mass marker λ HindIII (Invitrogen) was used in the electrophoresis at 80 V for 1 h to quantify the DNA.

DNA AMPLIFICATION AND SEQUENCING – Amplification of PCR products of three genomic regions, *ITS rDNA* (Internal Transcribed Spacer), *LSU rDNA* (Large Sub Unit) and *EF-1 alpha* (Elongation Factor-1 alpha) were conducted with primers ITS1-F (CTTGGTCATTTAGAGGAAGTAA) (Gardes and Bruns 1993), ITS4-R (TCCTCCGCTTATTGATATGC) (White et al 1990); specific primers CLA-F (5' GCATATCAATAAGCGGAGGA 3'), CLA-R (5' GACTCCTTGGTCCGTGTTTCA 3') (Currie et

al 2003); and EF1-983F (5' GCYCCYGGHCAYCGTGAYTTYAT 3'), EF1-2218R (5' GACTTGACTTCRGTVGTGAC 3') (Currie et al 2003), respectively.

All PCR reactions were performed in a total volume of 50 µL and contained 20 to 100 ng of genomic DNA, with buffer 1X (50 mM KCl, 10 mM Tris-HCl); 1.5 mM $MgCl_2$; 0.2 µM of each dNTP; 1 U of Taq DNA polymerase and 0.2 µM of the relevant primer. All reactions were done in a MJ Research PTC 100 thermocycler. For the ITS regions, PCR conditions were as follow: 5 min of denaturation at 95°C, followed by 30 cycles consisting of 30s at 95°C, 30s at 60°C and 90s at 72°C and finally 10 min of extension at 72°C. The CLA reactions were done starting with 2 min of denaturation at 95°C, followed by 40 cycles consisting of 30s at 95°C, 60s at 62°C, 90s at 72°C and finally 5 min of extension at 72°C. Meanwhile, the EF1-alpha reactions started with 2 min of denaturation at 95°C, followed by 40 cycles consisting of 30s at 95°C, 60s at 60°C and 90s at 72°C and then 5 min of extension at 72°C. PCR products were then purified using minicolumns according to the manufacturer's protocols (Roche-High Pure PCR Product Purification Kit).

Sequencing was carried out directly from purified PCR-amplified products using the automatic sequencer ABI Prism 3100. In order to place the *Escovopsis* isolates among the Hypocreales, additional sequences from other Hypocrealean species were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/nuccore). Representatives from three families (Nectriaceae, Hypocreaceae and Clavicipitaceae) as well as *E. weberi* and *E. aspergilloides* were sampled for a total of 21 hypocrealean taxa, including one *Glomerella* isolate (Glomerellales) for rooting the Hypocreales. Sequences are deposited in Genbank under the following accession numbers: (non-*Escovopsis* taxa: **LSU** *rDNA*, AF339530, AF543786-AF5437993, U00748, U00756, U17396, U17416, U57681; **ITS** *rDNA*, HM054156, EU559019, EU816393, FJ919229, EF495101, DQ119115, AJ292412, AF065611, AY755512, AY894979, HQ607386; **EF1-α**, AF543772, AF543774, AF543776-AF543780, AF543782–AF543784, AY489605, AY489615, EF468748, EF468783, FJ860712, EU401591; *Escovopsis* taxa: **LSU** *rDNA*, AY172606, AY172615; **ITS** *rDNA*, FJ948131; **EF1-α**, AY172623, AY172632).

Sequences were edited with the Staden Package (Staden 1996) and aligned with ClustalW (http://www.ebi.ac.uk/clustalw). Alignment was visually inspected

and edited manually in MEGA 4 program (Tamura 2007). We aligned all sequences and pruned their ends to eliminate fragments that we could not obtain for all taxa.

PHYLOGENETIC ANALYSES: Molecular phylogenies of ITS, LSU and EF-1α were used to describe and infer the relationship among *Escovopsis* isolates collected from the Atlantic Rainforest in Viçosa, Minas Gerais. Maximum parsimony (MP) and maximum likelihood (ML) analyses were conducted using PAUP 4.0b10 (Swofford 2002); Bayesian analyses were conducted using MrBayes 3.2 (Ronquist and Huelsenbeck 2003). For MP, heuristic searches with 1,000 random-addition sequence replicates and TBR (tree-bisection–reconnection) branch swapping were performed. Heuristic MP bootstrap analysis consisted of 1,000 pseudoreplicates (TBR branch swapping), with 10 random-taxon-addition replicates per pseudoreplicate.

For ML analysis, DNA sequence evolution model was established based on the Akaike information criterion (AIC) and likelihood ratio test implemented in the ModelTest 3.7 (Posada and Crandall 2001). Heuristic ML bootstrap analysis consisted of 100 pseudoreplicates (TBR branch swapping).

Bayesian analysis of the datasets was performed using MrBayes 3.2 (Ronquist and Huelsenbeck 2003). The DNA substitution model was determined based on the AIC criterion of MrModelTest (Nylander 2004). The general GTR+Γ+I model (general time reversible with a proportion of sites invariant and gamma-distributed rates) was used and included six separate runs, each consisting of 300K Markov Chain Monte Carlo (MCMC) generations and each with a "burn-in" of 100K generations. All runs converged on the same topology. The Markov Chain Monte Carlo (MCMC) analysis started with a heating parameter 0.1 from a random tree topology and lasted 5,000,000 generations. Trees were saved at each 100^{th} generation, resulting in 50,000 saved trees. Burn-in was set at generation 2,500 after which the likelihood values were stationary, leaving 12,500 trees from which the 50% majority consensus rule trees and posterior probabilities were calculated. The phylogenetic trees were constructed and edited with the Figtree program (http://tree.bio.ed.ac.uk/software).

RESULTS

Fungal sampling

Escovopsis spp. infection in *Acromyrmex* subcolonies is common. This mycoparasite emerged in all of *A. niger* subcolonies (n=2 subcolonies), 45.45% of *A. subterraneus molestans* subcolonies (n=11 subcolonies) and 66.66% of *A. subterraneus subterraneus* subcolonies (n=12 subcolonies). The results of the survey are presented in Table 1 (page 53) in which the total number of isolates that we have collected (n=38) are grouped into four morphotypes based on colony color, vesicle shape and spore morphology. These characters proved to be robust and were confirmed by the results of the molecular characterization. These are described as new species and keyed out from each other and from the other two species in the genus (see Table 2 –page 54).

Morphological data

Escovopsis moelleri H. C. Evans & J. O. Augustin sp. Nov. FIGS. Plate 1 (A-H page 55) and Plates 6-8 (A pages 60-62).

Etymology. Named in honour of A. F. W. Moeller who pioneered work on fungal gardens of ants and provided the first illustrations of *Escovopsis*.

Colonies on PCA at 25^{o} C after 7 days 5-6 cm diam, covering 9-cm diam plate within 14 days; greyish-white becoming brown as conidia mature; aerial mycelium dense reaching lid of plate, 8-10 µm diam; greyish-brown reverse; colony periphery uneven or feathery due to abundant, spreading stolons or rhizoids. Growth similar, but slightly faster on PDA; severely restricted on V8, remaining white and < 1 cm diam after 14 days. *Conidiophore* branches produced as lateral swellings from aerial hyphae, 55-90 x 10-16 µm, smooth, hyaline to subhyaline. *Vesicles* produced laterally and terminally on the branches; mainly clavate, 20-40 (-45) x 10-16 µm, also cylindrical and up to 80 µm long. *Phialides* arranged in rows along the vesicles; ampulliform, 6-9 x 4-5 µm, with a short (2 µm) neck: those on cylindrical vesicles longer and narrower, averaging 10 x 3 µm, tapering to a narrow neck up to 4 µm in length. *Conidia* produced in short, non-persistent chains; initially hyaline becoming

brown, thick-walled and conspicuously warty, oblong, 7-10 x 3.0-3.5 µm, with a truncate base and developing a distinct cap-like structure apically. Other spore forms not observed.

Specimens examined. BRAZIL, MINAS GERAIS, Viçosa, Mata do Paraíso, 700 m. Isolated from fungal garden of *Acromyrmex*

Escovopsis niveo-chlamydosporiformans H. C. Evans & J. O. Augustin sp. Nov. FIGS. Plate 2 (A-E page 56) and Plates 6-8 (B pages 60-62).

Etymology. In reference to the characteristic snow-white colonies with silvery chains or ropes of chlamydospores abundantly produced in culture and over the fungal gardens and middens.

Colonies on PCA growing rapidly, up to 6-7 cm diam after 7 days and reaching the edge of a 9-cm diam plate within 10 days; white, cotton-like aerial mycelium with abundant production of chains of chlamydospores, uniting into snow-white, silvery ropes or strands; white reverse: older colonies becoming creamish or dull yellow in color. Similar growth on PDA, but slower on V8, reaching 3.5-4 cm diam after 14 days; rhizoids formed around periphery, giving it an uneven appearance. *Chlamydospores* hyaline, smooth- and thin-walled, globose, 15-18 µm diam, guttulate, produced laterally from swollen hyphae in chains or clusters. *Blastoconidia* produced as lateral swellings from the walls of the aerial mycelium, abundant; subhyaline, sphaerical, 3-4 µm diam, smooth- and thick-walled. *Conidiophores* arising from aerial mycelium: hyaline, smooth-walled, multiseptate, 40-70 (up to 150) µm in length, 5 µm diam at base narrowing towards apex before swelling into a globose vesicle, 8-10 µm diam, often proliferating terminally to produce several vesicles in succession. *Phialides* formed in small groups (5-10) on vesicles, but also singly from small lateral swellings, septate at base, sometimes proliferating terminally and laterally; variable in shape and size: ampulliform, 10-20 x 2-3 µm, with a short neck; mostly cylindrical, 18-35 x 2.5-3.5 µm, tapering gradually to a blunt or long thin neck, 1 µm diam. *Conidia* catenate, hyaline, smooth- and thin-walled, limoniform to clavate, (3-) 5-8 x (1.5-) 2-3 µm, ending abruptly in or tapering to a truncate base.

Specimens examined. BRAZIL, MINAS GERAIS, Viçosa, Mata do Paraíso, 700 m. Isolated from fungal garden and middens of *Acromymex subterraneus subterraneus.*

Commentary. Paratype (*Acromymex subterraneus subterraneus* 2t) was distinguished initially as a morphotype since it produced only blastoconidia and chlamydospores. Subsequently, this was found to be a very variable character, as phialides and conidia were found to develop intermittently.

Escovopsis microsporum H. C. Evans & J. O. Augustin sp. Nov. FIGS. Plate 3 (A-F page 57) and Plates 6-8 (D pages 60-62).

Etymology. Distinguished from the closest morphotype, *E. moelleri*, by the similarly ornamented but much smaller conidia.

Colonies on PCA attaining 4.5-5.5 cm diam after 7 days, relatively low with sparse aerial mycelium; khaki brown centrally with a distinct snow-white periphery, reaching sides of 9-cm diam plate after 14 days and becoming uniformly brown with abundant dark brown exudates droplets; whitish-brown reverse; rhizoids absent, colony edge relatively even. No recordable growth on V8 after 14 days. *Conidiophores* produced as side branches from aerial mycelium; up to 200 µm long, 6-8 µm wide, forming vesicles laterally and terminally. *Vesicles* clavate, (20-) 28-40 x 8-13 µm, to cylindrical, 45-60 x 7-8 µm, producing phialides along the length. *Phialides* globose at base, 3 µm diam, with narrow (> 1 µm diam) needle-like neck, 1-3 µm in length. *Conidia* produced in persistent chains, white becoming thick-walled, brown and warty: globose, averaging 2-3 µm diam; to ovoid, 2.5 x 1.5 µm, with a truncate base.

Specimens examined. BRAZIL. MINAS GERAIS, Viçosa, Mata do Paraíso, 700 m. Isolated from fungal garden of *Acromymex.*

Escovopsis lentecrescens H. C. Evans & J. O. Augustin sp. Nov. FIGS. Plate 4 (A-D page 58) and Plates 6-8 (C pages 60-62).

Colonies on PCA very slow growing, no recordable growth after 7 days; reaching 0.6-0.7 cm diam after 14 days and 1.5-1.7 cm after 21 days; pinkish brown centrally with a white, even periphery of immature conidiogenous structures:

m*ycelium* sparse and colonies consisting of conidiophores arising directly from the agar or from aerial hyphae. No recordable growth on V8. *Conidiophores* hyaline, up to 200-300 µm in length and 9-10 µm diam. *Vesicles* produced laterally and terminally on short side branches, 30-40 x 6-8 µm, consistently 2-3 septate; variable in form, mainly globose and aspergilloid, (16-) 18-28 µm diam, to clavate, 23-29 x 20-23 µm. *Phialides* subglobose, 3.5-5.0 x 2.5-3.5 (-4.0) µm, with a short, abrupt, spike-like neck. Aberrant structures also produced on some vesicles, extending to form ampulliform to cylindrical phialides with long tapering necks. *Conidia* catenulate, becoming brown and echinulate to verruculose; ovoid to subglobose, 3.0-4.0 x 2.0-2.5 µm; older conidia with a loose, dark outer covering or cap.

Specimens examined: BRAZIL, MINAS GERAIS: Viçosa, Mata do Paraíso, 700 m. Isolated from fungal garden of *Acromyrmex.*

Commentary. Paratype (*Acromymex subterraneus subterraneus* 9f) slightly faster growing, 1.1-1.3 cm diam after 14 days; producing intercalary chlamydospores, globose (10-15 µm) to subglobose (15 x 10 µm).

Molecular characterization

We obtained partial sequences for the two non-protein coding genes (**ITS** 356 bp, conserved sites I = 210, variable sites (V) = 146 , parsimony informative sites (Pi) = 106, singleton (S) = 40; and **LSU** 528 bp, conserved sites I = 387, variable sites (V) = 141, parsimony informative sites (Pi) = 100, singleton (S) = 41), as well as the protein-coding gene (**EF1-α** 462 bp, conserved sites I = 300, variable sites (V) = 162, parsimony informative sites (Pi) = 110, singleton (S) = 52). Phylogenetic analyses using MP, ML and Bayesian methods all resulted in reconstructed trees with similar topology. Only trees obtained with Bayesian methods are presented. All three genes grouped the *Escovopsis* species within a consistent clade among the Hypocreales. For EF1-α and LSU, the Bayesian trees identify, with high posterior probability support, two clades within the *Escovopsis* group. One is made of the *Escovopsis weberi*-like species, comprising *E. weberi* and *E. microsporum*as well as the *Escovopsis aspergilloides*-like species, comprising *E. aspergilloides* and *E. lentecrescens.* The other clade comprises only one species, *Escovopsis niveo-*

chlamydosporiformans (FIGS. 1 (page 63) and 2 (page 64)). Such similar topology was also found for ITS, in which the Bayesian analysis places *E. niveo-chlamydosporiformans* away from the well supported clade comprising *Escovopsis* sp., *E. lentecrescens*, *E. microsporum* and *E. moelleri* (FIG. 3 (page 65)).

DISCUSSION

Our molecular results corroborate the morphological studies, enabling four new species of *Escovopsis* to be added to the genus. Based on our limited exploratory survey, this represents a start at assigning names to these distinctive morphotypes and clearly indicates that there may be many more undescribed species of *Escovopsis* throughout the geographic range of the Attini.

The phylogenetic analysis that we performed demonstrates the monophyly of *Escovopsis*, in agreement with the phylogenetic study on this specialized mycoparasite conducted by Currie et al (2003). However, our analysis also identifies two clades within the *Escovopsis* group, with good *posteriori*-probability support. Consistent with these results is the proposed description of the new fungus genus.

Moreover, our results are consistent with previously reported patterns of high incidence of *Escovopsis* in fungus gardens of attine ants (Currie et al 1999a; Gerardo et al 2004; Rodrigues et al 2008). Previous work has shown that infection rates in the Attini fungus gardens from Panama can vary between 33 and 51% for the three non-leaf cutter genera *Apterostigma*, *Cyphomyrmex* and *Trachymyrmex* and 42.9 to 51.4% among the leaf-cutters *Atta* and *Acromyrmex*, respectively (Currie et al 1999a). *Escovopsis* has emerged in 29% (n=90) of *C. muelleri* colonies and 60% (n=28) of *C. costatus* colonies, indicating that *Escovopsis* infection in sympatric colonies of those species is also common. A survey in southern Brazil revealed that 27% (n=37) of *Acromyrmex* colonies, comprising ten different sympatric species, were positively sampled for *Escovopsis* (Rodrigues et al 2008).

A distinctive feature of the hypocrealean fungi is their obligatory parasitic evolutionary history. Within the Hypocreales are numerous plant, fungal and animal pathogens (Spatafora et al 2007). As such, the evolutionary history of Hypocreales has been shown to be characterized by a shift in nutritional mode from plant-based

nutrition (Bionectriaceae and Nectriaceae) to animal and fungal-based nutrition (Hypocreaceae and Clavicipitaceae s.l.) (Spatafora et al 2007). The diverse morphology and growth patterns of the *Escovopsis* isolates we describe here hint at a different biology of the isolates, in particular their interaction with their hosts. The fact that *E. lentecrescens* grows much more slowly, at least *in vitro*, than the other fast-growing *Escovopsis* isolates suggests a more intricate co-evolutionary history between this mycoparasite and *Leucoagaricus*, so that the parasite may be more specialized on its fungal host. It might also represent a different life history strategy or potentially a strategy for evading detection.

Regarding the transmission of this mycoparasite, at present there is no evidence of how *Escovopsis* reaches its host. The few studies that have investigated this, including the present work, indicates that *Escovopsis* apparently does not rely on vertical transmission (Pagnocca et al 2008). There is preliminary evidence to support horizontal transmission between *Acromyrmex* nests (Chapter 3, this thesis). Ant middens, or refuse piles from fungal gardens, along the forest trail in our designated study area (see chapter three of this thesis for details), were found to bear at least two *Escovopsis* morphotypes: thus, the fungus may have an 'escape mechanism' whereby it can sporulate outside of the nest. In fact, higher Attini ants perform a series of task-partitioning behaviors that are crucial for colony health and maintenance. Midden building in *Acromyrmex lobicornis*, for example, has been described as part of such a behavioral repertoire (Farji-Brener 2000). At present, we are investigating the phenology of *Acromyrmex* middens in order to better elucidate the transmission of *Escovopsis* between Attini nests (Augustin et al in prep.).

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Table 1: *Escovopsis* isolates in fungus gardens of *Acromyrmex* spp. from Southeastern Brazil.

Fungal species	***Acromyrmex niger*** N=2; P=100%		***Ac. subterraneus molestans*** N=5; P=45%		***Ac. subterraneus subterraneus*** N=8; P=66%		**% of nests (=25) with the specified microfungi**
	Top *n*=2	Bottom *n*=2	Top *n*=11	Bottom *n*=11	Top *n*=12	Bottom *n*=12	
Escovopsis moelleri	_	_	1 (RC)	2 (RC) 2 (MP)	_	1 (RC)	24.0
Escovopsis niveo-chlamydosporiformans	1 (MP)	1 (RC) 1 (MP)	1 (RC) 1 (MP)	2 (RC) 2 (Rep)	3 (RC) 1 (Rep) 1 (MP)	1 (RC) 3 (MP)	72.0
Escovopsis microsporium	_	1 (RC)	1 (RC) 2 (Rep)	2 (RC) 2 (Rep) 2 (MP)	_	1 (Rep) 1 (MP)	48.0
Escovopsis lentecrescens	_	_	_	_	_	1 (RC) 1 (MP)	8.0

n Number of subcolonies sampled; N Number of subcolonies infected with *Escovopsis*; P Proportion of infected subcolonies with *Escovopsis*
RC = Recanto das Cigarras (UFV *campus*); Rep = Represa UFV (UFV *campus*); MP = Mata do Paraíso.

Table 2: Key to species of *Escovopsis*

1. Colonies predominantly white	*E. niveo-chlamydosporiformans*
1.Colonies turning yellow to brown	2
2. Colonies slow growing	*E. lentecrescens*
2.Colonies fast growing	3
3. Conidiophores mainly clavate	4
Conidiophores mainly aspergilloid	6
4. Conidia smooth	*E. weberi*
Conidia rugose	5
5. Conidia < 4 µm long	*E. microsporum*
Conidia > 7 µm long	*E. moelleri*
6. Colonies yellowish	*E. aspergilloides*
Colonies becoming brown	*E. lentecrescens*

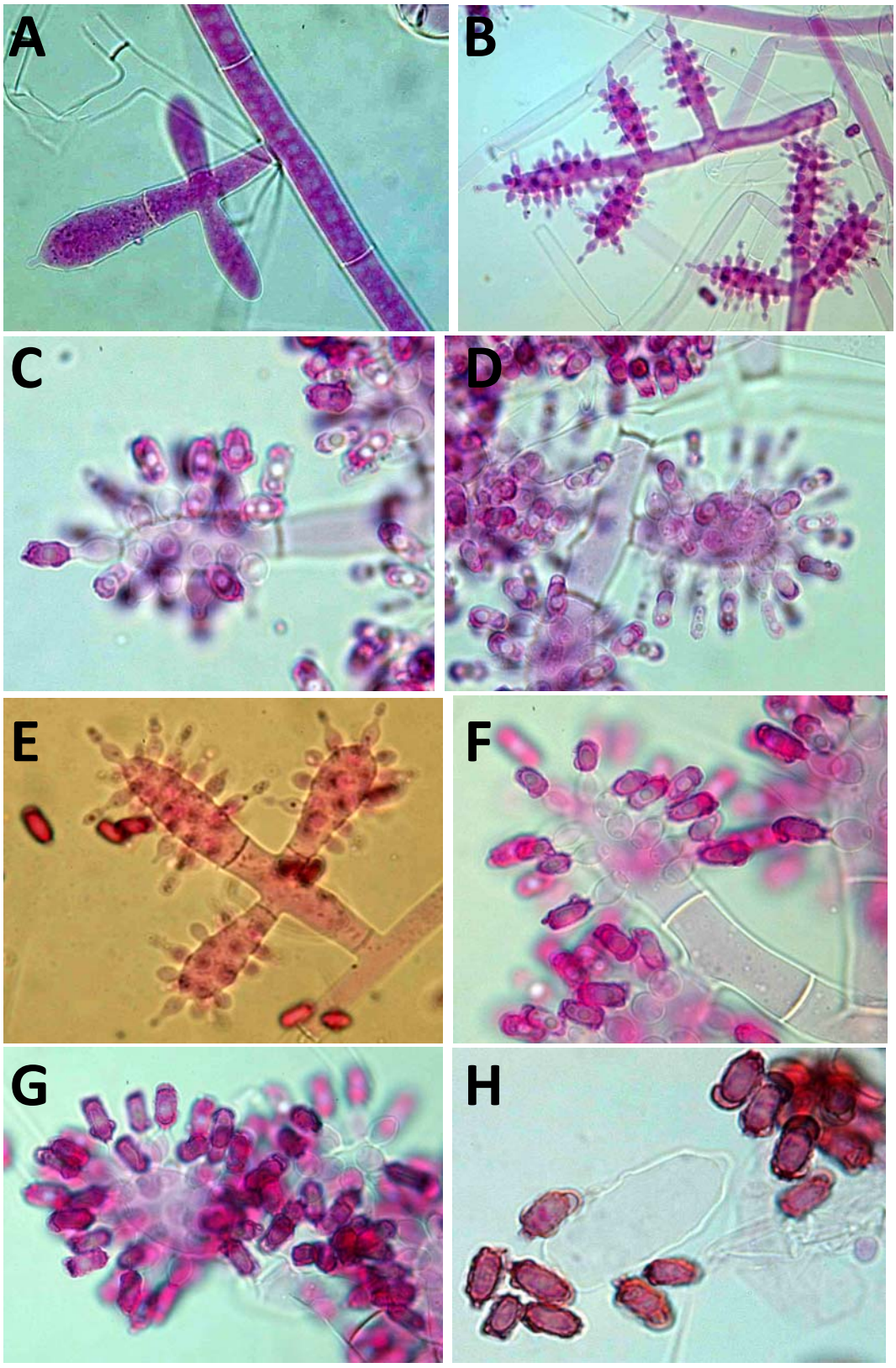

Plate 1 Light microscopy of *Escovopsis moelleri*. A-D. Details of conidiogenesis showing the clavate vesicles covered with swollen, short-necked phialides. **E-H.** Older stages in vesicle development showing the conidia darkening in colour and developing thickened rugose walls and cap-like structures; note the short-lived or evanescent vesicle (black arrow). Photos: H. C. Evans and J. O. Augustin

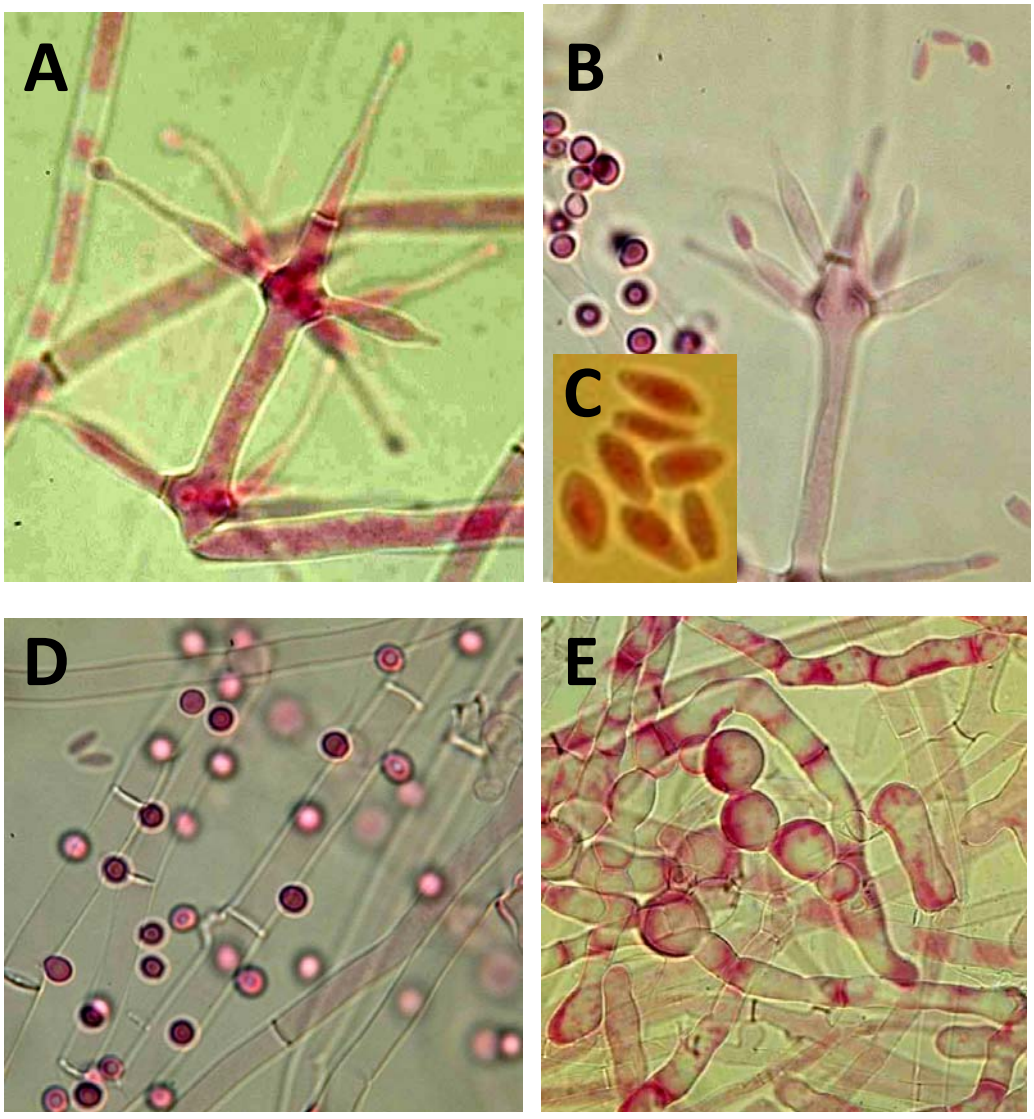

Plate 2 Light microscopy of *Escovopsis niveo-chlamydosporiformans*. A-B. Details of conidiogenesis showing both terminal and intercalary vesicles bearing few cylindrical, subulate phialides tapering gradually to a long neck region, and hyaline, thin-walled conidia (inset, **C**) distinguished from the sphaerical dark blastospores (**B**, left above inset); **D.** Blastospores emerging directly from mycelium; **E.** Chlamydospores formed in glistening white chains or ropes, densely guttulate.
Photos: H. C. Evans and J. O. Augustin

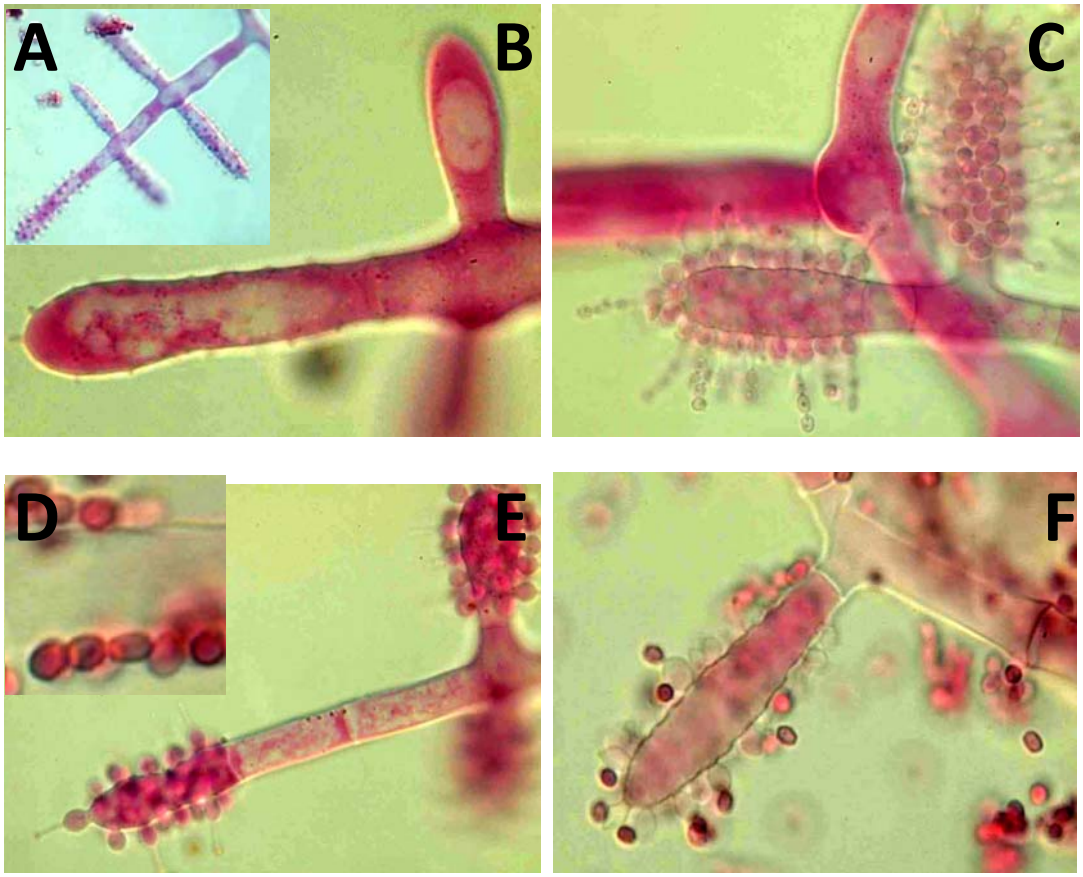

Plate 3 Light microscopy of *Escovopsis microsporum*. A-C. Details of conidiogenesis showing the clavate vesicles and swollen, short-necked phialides producing chains of conidia; **E-F.** Older vesicles becoming evanescent with darkening spores (inset, **D**).
Photos: H. C. Evans and J. O. Augustin

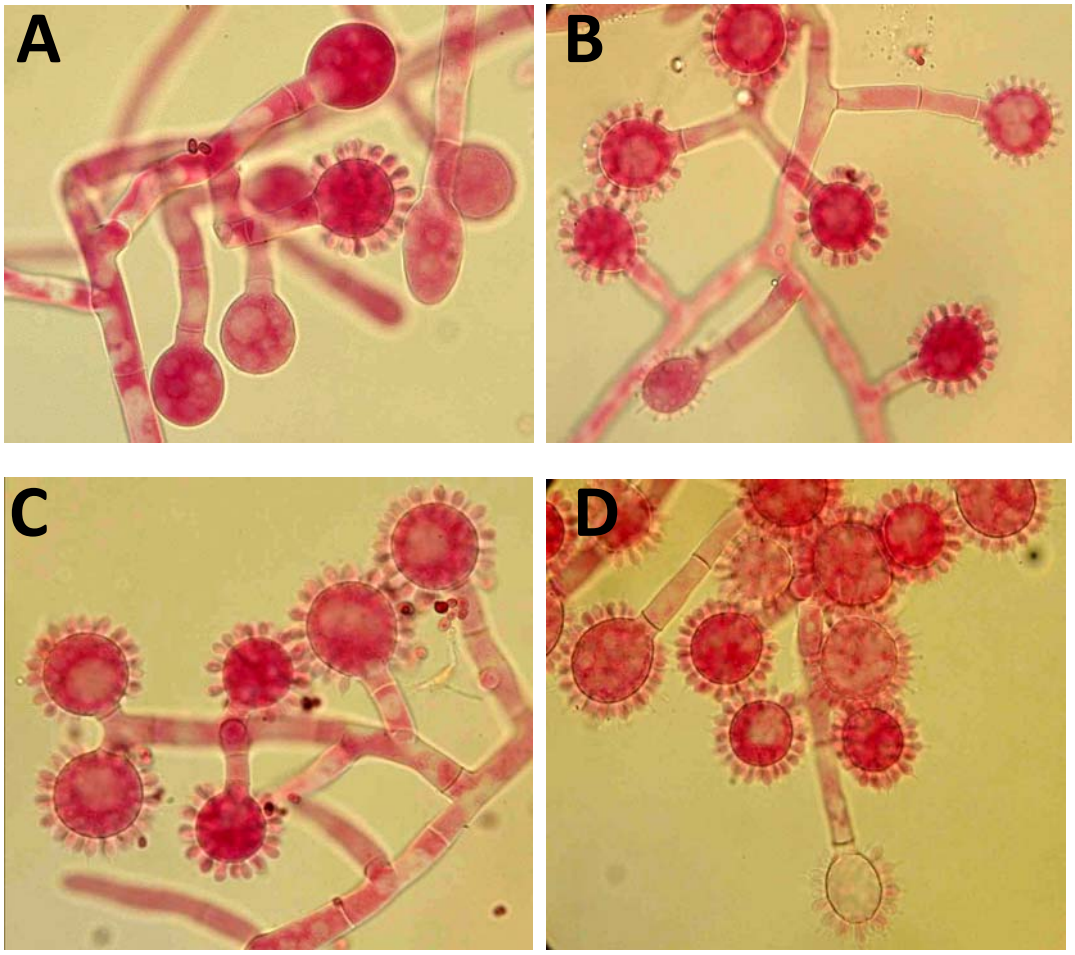

Plate 4 Light microscopy of *Escovopsis lentecrescens*. A-D. Details of conidiogenesis resulting in evanescent vesicles (**D**).
Photos: H. C. Evans and J. O. Augustin

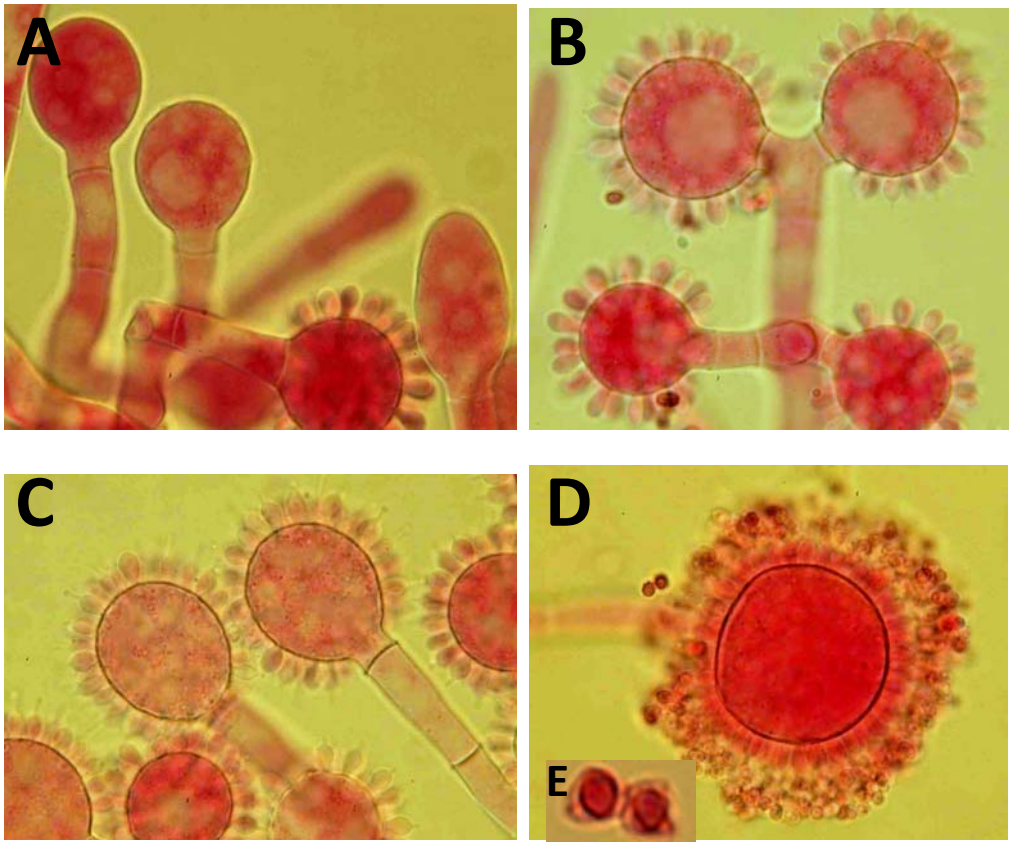

Plate 5 Light microscopy of *Escovopsis lentecrescens*. A-D. Paratype from attine ant midden, with slightly faster growth pattern; **E.** Conidia showing the elaborate coat or veil structure.
Photos: H. C. Evans and J. O. Augustin

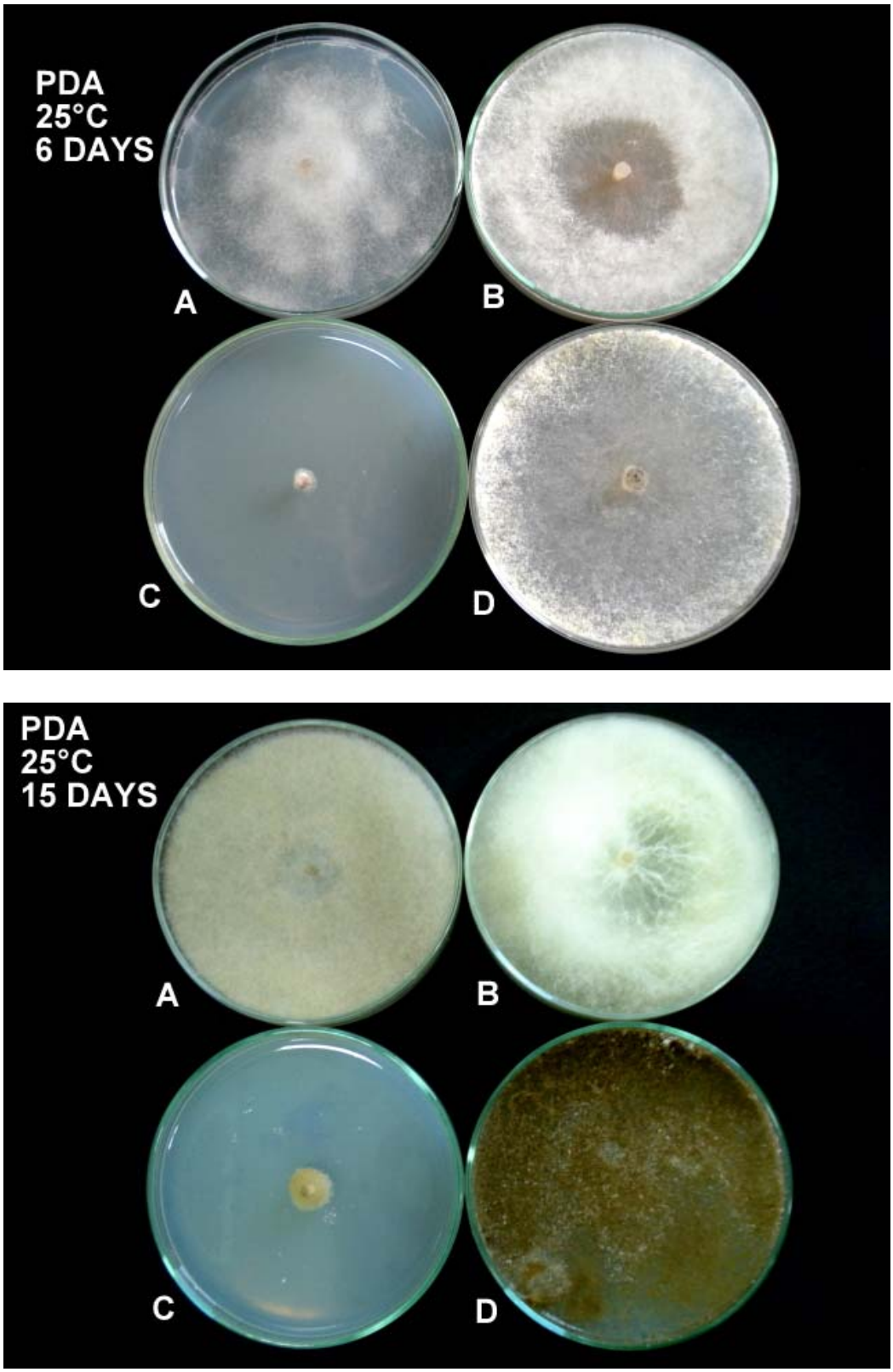

Plate 6 *Escovopsis* species grown on PDA (Potato Dextrose Agar) on 25°C for 6 days (top) and 15 days (bottom). **(A)** *E. moelleri*, **(B)** *E. niveo-chlamydosporiformans*, **(C)** *E. lentecrescens* and **(D)** *E. microsporum*. Photos: J. O. Augustin

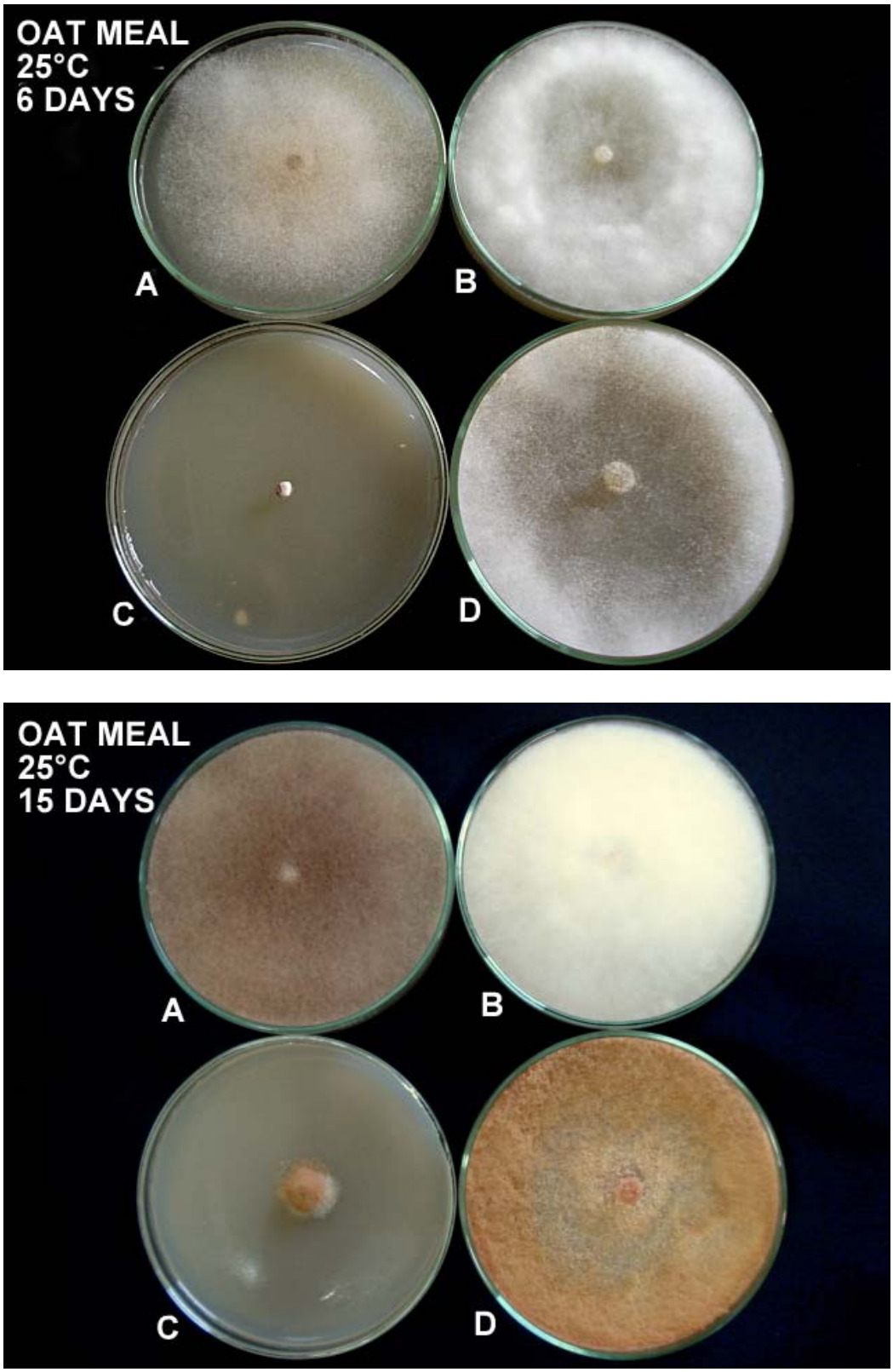

Plate 7 *Escovopsis* species grown on Oat Meal on 25°C for 6 days (top) and 15 days (bottom). **(A)** *E. moelleri,* **(B)** *E. niveo-chlamydosporiformans*, **(C)** *E. lentecrescens* and **(D)** *E. microsporum*. Photos: J. O. Augustin

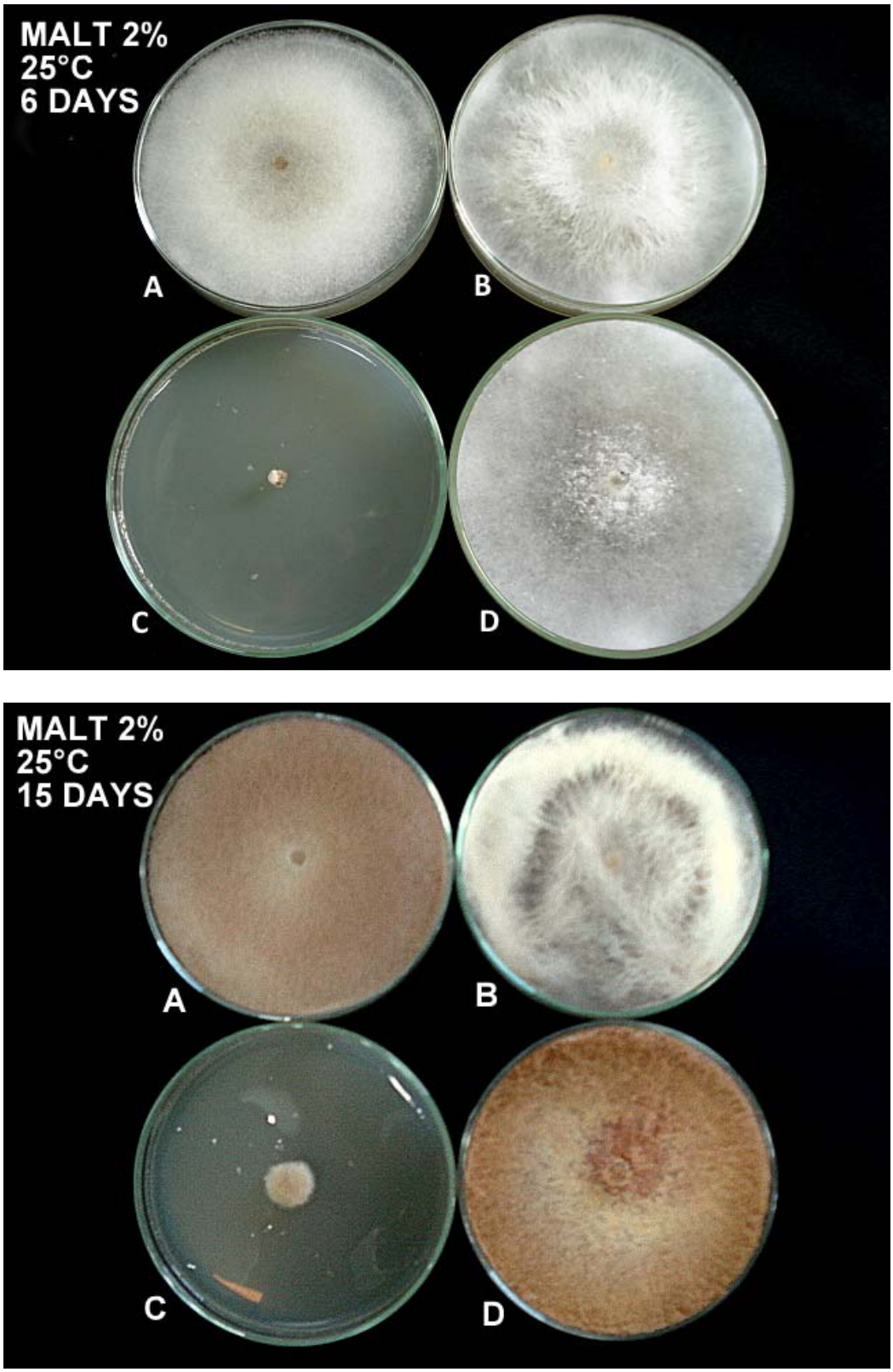

Plate 8 *Escovopsis* species grown on Malt Extract 2% on 25°C for 6 days (top) and 15 days (bottom). **(A)** *E. moelleri*, **(B)** *E. niveo-chlamydosporiformans*, **(C)** *E. lentecrescens* and **(D)** *E. microsporum*.
Photos: J. O. Augustin

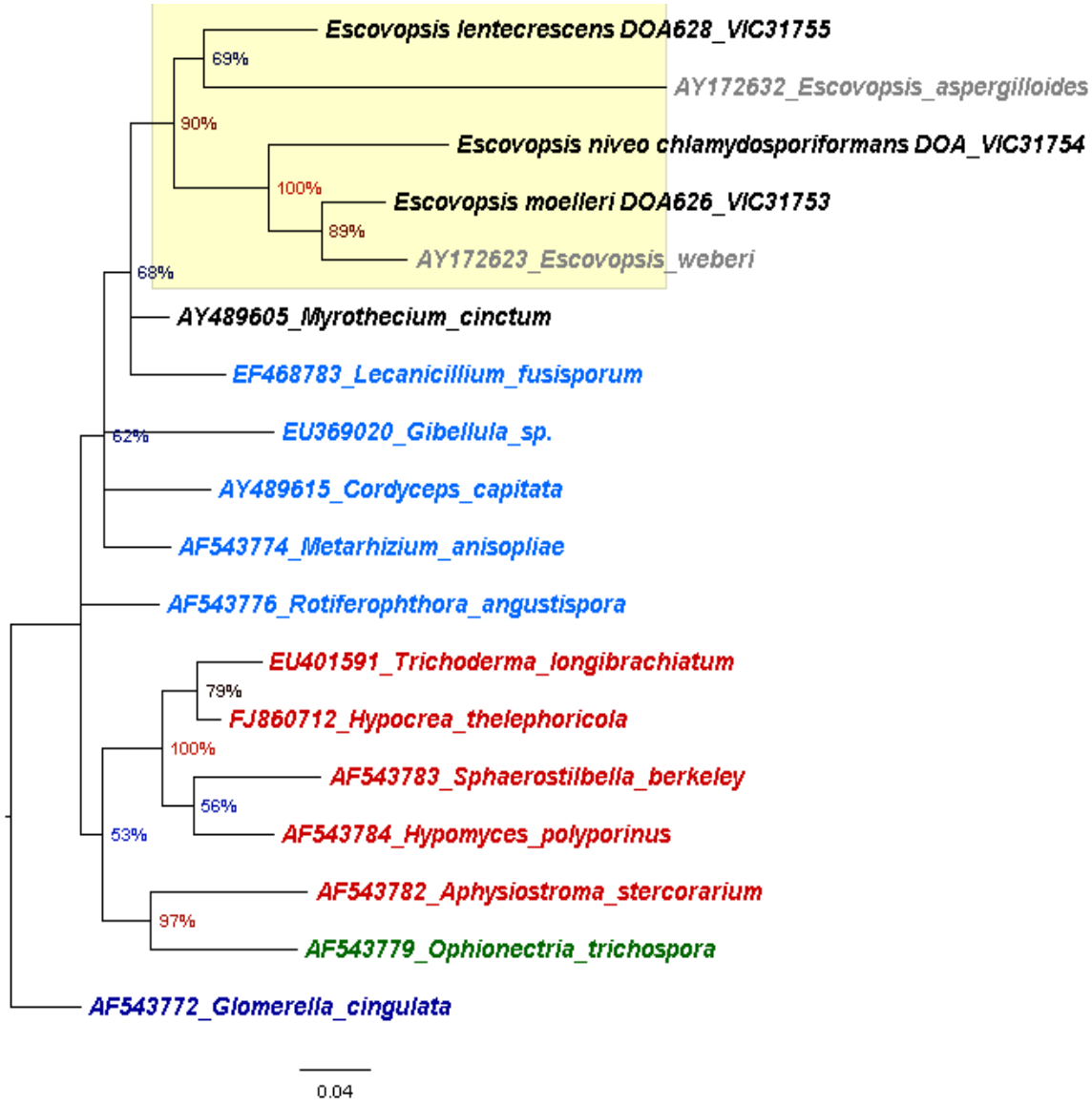

Figure 1 Phylogeny for 5 strains of *Escovopsis* ant garden parasites and 12 ascomycetous fungal outgroups based on 462 base pairs of DNA sequence data from EF-1 alpha. The *Escovopsis* clade is yellow highlighted. Grey taxa in the clade ascribe sequences derived from Genbank, which were included for the purpose of filogenetically situates the new *Escovopsis* species. This Bayesian consensus tree is topologically identical to trees obtained from maximum parsimony (MP) and maximum likelihood (ML) analyses. Numbers at tree nodes are bootstrap support values obtained from Bayesian analyses encompassing 5 million markov chain Monte Carlo generations (GTR+Γ+I model). Color taxa indicate representatives from three hypocrealean families: Nectriaceae (green), Hypocreaceae (red), and Clavicipitaceae (light blue), giving a total of 17 hypocrealean taxa.

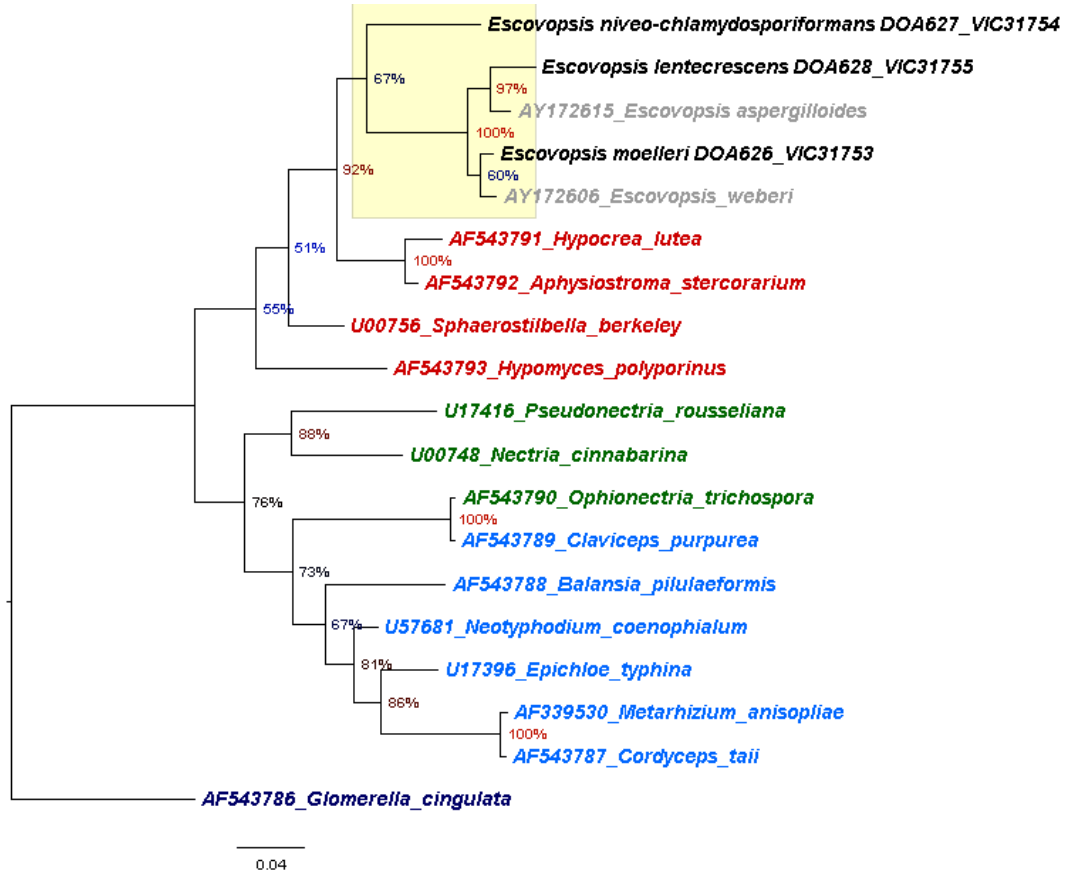

Figure 2 Phylogeny for 5 strains of *Escovopsis* ant garden parasites and 12 ascomycetous fungal outgroups based on 528 base pairs of DNA sequence data from LSU. The *Escovopsis* clade is yellow highlighted. Grey taxa in the clade ascribe sequences derived from Genbank, which were included for the purpose of filogenetically situates the new *Escovopsis* species. This Bayesian consensus tree is topologically identical to trees obtained from maximum parsimony (MP) and maximum likelihood (ML) analyses. Numbers at tree nodes are bootstrap support values obtained from Bayesian analyses encompassing 5 million markov chain Monte Carlo generations (GTR+Γ+I model). Color taxa indicate representatives from three hypocrealean families: Nectriaceae (green), Hypocreaceae (red), and Clavicipitaceae (light blue), giving a total of 19 hypocrealean taxa.

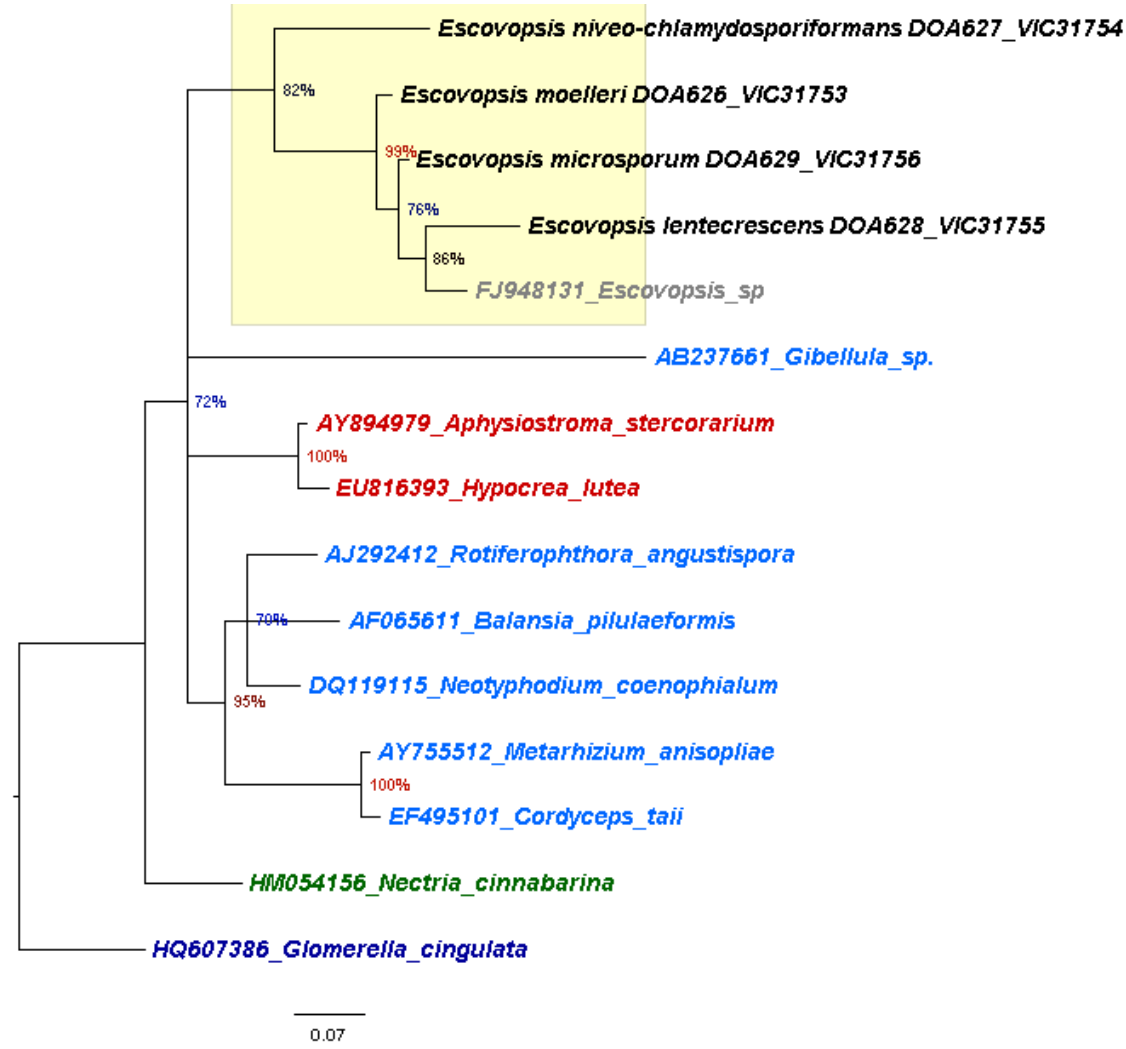

Figure 3 Phylogeny for 5 strains of *Escovopsis* ant garden parasites and 12 ascomycetous fungal outgroups based on 356 base pairs of DNA sequence data from ITS. The *Escovopsis* clade is yellow highlighted. Grey taxa in the clade ascribe sequences derived from Genbank, which were included for the purpose of filogenetically situates the new *Escovopsis* species. This Bayesian consensus tree is topologically identical to trees obtained from maximum parsimony (MP) and maximum likelihood (ML) analyses. Numbers at tree nodes are bootstrap support values obtained from Bayesian analyses encompassing 5 million markov chain Monte Carlo generations (GTR+Γ+I model). Color taxa indicate representatives from three hypocrealean families: Nectriaceae (green), Hypocreaceae (red), and Clavicipitaceae (light blue), giving a total of 15 hypocrealean taxa.

CHAPTER 3

EVIDENCE FOR CRYPTIC HORIZONTAL TRANSMISSION OF *ESCOVOPSIS*, A FUNGAL PARASITE OF ATTINI COLONIES

Abstract: A remarkable example of symbiosis is the attine ant-microbe system, an emerging model system for studying parasitism in insect societies due to its multiple interactions between different coevolving living organisms. An antagonistic partner within this symbiosis is the specific mycoparasite *Escovopsis* (Ascomycota: Hypocreales), a microfungus that inhabits healthy attine colonies throughout their geographic range. *Escovopsis* is present in the diverse cultured fungal lineages (Leucocoprineae and Pterulaceae) of attine ants and it can easily be isolated from dead and even healthy colonies. It has been a puzzle, however, how *Escovopsis* reaches its host. Apparently it is not vertically transmitted nor is there evidence for horizontal transmission. Here we report evidence for horizontal transmission of *Escovopsis*. We found it sporulating continuously during 15 weeks of sampling on midden material deposited outside 30 nests entrances by workers of *Acromyrmex* colonies in remnant Atlantic Rainforest in Brazil. Two new *Escovopsis* species were found and are currently been described. Our discovery is evidence for the dispersal mechanism by which the specialized mycoparasite *Escovopsis*, in natural conditions, may reach its host. This is because sporulating consistently may favor rain, wind or an invertebrate vector to transmit the mycoparasite from infected colonies to uninfected ones.

Key words: Leaf-cutting ants, disease transmission, midden piles, fungal inoculum, Attini.

INTRODUCTION

Fungus-growing ant-microbe symbioses have emerged as a model system for the study of parasitism in complex insect societies. In these systems, attine ants tend the fungus (Basidiomycota, Agaricales: Pterulaceae and Leucocoprinae), providing it with optimal conditions for growth, while the fungus has become the ants' main food source, on which larvae and the queen feed exclusively. This obligate mutualism is exploited by a fungal parasite of the genus *Escovopsis* (Ascomycota: Hypocreales), one of the non-mutualistic fungi most frequently isolated from attine nests (Currie et al 1999a; Rodrigues et al 2005a) [Chapter 2, this thesis]. Despite of a steady flow of high-profile publications on the biology, ecology and evolution of *Escovopsis* (Currie et al 1999b; Currie 2001; Currie and Stuart 2001; Gerardo et al 2004; Gerardo et al 2006a, b; Gerardo and Caldera 2007; Kost et al 2007; Taerum et al 2007; Mueller et al 2008; Schultz and Brady 2008; Caldera et al 2009; Taerum et al 2010), there is still no evidence on how this fungus reaches its host.

Infected colonies can transmit a pathogen to adult colonies (horizontal transmission) and/or descendent colonies when these reproduce (vertical transmission). The mechanisms of these two routes of transmission diverge and can ultimately dictate selection upon virulence of a pathogen. Models for explaining the evolution of virulence integrate ideas from theoretical evolutionary ecology and epidemiological studies (Ewald 1987; Bull et al 1991; Galvani 2003). This suggests that the term virulence is a result of complex evolutionary, ecological and epidemiological interactions. However, if the mode of transmission is to be considered the only factor acting on virulence evolution, theory predicts that pathogens that are transmitted vertically should evolve reduced virulence, relative to those horizontally transmitted, because vertically transmitted pathogens depend on host survival and reproduction to reach the next generation (Ewald 1987; Bull et al 1991). The current lack of knowledge of *Escovopsis*' mode of transmission is one

of the biggest gaps of this system, since it limits our understanding of the ecological and evolutionary dynamics of the attine ant-microbe symbiosis.

Although there is still no evidence for horizontal transmission of *Escovopsis*, a possible mechanism is that ants accidentally pick up fungal spores while foraging, or that another arthropod, for example mites that frequently inhabit the colonies, could potentially move between colonies and take *Escovopsis* spores with them. This hypothesis is supported by the fact that empirical data demonstrates that young newly-founding *Atta* colonies do not harbor *Escovopsis*, suggesting that vertical transmission of the mycoparasite does not occur, thus implying horizontal transmission (Pagnocca et al 2008). However, the horizontal transmission hypothesis predicts either some phoretic capability of the mycoparasite, which would then move horizontally and/or vertically between nests. Dispersing individuals, such as reproductive founding females, may carry *Escovopsis* spores either on their cuticle, or within their fungal pellets, allowing the parasite to be vertically transmitted or at least the capability to use wind or rain to disperse. These possibilities hypothetically require a minimal sporulating activity of the microfungus within the symbiosis, either inside or outside nests.

The occurrence of sporulating *Escovopsis* has previously been documented on external waste piles of a mature (~5 year old) *Atta colombica* colony in Panama (Hart 2002), followed by colony emigration. This is the only published evidence so far that *Escovopsis* is able to reach the outside of an attine colony, in natural conditions. However, this observation was limited to a single colony under unusual circumstances.

Aiming at elucidation the mode of transmission of *Escovopsis*, we evaluated the natural infection rates of midden piles produced by 30 sympatric *Acromyrmex subterraneus subterraneus* colonies in Brazil.

MATERIALS AND METHODS

We identified and marked 30 *A*. *subterraneus subterraneus* colonies in remnant, fragments of secondary forest (UTM: 23K 0722867 7698209) in the southeastern region of the Zona da Mata Mineira, State of Minas Gerais, Brazil. Colonies were located along a 246m walking trail and separated 12.55 ± 8.43m (SEM) from each other. Colonies were then inspected weekly from late February to early June 2011 for the presence of *Escovopsis* on the midden piles.

Midden samples (~1mL) were brought to the laboratory, and examined using an Olympus RX51 microscope with MicroPublisher 3.3 RTV Q imaging camera. During field inspections, we observed conspicuous cotton-like white mycelium covering the top of several midden piles. After evaluating these samples microscopically, they were characterized as blooming *Escovopsis*. Microscopy also revealed that even without blooming, it was still possible to detect sporulating *Escovopsis* on the midden samples. This was characterized as cryptic.

RESULTS

Escovopsis was found in all (n = 115) midden samples. The number of blooming *Escovopsis* increased with time, as did rainfall (data not shown).

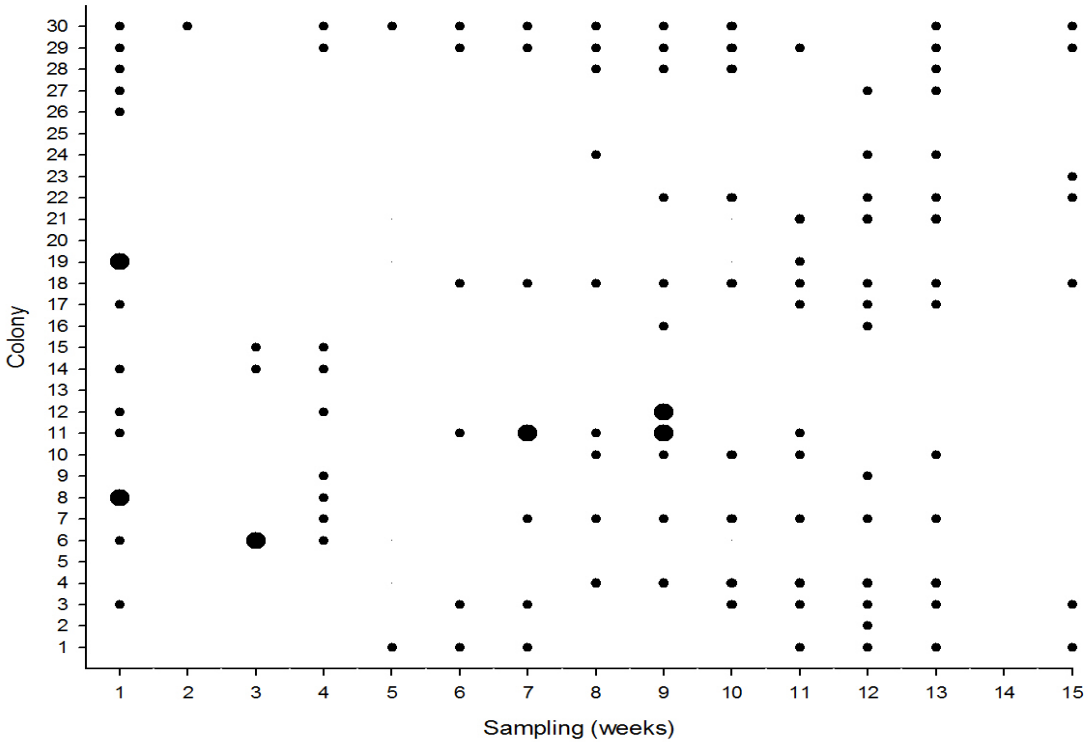

Figure 1 Frequency of midden piles (n= 115) produced by 30 field colonies of *Acromyrmex subterraneus subterraneus* for a 15 week period, comprising the period from late February to late June 2011. Small dots represent midden samples from which we observed *Escovopsis* sporulating cryptically (cryptic *Escovopsis*). Large dots represent midden samples from which we observed *Escovopsis* sporulating conspicuously (blooming *Escovopsis*).

DISCUSSION

This is the first evidence for a consistent presence of *Escovopsis* outside underground attine nests. The evidence that the mycoparasite is able to sporulate outside nests strongly indicates that *Escovopsis* relies on horizontal transmission to reach its host. The horizontal mode of transmission in this system could occur through several routes.

First, rain, almost a daily phenomenon in the area in that period of time, as well as wind, may favor *Escovopsis* propagules to be dispersed. Also, the proximity

of nests (mean distance between nest entrances = 12 ± 1 m) located along the single common trail where the colonies were distributed may explain the fact that all middens sampled harbored sporulating *Escovopsis*, whether cryptic or blooming. It is possible that the 30 *Acromyrmex* colonies were not all taking *Escovopsis* infected midden particles out of their underground nest chambers. However, such consistency of *Escovopsis*-infected middens probably means that all colonies got infected by the propagules of the mycoparasite that sporulated in a few nearby infected colonies.

Secondly, sporulating outside nests probably facilitates vectoring by foraging ants, mites or other invertebrates that frequently inhabit attine colonies. They could accidently pick up *Escovopsis* propagules from a midden pile and inadvertently transfer them to another midden pile or even bring them to the underground colony, thus contributing to the horizontal transmission of the mycoparasite.

Our results indicate that *Escovopsis* may be naturally favored to bloom in response to suitable humidity. Indeed, this blooming response to humidity occurred within only four days after ants took the very first midden fragments out. This observation is based on additional sampling that was done but not shown here. Also, blooming *Escovopsis* occurred when cumulative precipitation data was over 61.2mm. Below this value, only cryptic *Escovopsis* were found.

Our discovery reveals the persistent presence - in time and number of colonies - of *Escovopsis* in naturally produced midden piles. From late February to early June, all middens had either blooming (5.22%, n=6) or cryptic (94.78%, n=109) *Escovopsis*, comprising two new *Escovopsis* morphotypes. These fall into either the aspergilloides group (Chapter 2, pages 65, 66) or the new niveo-chlamydosporiformans group (Chapter 2, page 63).

The occurrence of blooming *Escovopsis* on a waste pile has previously been documented for a mature (~5 year old) *Atta colombica* colony in Panama (Hart 2002), followed by colony emigration. Compared to that particular *Atta colombica* colony, the *Acromyrmex* colonies we observed were much smaller (estimated at

109.46 ± 8.79 cm^3 of nest entrance mound), producing small midden piles (3.04 ± 1.17 cm^3).

Thirdly, horizontal transmission of *Escovopsis* is further supported by the fact that the two *Escovopsis* morphotypes we found on the middens produced dormant spores. Attempts were made to induce spore germination on agar, but we were unable to get the two morphotypes in pure culture. We concluded those were dormant spores, unable to germinate in the absence of its host, the ant's fungus garden. Once within the fungus garden and – as we hypothesize - under specific environmental and/or intracolonial conditions, *Escovopsis* may then germinate.

Our observation is not only evidence for the dispersal mechanism by which the specialized mycoparasite *Escovopsis*, in natural conditions, may reach its host but also unveil a possible avenue for investigating the crucial yet quite unknown aspect of the nature of virulence of this pathogen. In order to explore this, it is important to know how *Escovopsis* gets into the colony, because the mode of transmission is predicted to be one of the factors that will ultimately influence virulence evolution of a pathogen (Ewald 1987; Bull et al 1991; Galvani 2003). In this context, virulence is theoretically expected to decrease in vertically transmitted pathogens, because they need their hosts to survive in order to reach to the next host's generation (Lipsitch et al 1995; Ewald 1996; Frank 1996). Although our results do not refute vertical transmission of *Escovopsis*, it is strong evidence that the mycoparasite relies on horizontal transmission to reach its host. It remains to be elucidated whether *Escovopsis* relies solely or not on horizontal transmission. We are currently investigating the possibility of vertical transmission of *Escovopsis* among attine colonies.

The main conclusions we can draw from this study are that *Escovopsis* spp. were able to sporulate naturally on middens of *A. subterraneus subterraneus* colonies; sporulation were mostly inconspicuous (cryptic sporulation); conspicuous sporulation (blooming) occurred rarely and in response to precipitation; although it remains to be elucidated if *Escovopsis* is coming out of the underground nests, it is

safe to conclude that it infects middens consistently in time and number of colonies; and sporulating outside nests can strongly favor horizontal transmission of *Escovopsis*.

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CHAPTER 4

THE NATURE OF VIRULENCE OF *ESCOVOPSIS*, A PARASITE IN THE ATTINE-ANT MICROBE SYMBIOSIS

Abstract: Leaf-cutting ants (Hymenoptera: Formicidae: Attini) depend almost entirely on their fungus garden (Lepiotaceae) for food. This fungus garden hosts the fungal parasite Escovopsis, which is only known from attine fungus gardens. It is commonly assumed that Escovopsis is highly virulent to its host. Nevertheless, empirical evidence for this is misleading. A common symbiont in the fungus-ant system, Escovopsis can indeed destroy the fungus garden because it consumes the hyphal body of the ant's cultivar, being a potential threat to the colony. However, the frequency with which Escovopsis is found in healthy laboratory and field attine colonies suggests that this mycoparasite is not a highly virulent pathogen to its host. The aim of the present study was to evaluate the impact of Escovopsis in laboratory colonies of Atta sexdens rubropilosa. We used spore suspension of E. niveo-chlamydosporiformans and E. microsporum as treatments; water and spore suspension of Metarhizium sp. were used as negative and positive controls. Suspensions of up to 3.6 x 10^{10} spores of Escovopsis were sprayed directly on top of fungus gardens. We found that the Escovopsis treatments did not cause significant negative impact on the colonies. In contrast, the most dramatic responses were observed in the Metarhizium-treated colonies, which had their fungus garden volumes severely reduced and colonies died after a 2 month period post spraying. All of the E. niveo-chlamydosporiformans survived up to a 8 month-period. Our results represent empirical evidence for a non-virulent nature of Escovopsis, in stark contrast to the widespread assumption of a highly virulent parasite within the attine ant-microorganism symbiosis.

Key-words: Attini, mutualism, *Metarhizium*, symbiosis, parasitism, *Atta*

INTRODUCTION

Parasites are a common and constant threat to organisms at all levels of organization (Thrall et al 2007). In fact, parasites are considered a shaping force in the evolution of almost every organism, with effects not only on host population size (Price et al 1986) but even the maintenance of sexual reproduction (Hamilton 1980). Parasites have these inherent effects due to their virulence, defined as the harm they can cause on their hosts (Frank 1996). Theory of evolution of virulence suggests that natural selection may favor a range of virulences, depending on the mode of parasite transmission (Ewald 1987), with mostly horizontally transmitted parasites tending to higher levels of virulence, while mostly vertically transmitted parasites tending toward decreased virulence(Bull et al. 1991; Jensen et al. 2006).

Leaf-cutting ants (Hymenoptera: Formicidae: Attini) depend almost entirely on their Lepiotaceae fungus garden (Schultz and Brady 2008) for food (Quinlan and Cherrett 1979; Bass and Cherrett 1995, 1996; Silva et al 2003). This fungal cultivar is host of the specialized fungal pathogen *Escovopsis* (Seifert et al 1995). It has become a common assumption that *Escovopsis* is highly virulent to its host (Currie et al 1999a,b; Currie 2001a, b; Currie and Stuart 2001; Currie et al 2003; Little et al 2003, 2006; Reynolds and Currie 2004; Currie et al 2006; Gerardo et al 2006; Little and Currie 2007; Caldera et al 2009; Fernández-Marín et al 2009; Haeder et al 2009; Hölldobler and Wilson 2009; Taerum et al 2010). Although a number of studies reveal its prevalence among the wide range of non-mutualist fungi in the attine ant symbioses (Currie et al 1999b; Gerardo et al 2004; Rodrigues et al 2005a; J. O. Augustin personal observation), there are currently no data to support the general belief that *Escovopsis* is virulent to its host.

Since Currie's publication in 1999, it has generally been assumed that *Escovopsis* is a highly virulent fungus for symbiont fungus gardens of attine ants. It has been previously demonstrated, however, that *Escovopsis* is a common inhabitant of attine fungus gardens (Currie et al 1999a; Gerardo et al 2004; Rodrigues et al 2005a) (J. O. Augustin, personal observation), occurring very

frequently in different parts of healthy fungus gardens (Rodrigues et al 2005b; Rodrigues et al 2008; J. O. Augustin, personal observation). Despite this, colonies still forage well and grow ergonomically (J. O. Augustin, personal observation). This is an important observation because it indicates that the ants manage to overcome the potential negative effects of *Escovopsis* which in turn strongly suggests that this is not such a virulent parasite.

We hypothesize that *Escovopsis* is a common inhabitant of healthy attine fungus gardens because it is not a virulent antagonist in the symbiosis. The aim of the present study was to test this hypothesis. Because numerous previous assays suggested that whole healthy colonies could support greater amounts of spores of *Escovopsis* without significant negative impact, we tested our hypothesis by means of spraying an high dosage ($6.5x10^{12}$ spores per colony in the case of *E. microsporum*) of the parasite on fungus gardens of healthy young (9 months old and approx.. 180mL of fungus garden) *Atta sexdens rubropilosa* colonies.

MATERIALS AND METHODS

Collection and maintenance of ant colonies

Wingless newly-mated reproductive females of *Atta sexdens rubropilosa* queens were collected on 14th October 2010 immediately after their nuptial flight in an open field area surrounded by a secondary forest in the Zona da Mata Mineira, Brazil. They were collected and deposited in individual plastic pots (250 mL) before being brought back to the laboratory. Each pot contained a 1 cm layer of dampened plaster in its base to keep humidity inside the chambers, which were watered every other day. During the colonies' founding stage, laboratory conditions were 25 ± 2°C, 75 ± 3% R.H. and constant darkness. When the first adult workers emerged in the colonies, leaf fragments of *Acalypha wilkesiana* (Euphorbiaceae) were collected daily in the UFV campus and offered as foraging substrate to the ants.

All ant colonies used in this experiment were nine months old and queen-right, each with its own claustral founding queen. Their fungus gardens were allowed to grow up to approximately 180 mL (estimations as a cylinder varied from 85 to 233 mL; 178.42 ± 12.31), so as to make an attempt to maximize any effects of the spore suspensions on such small colonies. These were randomly assigned to groups of four, with one colony from each block randomly receiving one out of four spraying treatments: *E. microsporum*, *E. niveo-chlamydosporiformans, Metarhizium* sp. or water (control).

Sampling for *Escovopsis*

As attine ant colonies may be infected by multiple *Escovopsis* strains (Taerum et al 2010; Chapter 2 this thesis), sampling of nonmutualistic fungi was conducted in order to verify the presence of *Escovopsis* spp. in the colonies. So prior to the experiment, we took 10 small pieces (~5 mm^3) of the fungus gardens from throughout the gardens, comprising top and bottom, and placed them individually on nutrient agar under aseptic conditions. We used Potato Carrot Agar (PCA) (Tuite 1969), with antibiotic (either rifamycin or chloramphenicol). The pieces were monitored daily for the growth of *Escovopsis* spp., which were then isolated into pure culture.

In vitro assay

Simultaneously to spraying whole colonies with the treatments mentioned, we also performed an *in vitro* experiment in order to check the response of subcolonies towards alien fungi suspension under an artificial condition, in which a colony was fragmented into a subcolony. Subcolonies of attine ants consist of the symbiont fungus garden and worker ants. We used spare colonies for this, not the ones we used in the assay. Because a subcolony is deprived of its functional queen and therefore may respond differently from a queen-right colony (whole colony), we did this *in vitro* experiment so as to compare the responses of each colony towards the treatments. The *in vitro* experiment consisted of 24 small subcolonies

(~5 cm^3 of fungus garden with 3 ants each, or no ant at all, three repetitions each) placed in a 9 cm Petri dish fitted with wet sterile filter paper so as to assure humidity inside Petri dishes. Each group of six subcolonies (three with ants and three without) received a particular spray of spore suspension as follows: Treatment 1: 1 mL of $9.2x10^3$ spores of *E. microsporum*; Treatment 2: 1 mL of $7x10^4$ spores of *E. niveo-chlamydosporiformans*; Treatment 3 (control): 1 mL of $1x10^2$ spores of *Metarhizium*; Treatment 4 (blank): 1 mL of sterile distilled water and nonionic detergent Tween 80® 0.01%. Each of these treatments was blind.

Inoculating colonies with *Escovopsis* spp.

The fungal isolates used in the assay were: *E. microsporum* (DOA629-VIC31756), *E. niveo-chlamydosporiformans* (DOA627-VIC31754) and *Metarhizium* sp. (AUJ11). We used a *Metarhizium* isolate in the assay so as to get baseline data for comparison to a known entomopathogenic fungus. This isolate was not *M. anisopliae*, but an undescribed *Metarhizium*, which was isolated from a sympatric attine colony (see below).

All isolates used in this experiment represented alien fungi in the *Atta* colonies, because they were not isolated from them, but rather from sympatric non-*Atta* colonies. The *E. microsporum* strain was isolated from the fungus garden of an *Acromyrmex subterraneus molestans* colony, and the *E. niveo-chlamydosporiformans* strain was isolated from the fungus garden of an *A. subterraneus subterraneus* colony. The *Metarhizium* sp. strain used as the experimental entomopathogen was isolated from a *Trachymyrmex* sp. colony (J.O. Augustin, H.C. Evans and S. L. Elliot, unpublished data).

The isolates were allowed to grow in agar for about 10 days prior to the experiment. The two *Escovopsis* isolates were grown on organic Pagnocca media A (Pagnocca et al 1990) supplemented with 45g/L flaked oats (Granum, Contagem, MG, Brazil). The *Metarhizium* isolate was grown on PCA (Potato Carrot Agar) media

(Tuite 1969) added by 400mg of Cu/mL. These culture media were used because they allow for massive spore production (J. O. Augustin, personal observation).

During the experiment, laboratory conditions were 22.93 ± 0.29°C, 64.06 ± 0.92% R.H. and L8:D16 photoperiod. Colonies were inoculated by spraying the spore suspensions directly on top of the fungus gardens. Because previous assays suggested that whole healthy colonies can support great amounts of spores of *Escovopsis* without perishing, we used the maximum quantity of *Escovopsis* conidia we could obtain for each isolate. This allowed for an approximately 10 times more *Escovopsis* conidia per mL of fungus garden than what the literature prelude (Currie 1999b). This was not the case with the *Metarhizium* treatment, which followed approximately the amount of the literature for individual leaf-cutting ants (Hughes et al 2002; Hughes and Boomsma 2004). Colonies were sprayed with $11.5x10^{3}$ - $6.5x10^{12}$ spores in 10 mL of water on the top of the fungus gardens. Each spore suspension of *Escovopsis* represented the highest quantities of spores we could prepare and we decided to use those high *Escovopsis* spore suspensions since we aimed at quantifying negative effects arising from that intervention. Therefore, the spore concentrations of each suspension were different.

Because *Escovopsis* can produce dormant conidia, we tested the viability of each spore suspensions so as to evaluate if we were indeed spraying viable conidia on the ant colonies. This test followed methodology described in Alves (1998) and was performed by preparing ten-fold dilution series of each spore suspensions. Aliquots of 1 mL were deposited on Agar-Water culture media and incubated for 3-5 days at 25°C. After that we counted the number of spores that germinated under a stereomicroscope and then the number of viable conidia was determined. Three plates of each fungus species were used as replicates for this test. Viability was estimated as 95-100% for *E. niveo-chlamydosporiformans*, 80-83% *E. microsporum* and 80-100% *Metarhizium* sp.

Colony responses to treatments

Based on pilot tests we determined the most practical biological and behavioral parameters that best served to monitor the responses of the colonies to each treatment. Therefore, for each colony, the biological variables evaluated were changes in fungus garden volume, number of workers carrying midden fragments, number of foragers processing vegetal substrate in the foraging arena, number of leaf fragments in garden chamber, number of workers performing broodcare behaviors and the cumulative number of dead workers found in the midden piles on each day. Data collection began seven days prior to inoculation, when the colony responses were evaluated daily on a four hour interval, to obtain baseline data for comparison for each colony. Changes in fungus garden volume were not evaluated daily but rather one week prior to spraying and then every week for five weeks post spraying. Waste material was collected in a 24h interval, using a clean spoon, which was clean with alcohol 70% between sampling.

In order to check for the ability of the ants to remove the spores of the alien fungi that we sprayed, we stored all waste samples in clean plastic pots or 5 cm Petri dishes which were kept in 3°C until they were sampled for micro-symbionts in agar plates, using two types of culture media: Potato Carrot Agar – PCA (Tuite 1969) and PCA media added with 400mg of Cu/mL of media prepared. Sampling for micro-symbionts was done simultaneously to the experiment and five weeks post-spraying. Ten midden fragments (~1mm^3) were sampled for each midden sample.

RESULTS

Sampling for *Escovopsis*

The methodology used for sampling for *Escovopsis* revealed the presence of a single *Escovopsis* species, namely, *E. microsporum*. This was present in all of the tweve *A. sexdens rubropilosa* colonies used in the experiment.

In vitro assay

Sub-colonies with and without workers treated with *E. microsporum* sporulated this microfungi within four and two days, respectively. The *E. niveo-chlamydosporiformans* - treated subcolonies with and without workers sporulated this microfungi within seven and one day, respectively (Table 2).

Table 2: Mean time (days) it took *Escovopsis microsporum* and *E. niveo-chlamydosporiformans* to sporulate in fungus garden fragments - with and without ants - of colonies of *Atta sexdens rubropilosa* (shown are means of three subcolonies, followed by standard errors).

Treatment	**Fungus garden fragments**	
	With workers	No workers
E. microsporum	4.333 ± 0.666	2.000 ± 0.577
E. niveo-chlamydosporiformans	7.000 ± 1.155	1.333 ± 0.333

Colony responses to treatments

Compared with the previous 72 hour pre-spray period, the *Escovopsis* treated-colonies suffered a behavioral change following the 72 hour post-spray period. However, these changes reflect no apparent negative effect on the growth and health of the *A. sexdens rubropilosa* colonies.

Workers engaged in licking the top of the garden surfaces within approx. 10 minutes after the suspensions were sprayed, whether these were water or spore suspensions (data not shown). After the first 15 minute post-spraying, the biological parameters were measured. The number of leaf-fragments in garden chambers were recorded to be greater at approx. 15 hours post-spraying (Figure 1 A), when the ants engaged more frequently in processing leaf material inside garden chambers as well (data not shown), even though the number of workers on foraging arena apparently did not increase significantly, apart from one colony that was treated with *E. niveo-chlamydosporiformans* (Figure 2 A). In contrast, the number of leaf fragments in the fungus garden chamber apparently did not change

significantly in colonies treated with *Metarhizium* sp., apart from a single colony (Figure 1 A).

The number of workers performing midden tasks increased for colonies treated with water, *E. microsporum* and apparently more significantly *Metarhizium* sp.; treatment with *E. niveo-chlamydosporiformans*, on the other hand, did not seem to influence in the number of workers performing engaged in midden work (Figure 1 B).

The number of dead ants as a cumulative function was greatest for colonies treated with *E. microsporum* and *Metarhizium* sp. Similarly, one colony treated with water also had a great number of their workers died gradually over time. Contrary to this trend, treatment with *E. niveo-chlamydosporiformans* did not seem to trigger any increase in the number of dead ants found in midden piles, together with other two water treated colonies (Figure 3 A).

Apart from a single water-treated colony, all colonies lost fungus garden volume immediately after the first week of treatment (Figure 3 B). This trend continued until the second week post-spray for all treated colonies, apart from those sprayed with *Metarhizium*. These lost fungus gardens volumes following the five week period post-spraying. Indeed, colonies treated with water, *E. microsporum* and *E. niveo-chlamydosporiformans* recovered fungal garden volume after three weeks post-treatment.

Midden sampling in agar plates revealed that ants were not effective in removing spores of *E. microsporum* nor *E. niveo-chlamydosporiformans*. This is because the presence of each *Escovopsis* isolate could only be detected in a very small number of midden fragments sampled (3-12 out of 1,350 midden pieces sampled daily by the end of 8 weeks post-treatment). Comparatively, removing *Metarhizium* sp. spores seemed to be more effective (Table 1).

Table 1: The presence (total number of midden pieces infected) of *E. microsporum*, *E. niveo-chlamydosporiformans* and *Metarhizium* sp. in midden material during in an eight week post treatment period. The values in parenthesis are percentages of colonies infected. Numbers in brackets shows the mean time post-treatment (days) followed by standard errors it took *E. microsporum*, *E. niveo-chlamydosporiformans* and *Metarhizium* sp. to emerge from midden samples in agar plates.

Treatment	*E. microsporum*	*E. niveo-chlamydosporiformans*	*Metarhizium* sp.
	Presence	Presence	Presence
E. microsporum	11 (100)[1.625±0.321]	0	0
E. niveo-chlamydosporiformans	3 (100)[3.742±0.735]	7 (100)[5.782±3.984]	0
Metarhizium sp.	5 (100)[13.648±3.837]	0	97 (100)[1.906±0.732]
Control	12 (100)[5.937±0.902]	0	0

Finally, the life span of the colonies appeared to be rather different among treatments. Seven weeks post-spraying, the queens in two of the *Metarhizium* sp. treated colonies died, while the one that survived seems healthy because the fungus garden filled up completely the 200 mL plastic chamber and the forager workers were foraging well. One of the water-treated colonies lost its fungus garden completely by the end of the tenth week post-spraying, while its queen and offspring remained alive; the other two water-treated colonies remained healthy (based on the fungus garden volume and foraging activity as described above). This was also the case of the colonies treated with *E. microsporum*: six weeks post-treatment the queen died in one colony and the colony as a whole began to decline soon after that. The other two *E. microsporum*, however, remained healthy. None of the *E. niveo-chlamydosporiformans* –treated colonies died and they remained healthy 32 weeks post-treatment.

In the case of subcolonies with ants treated with *Metarhizium*, the fungus *Syncephalastrum* sp. sporulated within 13 days while from those without workers, the fungus *Syncephalastrum* sporulated within 9 days. As regards the ants themselves from these colonies, they died and *Metarhizium* from them after 15 days post-spraying. The water-treated colonies with ants all sporulated *Syncephalastrum* within 10 days while the subcolonies with no ants sporulated *Syncephalastrum* within 3 days.

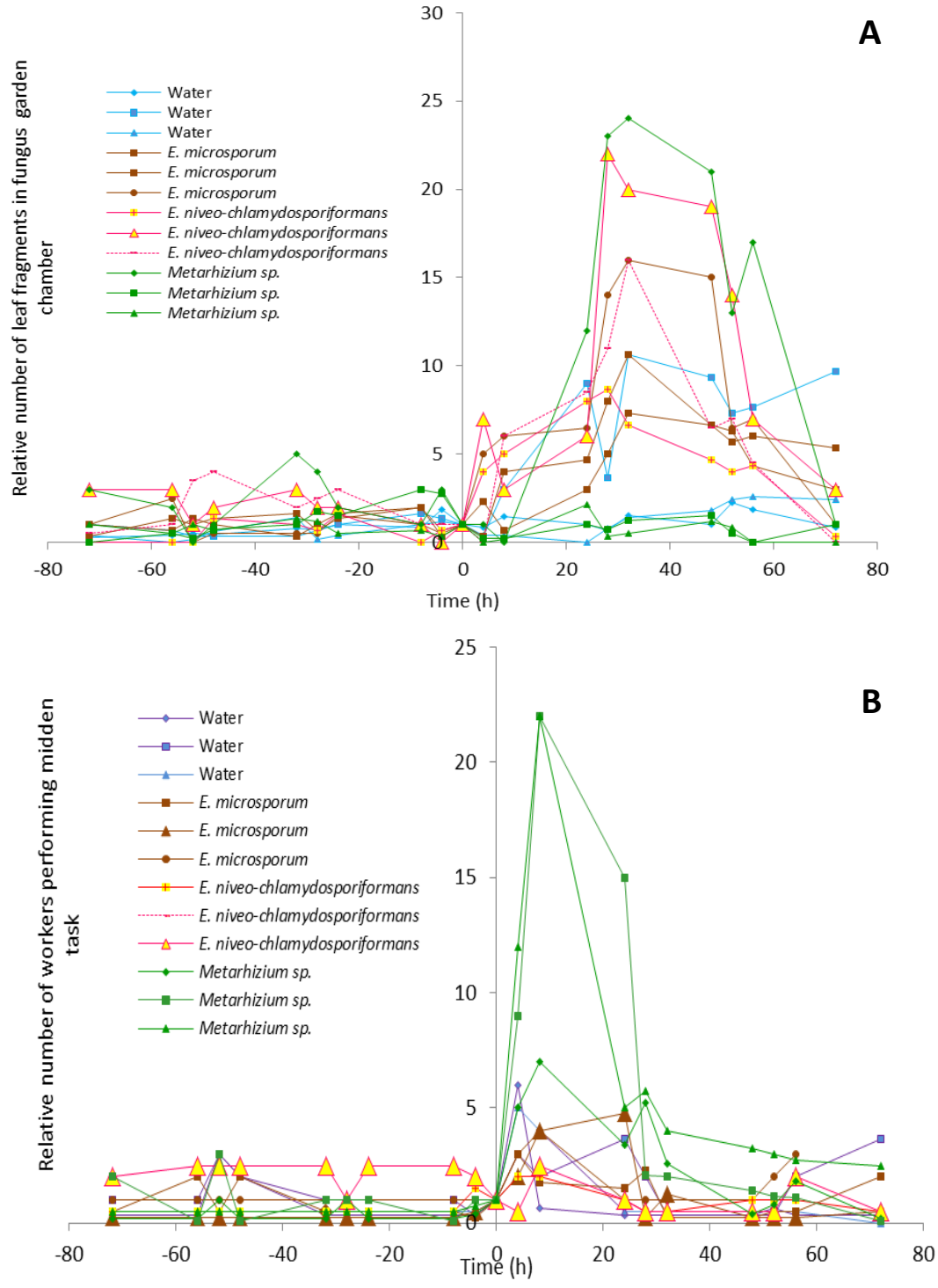

Figure 1: **Colony behavioral responses over the 72 h following treatment with water, *Escovopsis microsporum*, *E. niveo-chlamydosporiformans* and *Metarhizium* sp. in young colonies of *Atta sexdens rubropilosa*. A**. Relative number of leaf fragments in fungus garden chamber; **B**. Relative number of workers performing midden task. Interception between X and Y axes indicates Day 1 of treatments.

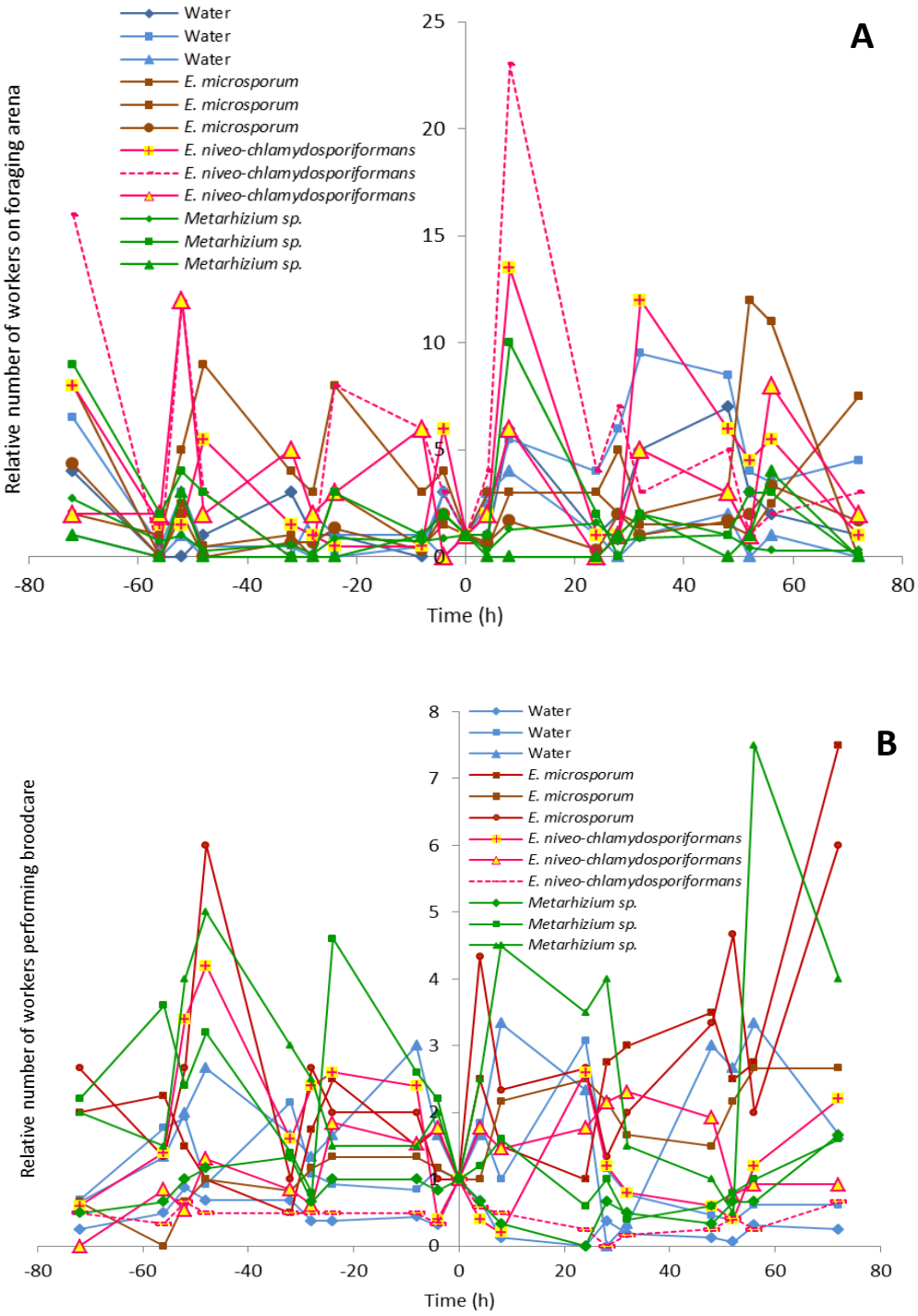

Figure 2: **Colony behavioral responses over the 72 h following treatment with water, *Escovopsis microsporum*, *E. niveo-chlamydosporiformans* and *Metarhizium* sp. in young colonies of *Atta sexdens rubropilosa*. A.** Relative number of workers processing leaf material on foraging arena; **B.** Relative number of workers performing broodcare. Interception between X and Y axes indicates Day 1 of treatments.

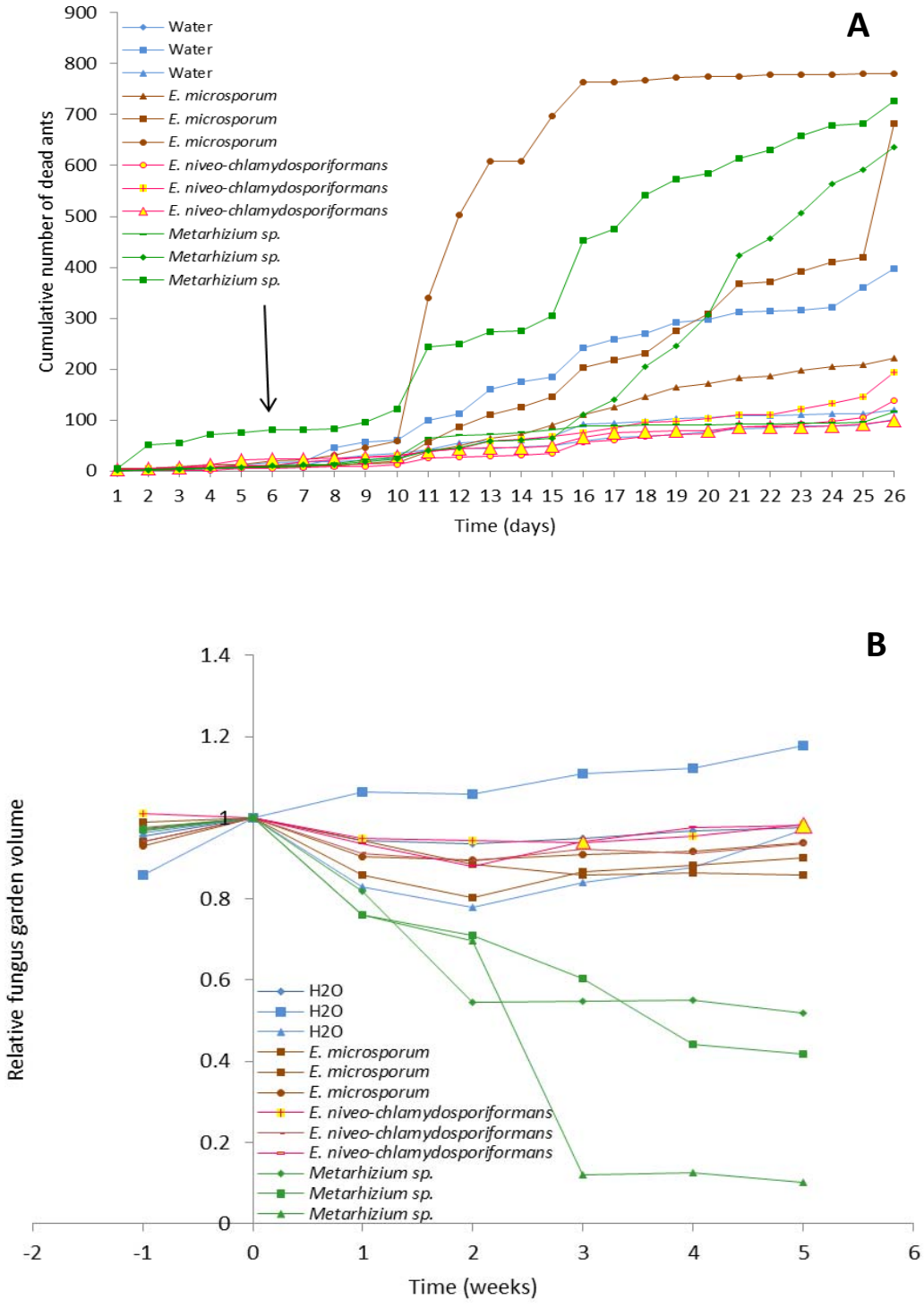

Figure 3: **Colony responses following treatment with water, *Escovopsis microsporum*, *E. niveo-chlamydosporiformans* and *Metarhizium* sp. in young colonies of *Atta sexdens rubropilosa*. A.** Cumulative number of dead ants counted daily from midden piles. Arrow indicates Day 1 of treatment; **B.** Relative fungus garden volume. Interception between X and Y axes indicates Day 1 of treatments.

DISCUSSION

Our study demonstrates that *Escovopsis* does not cause negative impact on whole healthy colonies of *A. sexdens rubropilosa*. Despite being artificially subjected to massive amounts of viable spores, it was only the *Metarhizium*-treated colonies had their fungus garden biomass reduced with subsequent death of the colonies within a two month post treatment period. These results do not corroborate with the literature (Currie 2001a).

The results of the *in vitro* assay showed that *Escovopsis* was able to sporulate on fungus garden fragments with and without *A. sexdens rubropilosa* workers. However, sporulation occurred more quickly in the fragments deprived of workers. This information together with the results of the experiment on whole colonies support the argument that the specialized mycoparasite *Escovopsis* is unable to overcome or at least have some difficulty to do so in gardens of whole healthy colonies of *A. sexdens rubropilosa*. We hypothesize that this is because ants of eusocial colonies can defend themselves against pathogens not only physiologically, through numerous glands with antibiotic properties (Poulsen et al 2002; Fernández-Marín et al 2006), but also behaviorally, through hygienic and prophylactic behaviors (Hart and Ratnieks 2001; Fernández-Marín et al 2007; Augustin et al 2011), and, in the particular case of the fungus-growing ants, also symbiotically, through the association with mutualistic bacteria that secrete antibiotics with potent antagonistic properties against micro-organisms, including *Escovopsis* (Currie et al 2006; Kost et al 2007; Sen et al 2009; Cafaro et al 2010).

The behavioral response relative to foraging activity changed post-treatment in the *Escovopsis*-treated colonies. The marked increase in the number of leaf fragments inside garden chamber within 50 hours post-spraying for the treatments with *E. microsporum* and *E. niveo-chlamydosporiformans* probably indicates a mechanism to overcome potential negative effects caused by the massive presence of spores of the mycoparasites. Interestingly, this increase was not shared for the control and *Metarhizium* treatments, apart from a single each *Metarhizium* and

water treated colony. We speculate that the more vegetal is incorporated in the garden matrix the more diluted would the potential negative effects of parasitism by *Escovopsis* be.

Treating colonies with *E. microsporum* and *Metarhizium* sp. caused a sharp increase in the number of dead ants counted daily from midden piles. We observed that the colonies that showed the greater number of dead workers was the ones treated with *E. microsporum* which also engaged more in foraging (as for the number of leaf fragments in garden chamber) and midden work. It seems that the ants would try to get rid of *Escovopsis* by incorporating more leaf fragments into the fungus garden and removing infected garden pieces, which on the other hand would cause a significant stress to the colonies, resulting in death to the workers. Even if workers died more with this treatment, it was not enough to let the fungus garden to shrink. It seems that the *E. microsporum*-treated colonies overcame the artificially massive introduction of spores of the alien fungi. This is also evidence for a non-virulent nature of *Escovopsis*.

The *Metarhizium*-treated colonies, on the other hand, did not invest in foraging behavior but rather in midden work, apart from being the group of colonies which also registered the greater number in dead ants. Indeed, the most negative effect on the colonies was caused by *Metarhizium* sp. and not by *Escovopsis* spp. It seems that workers from these treated colonies engaged more (and also more effectively, as Table 6 shows) than the others in taking midden fragments out of their garden chambers particularly with the 24h post spraying but their effort were not enough to avoid a sharp decrease in fungus garden volume over the 5 week post spraying.

Importantly, none of the colonies sporulated *Escovopsis* spp. in their respective fungus gardens while they were alive. This is evidence that the massive spore suspensions sprayed directly on the fungus gardens were unable to overcome the defense strategies of the colonies.

Compared to the *Metarhizium*-treated colonies, we show here that consistent negative effects on the *Escovopsis*-treated colonies were not detected, even with high dosages of two *Escovopsis* species. This is sharply divergent from the prevailing assumption of high virulence for *Escovopsis latu senso*. Indeed, none of the *E. niveo-chlamydosporiformans* treated colonies died after 7 months post spraying, and the single *E. microsporum* treated colony had its queen die before colony decline, indicating that the mycoparasite was not the cause of colony death.

We expected workers to successfully remove the inoculated *Escovopsis* spores from their colonies, however, as the re-isolation of the mycoparasite from midden piles shows, this did not occur. Only *Metarhizium* sp. was re-isolated frequently in culture media. This indicates that the workers were more effective in removing *Metarhizium* than *Escovopsis* spores. A similar result has been recorded for *A. colombica* colonies, which were found to eliminate *Trichoderma* sp. more effectively than *Escovopsis* sp. (Currie and Stuart 2001). However, in contrast to Currie and Stuart (2001), who suggested that the specialized mycoparasite has probably evolved counter-adaptations in order to overcome the defense barriers of attine ants, we argue that *Escovopsis* simply does not impose a great threat to *A. sexdens rubropilosa* colonies, so that workers do not need to employ great effort to remove it, at least compared to *Metarhizium*.

The first empirical data on the impacts of *Escovopsis* on attine colonies was performed by Currie (2001a), who used incipient 10-12 weeks old *A. colombica* founding colonies. We argue that it is already expected that any disturbance in an ant's colony during their founding process will inevitably cause negative effects on them. That is because the colony founding state of any ant species, particularly those which start up their colonies claustrally, which is the case of all *Atta* species, is the most vulnerable stage of an ant's life cycle (Autuori 1942; Hölldobler and Wilson 1990; Augustin 2007). That is to say that the negative impact on colony growth found by the author (Currie 2001a) was not probably caused by *Escovopsis* itself but rather by the intrinsic vulnerability of the colonies themselves. Our results are then

the first empirical evidence for a non-virulent nature of the specific mycoparasite of the attine fungus gardens and do not corroborate with previous assumptions of high virulence of *Escovopsis* prevailing in the literature (Currie et al 1999a,b; Currie 2001a, b; Currie and Stuart 2001; Currie et al 2003; Little et al 2003, 2006; Reynolds and Currie 2004; Currie et al 2006; Gerardo et al 2006; Little and Currie 2007; Caldera et al 2009; Haeder et al 2009; Fernández-Marín et al 2009; Taerum et al 2010). In our work we used colonies of 9-10 months old, when they exhibited visible signs by healthy ergonomic colonial growth despite being young in age and small in size.

It would be interesting to repeat this experiment with older colonies, at their full mature stage of colony development, as a comparative study. Likewise, future avenues for research include extending the presently described experiment in laboratory and field conditions, using representatives of the lower as well as the higher attine ants.

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CONCLUSION AND PERSPECTIVES

The attine-ant microbe symbiosis is a complex system and we are yet to elucidate the ecological role of each microorganism within the symbiosis. Based on the present study we conclude that leaf-cutter colonies can house more than 40 different taxa of microfungi, not to mention the numerous yeasts, bacteria and actinomycetes whose function within the colony are as yet unclear. One hypothesis is that these microorganims may be only temporary or transient symbionts without establishing specific roles in the system. Alternatively, if one considers that the fungus garden biomass is a potential source of nutrients for microorganisms in general, then these may be antagonists of the ants' mutualist fungus, either parasitizing it – which seems to be the case of the prevalent *Syncephalastrum* sp. (J. O. Augustin, H. C. Evans and S. L. Elliot unpublished data) – or competing for vegetal substrate in the gardens. Future work should elucidate this.

Escovopsis is an example of a mycoparasite within the symbiosis. Each new species of *Escovopsis* described in this thesis seems to have a distinct biology, according to their particular morphological structures and behaviors in agar plates. For example, it is possible that *E. moelleri* has some phoretic ability, because of the thickened rugose walls and cap-like structures of its conidia. We are presently trying to obtain SEM images of this species, in order to understand what these ornamentations are. Although ornamentation may contribute to dispersal of *Escovopsis*, there is another biological aspect of the mycoparasite that suggests capability of dispersion in the environment. This species, together with *E. microsporum* and *E. lentecrescens*, form melanized dormant conidia, which presumably may help them keep their viability in the environment, outside attine nests, until they are picked up by a potential vector that could move between colonies. We have field data that corroborates this hypothesis: the two *Escovopsis* morphotypes that were found to sporulate naturally and consistently in external midden piles of *Acromyrmex* colonies produced dormant spores. Despite numerous

attempts to make pure culture media of those morphotypes, their spores never germinated.

Even with evidence suggesting a reliance on passive dispersion of *Escovopsis* conidia (rain and wind may disperse the dormant *Escovopsis* propagules in field conditions), only experimental manipulation will tell us if these *Escovopsis* morphotypes can indeed rely on phoretic ability to reach its host, the attine ant fungus gardens. What is clear however is that colonies of *A. subterraneus subterraneus* are consistently infected by *Escovopsis* in a natural condition.

The ever-white *E. niveo-chlamydosporiformans*, on the other hand, forms three different types of propagules, whose functions are quite puzzling, although it is intriguing that the chlamydospores resemble gongylidia of the ants' mutualist fungus. We hypothesize that this species mimics gongylidia chemically and physically, which would favour its persistence within the colony and potentially lead foraging ants to pick it up and bring it to the colony. This too will need to be explored with carefull experimentation.

Escovopsis lentecrescens was distinctive in that it grew on agar plates much more slowly than the other *Escovopsis* species described here. We hypothesize that this reflects a greater degree of specialization on the host fungus, and potentially a longer evolutionary association of this mycoparasite within the symbiosis. The fact that it grows so slowly in the absence of its host indicates that this species may be more dependent on its host for growth than the other species of *Escovopsis* described here.

Despite numerous attempts, we were unable to induce the formation of the teleomorph of *Escovopsis*. It is possible that *Escovopsis* lost its sexual cycle/stage after it entered the attine-ant symbiosis, but this is unlikely given that parasites rely on genetic variation to keep up with their hosts over evolutionary time scales.

The fungal order Hypocreales has a broad host range and diverse ecology that is unique within the Ascomycota. It includes more than 500 species of arthropod-pathogenic fungi, more than any other order of Kingdom Fungi (Sung et

al 2007; Spatafora et al 2007). Major families and ecologies of Hypocreales include Hypocreaceae, with most species being parasites of fungi; Nectriaceae and Bionectriaceae, which comprise numerous plant pathogens and saprobes of woody and herbaceous plants; and Clavicipitaceae, which comprises a diverse assemblage of fungi characterized by symbioses and associations with other eukaryotes including pathogens of animals and parasites of other fungi, and plants. As such, the evolutionary history of Hypocreales has molecularly been shown to be characterized by a shift in nutritional mode from plant-based nutrition (Bionectriaceae and Nectriaceae) to animal and fungal-based nutrition (Hypocreaceae and Clavicipitaceae s.l.) (Spatafora et al 2007). Although *Escovopsis* sp. was not included in the analyses performed by Spatafora et al. (2007), this result suggests that *Escovopsis* spp. took this evolutionary path, shifting from a plant host association - either pathogens of plants, decomposers of plant debris or endophytes -to a fungal-based nutrition. Both lifestyles are reliant on plants or vegetal material as an immediate food source.

Whether *Escovopsis* was/is an endophyte we do not know. Much (not to say all) of the biology of this fungus still needs to be worked out. What we know is that this fungus has only been isolated from fungus-growing ant colonies so far. *Escovopsis* is a necrotrophic mycoparasite (Reynolds and Currie 2004) and apparently does not produce spores within the garden matrix unless it has completely overwhelmed the colony (J. O. Augustin, personal observation). This is to say that *Escovopsis* virulence could vary depending not only on the life stage and health of an attine colony but also on environmental factors, such as temperature or humidity. Colonies eventually overwhelmed with *Escovopsis* in the field (Currie 2001; J. O. Augustin, personal observation) and laboratory (Muchovej & Della Lucia 1990) may have already been in decline for other reasons, giving *Escovopsis* the chance to overtake the fungus garden.

Within the gardens of *Atta sexdens rubropilosa* as well as *Ac. subterraneus subterraneus*, *Escovopsis* is most abundant in the bottom (older) portion of the

gardens (J. O. Augustin, personal observation), as previously documented for other leaf-cutter species (Currie 2001). *Escovopsis moelleri*, *E. niveo-chlamydosporiformans* and *E. microsporum* readily grow (~1.5 cm/day) and sporulate in laboratory culture media at 15, 20, 25 and 30°C on PDA, PCA, Oat meal and Malt Extract 2%, apart from *E. lentecrescens* – which grows comparatively slowly (~0.5 cm/day) in those conditions (J. O. Augustin, personal observation).

Previous work indicates the phylogenetic proximity between 17 strains of *Escovopsis* to Hypocreaceae (Currie et al 2003). As noted above, the common ancestor of Hypocreaceae corresponds to a departure from plant-based nutrition to one that specializes on fungi (Spatafora et al., 2007). This could likely be the case of the specialized mycoparasite *Escovopsis*, which could have found, in the course of evolution, no better place to settle than the ant-fungus symbioses. *Escovopsis* could have entered the ant-fungus symbioses simply by chance, because, hypothetically, it was originally an endophyte and found a suitable host to exploit, the leucocoprineaceous fungi of the leaf-cutters. If this is true, then this window of opportunity that *Escovopsis* encountered for host shifting occurred together with the origin of ant agriculture, approximately 50 million years ago, a period of global warming in which an extraordinary diversity of plants with tropical affinities occurred at middle and high latitudes in South America (Shultz and Brady 2008). How *Escovopsis* reached the lower attine symbiosis seems to be another puzzle. Also, it is also possible that the teleomorph of *Escovopsis* is out there, disguised in a different name, exhibiting a completely different morphotype.

With respect to the mode of transmission of *Escovopsis*, we conclude it has the potential to be horizontally transmitted among nests. Some of the species appear to have phoretic potential – through conidial ornamentation and the presence of dormant conidia; additionally we have shown that *Escovopsis* is abundant in middens, indicating that environmental conditions, such as rain and air currents may help it to disperse in its natural habitat. Although we now know that *Escovopsis* is able to sporulate consistently outside nests, future work should

elucidate if this microfungus is actually coming out from the underground *Acromyrmex* nests.

A deeper understanding of the life cycle of *Escovopsis* and its basic biology are crucial to help us elucidate the evolutionary history of this mycoparasite, which in turn, may lead us to a better understanding of the complex attine ant-microbe symbiosis.

Future topics for research are:

1. Determining the parasitic nature (or otherwise) of isolates of genera other than *Escovopsis* that we have found in *Acromyrmex* colonies, like *Metarhizium* sp. and *Syncephalastrum* sp. Because these strains were isolated from the fungus gardens of the *Trachymyrmex* sp. and *Ac. subterraneus subterraneus* colonies we sampled, we hypothesize that these may also be specific antagonists - possibly new species - in the fungus garden of these attine ants;
2. Testing for endophytic *Escovopsis*;
3. Studying the biology behind each new species of *Escovopsis* here described, as well as their parasitic nature;
4. Testing for a selective media for isolating *Escovopsis* from attine colonies to assist in further field studies;
5. Testing different methodologies so as to induce the formation of a sexual cycle of *Escovopsis*;
6. Testing a methodology for breaking with the dormancy of *Escovopsis* conidia;
7. Testing the hypothesis that only under stressing colony conditions, such as low temperature (lower than 20°C) or suboptimal relative humidity preferences (below 98% R.U.) (Roces and Kleineidam 2000), will *Escovopsis* be favoured to grow and sporulate on the colonies because it is not a virulent symbiont.

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Thesis appendix

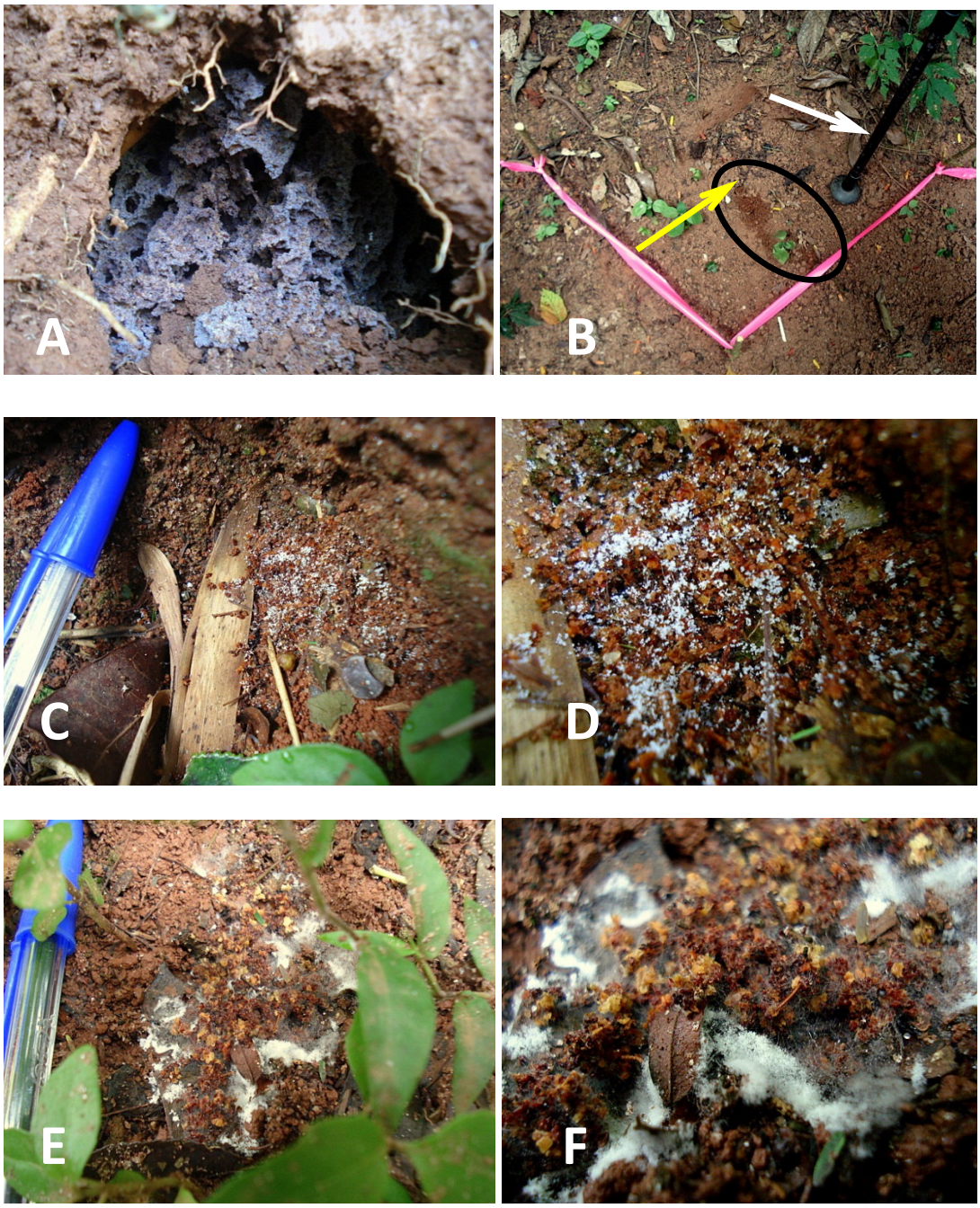

Plate 1 Field site, Mata do Paraíso, Viçosa, Minas Gerais, Brazil. A. Exposed nest of *Acromyrmex subterraneus subterraneous* revealing fungal garden; **B.** Middens or waste piles - characterized by a reddish-brown, yellowish or even blackish colour - from *A. subterraneus subterraneous* (black ellipse) below nest entrance (yellow arrow) on bank above (white arrow shows base of trekking pole, for scale reference); **C.** and **E.** *Escovopsis* blooming on midden piles 3-4 days after the ants brought the first midden particles to the outside of the nest; Details of blooming *Escovopsis* eliciting aspergilloid sporulation type **(D)** and niveo-chlamydosporiforman sporulation type **(F)**.

Photos: J. O. Augustin

Thesis appendix

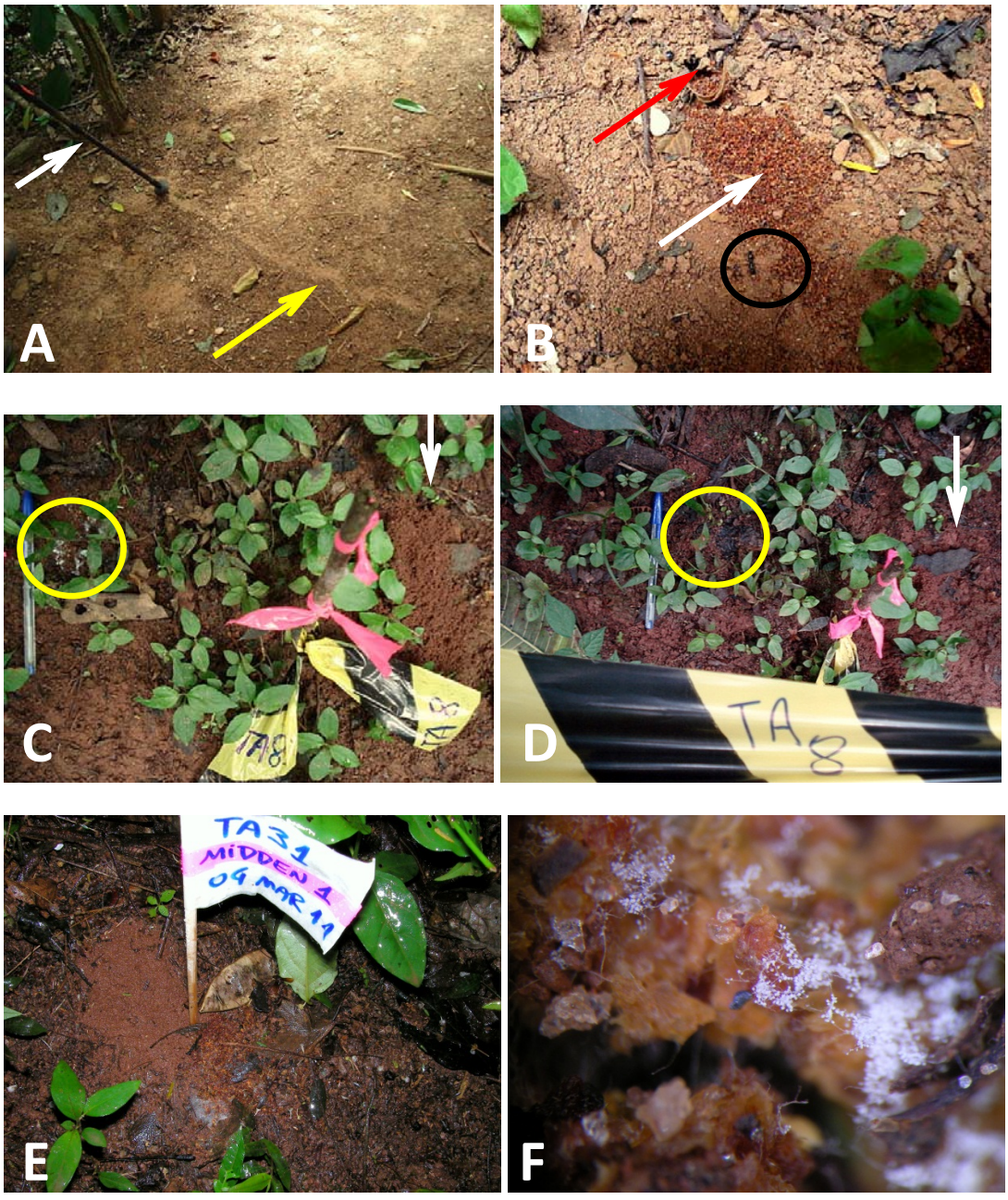

Plate 2 Field site, Mata do Paraíso, Viçosa, Minas Gerais, Brazil. A. An ant's foraging trail (yellow arrow) on the same trail where the 30 *A. subterraneus subterraneous* were located (white arrow shows base of trekking pole, for scale reference). **B.** A reddish-brown midden pile (white arrow) deposited just outside nest entrance (red arrow) and visited by a Poneromorph ant (black circle); **C.** a niveo-chlamydosporiforman type of *Escovopsis* blooming (yellow circle) on a midden pile (white arrow shows nest entrance); **D.** three days after heavy rain we can still find midden fragments (yellow circle) but no sporulating *Escovopsis* (white arrow shows the nest); **E.** Detail of a rain washed midden pile produced by colony TA 31; **F.** Cryptic *Escovopsis* on tiny midden fragments.

Photos: J. O. Augustin (A-D) and H. C. Evans (E-F)