SILMA LEITE ROCHA

INTERACTIONS BETWEEN LEAFCUTTING ANTS, ENDOPHYTIC FUNGI AND PLANTS

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: 28 de fevereiro de 2011.

Prof. Angelo Pallini

Prof. Eraldo Rodrigues de Lima

Pesq. Harold C. Evans

Pesq. Tathiana Guerra Sobrinho

Prof. Simon Luke Elliot
(Orientador)

Aos meus pais, dedico...

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BIOGRAFIA

SILMA LEITE ROCHA, filha de Ademar Rocha Xavier e Silma Leite Rocha Xavier, nasceu em Montes Claros, estado de Minas Gerais, Brasil em 22 de novembro de 1980. Em fevereiro de 2000 ingressou no curso de graduação em Agronomia, na Universidade Federal de Minas Gerais, graduando-se em janeiro de 2005. Em março de 2005 ingressou no Programa de Mestrado em Entomologia na Universidade Federal de Viçosa defendendo a dissertação em fevereiro de 2007. Em seguida, ingressou no Doutorado em Entomologia na mesma instituição defendendo tese em fevereiro de 2011.

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RESUMO

ROCHA, Silma Leite. D.Sc., Universidade Federal de Viçosa, fevereiro de 2011. **Interações entre formigas cortadeias, fungos endofíticos e plantas.** Orientador: Simon Luke Elliot. Coorientadores: Olinto Liparini Pereira, Robert Weingart Barreto e Terezinha Maria Castro Della Lucia.

O objetivo deste trabalho foi estudar a interação entre dois pares de organismos simbióticos e mutualistas: as plantas florestais (neste caso, Mata Atlântica) e fungos endofíticos por um lado, e as formigas cortadeiras e seus fungos simbiontes no outro. Neste contexto, a primeira parte desta tese é uma análise e síntese de interações (conhecida ou presumida) entre o mutualismo planta-endófito e o mutualismo formiga-fungo. Na segunda parte, verificou-se a presença de fungos endofíticos em fragmentos vegetativos que estavam sendo carregados ou que estavam no lixo de *Atta laevigata* em cinco coletas quinzenais. A elevada diversidade fúngica foi encontrada e alguns gêneros de fungos foram encontrados mais ou menos do que o esperado em material rejeitado. Os gêneros *Glomerella*, *Mycelia sterilia* sp. 22, *Pestalotiopsis*, *Phomopsis* sp. 1 e *Xylaria* foram mais prevalentes em fragmentos carregados enquanto que os gêneros *Cylindrocladium*, *Drechslera, Epicoccum, Fusarium*, *Mycelia sterilia* sp. 21, *Trichoderma* e *Ulocladium* foram encontrados mais em fragmentos rejeitado do lixo. A nova descoberta foram os gêneros *Ulocladium* e *Trichoderma* como endofíticos em folhas de plantas tropicais. Discutimos os potenciais riscos desses endofíticos, em particular os micoparasitas *Trichoderma* e *Epicoccum*. Na terceira parte, dez coletas noturnas foram feitas de acordo com o mesmo esquema relatado anteriormente, mas com a formiga *Atta sexdens rubropilosa*. Neste exemplo, *Trichoderma* prevaleceu em material rejeitado, indicando possível rejeição ativa desses fragmentos. Os isolados de *Trichoderma* encontrados foram colocados em cultivos pareados em experimentos *in vitro* com *Leucoagaricus gongylophorus*, fungo mutualista das formigas cortadeiras.

Descobrimos que *L. gongylophorus* inibiu o crescimento de todos os isolados de *Trichoderma*, indicando uma linha potencialmente importante na defesa em colônias de formigas cortadeiras. Também foi investigado o endofitismo de dez isolados de *Trichoderma* em plantas de *Ligustrum japonicum* e de *Eucalyptus grandis*. O endofitismo foi fraco em *L. japonicum*, embora uma observação importante foi feita em função da presença de alguns isolados na gema apical e que não foi encontrado em *E. grandis*, porem o endofitismo foi mais expressivo. Alguns isolados foram re-isolados de folhas acima da área inoculada, indicando o potencial para infecção sistêmica de plantas. Na parte final da tese, um isolado de *Trichoderma* supostamente micoparasita foi oferecido com arroz para mini-colônias sem rainha de *A. sexdens rubropilosa*. Nenhum efeito foi encontrado na atividade de forrageamento, na quantidade de arroz ou folhas forrageadas e a quantidade de lixo produzido. Embora nenhum efeito tenha sido encontrado no tempo de sobrevivência das colônias, houve, no entanto uma redução no tempo de sobrevivência das operárias em colônias infectadas com *Trichoderma*. Para concluir, há perspectivas quanto estudos futuro deste sistema, concentrando-se em particular sobre o uso potencial de *Trichoderma* no controle biológico de formigas cortadeiras.

ABSTRACT

ROCHA, Silma Leite. D.Sc., Universidade Federal de Viçosa, February, 2011. **Interactions between leafcutting ants, endophytic fungi and plants.** Adviser: Simon Luke Elliot. Co-advisers: Olinto Liparini Pereira, Robert Weingart Barreto and Terezinha Maria Castro Della Lucia.

The aim of this research was to study the interaction between two pairs of mutualistic and symbiotic organisms: forest plants (in this case Atlantic forest) and endophytic fungi on the one hand, and leafcutter ants and their fungal symbiont on the other. In this context, the first part of this thesis is a review and synthesis of interactions (known or predicted) between the plant-endophyte mutualism and the ant-fungus mutualism. In the second part, we verified the presence of endophytic fungi in vegetative fragments that were being carried and were in middens of *Atta laevigata* in five fortnightly collections. A high fungal diversity was found and some fungal genera were found more or less than expected in rejected midden material. The genera *Glomerella*, *Mycelia sterilia* sp. 22, *Pestalotiopsis*, *Phomopsis* sp. 1 and *Xylaria* were more prevalent in carried fragments while the genera *Cylindrocladium*, *Drechslera, Epicoccum, Fusarium*, *Mycelia sterilia* sp. 21, *Trichoderma* e *Ulocladium* and *Ulocladium* were found more in rejected fragments from middens. A new finding was the genera *Ulocladium* and *Trichoderma* as endophytes in leaves of tropical plants. We discuss the potential risks of these endophytes, in particular the mycoparasites *Trichoderma* and *Epicoccum*. In the third part, ten nocturnal collections were made according to the same scheme but with the ant *Atta sexdens rubropilosa*. In this instance, *Trichoderma* was found to be more prevalent in rejected midden material than expected, possible indicating active rejection of these fragments. The *Trichoderma* isolates found were paired off in *in vitro* experiments against the ants' mutualist fungus *Leucoagaricus gongylophorus*. We found that *L. gongylophorus* inhibited the growth of

all of the *Trichoderma* isolates, indicating a potentially important line of defence for the colonies. We also investigated the endophytism of ten of these *Trichoderma* isolates in the plants *Ligustrum japonicum* and *Eucalyptus grandis*. Generally, endophytism was weak in *L. japonicum* although an important observation was made of the presence of some isolates in the bud. This was not found in *E. grandis* but in this plant endophytism was more expressive. Importantly, some isolates were re-isoltaed from leaves above the inoculated area, indicating the potential for systemic infection of plants. In the final part of the thesis, an isolate of *Trichoderma* supposed mycoparasite was offered with rice to queenless mini-colonies of the *A. sexdens rubropilosa*. No effects were found of the presence of this fungus on foraging activity, the amount of rice or leaves harvested or the amount of waste produced. Although no effect was found on survival time of the colonies, there were few of these. There was however a reduction in the survival time of the workers from *Trichoderma*-infected. To conclude, perspectives are offered for future study of this system, focussing in particular on the potential use of *Trichoderma* in biological control of leafcutter ants.

General Introduction

Leafcutter ants, belonging the tribe Attini (subfamily Myrmicinae), are among the dominant herbivores in the Neotropics (Chapela et al. 1994), where *Atta* alone cuts between 12 and 17% of leaf production in some forest ecosystems (Cherrett 1968, Farji-Brener 2001). Although they are considered agricultural pests, they play an important role and have a huge impact on natural ecosystems, being considered a keystone species (Fowler et al. 1989). Additionally, leafcutter ants contribute to non-trophic effects on the ecosystem such as enhancing soil nutrients and moisture content at the nest surface (Farji-Brener and Ghermandi 2000, Moutinho et al. 2003), and altering the soil horizon through removal of soil and re-dispersal and concentration of nutrients released from refuse material (Farji-Brener and Medina 2000). They also disperse seeds (Dalling and Wirth 1998) and increase plant diversity at the nest site (Farji-Brener and Ghermandi 2000).

Attine ants farm a fungus with which they maintain an obligatory mutualism that originated some 45 to 65 million years ago (Mueller et al. 2001). The tribe Attini is represented by 13 genera and approximately 210 species (Schultz and Meier 1995). It is subdivided into two groups: higher attines, *Atta* and *Acromyrmex* which predominantly use fresh-cut plant material to grow the fungus (with the exception of *Trachymyrmex* and *Sericomyrmex* that use mainly dead material) and lower attines, that use exclusively dead vegetable matter (Pacheco and Berti Filho 1987). The cultivated fungus can be likened to an external stomach of the leafcutter ants that serves to degrade cellulose, xylan and starch from vegetative fragments, thereby mediating the transfer of carbon to the ants (De Siqueira et al. 1998, Silva et al. 2006a). Thus, the role of the cultivated fungus in the relationship with leafcutter ants is to supply food (Bass and Cherrett 1995) that has a high nutritional value in the form of easily assimilable carbohydrates, together

with high protein content and essential amino acids (Febvay et al. 1984). In addition, the cultivated fungus detoxifies vegetative fragments that are otherwise detrimental to the ants (Dowd 1992) and provides sterols, which the ants use as precursors of moulting hormones and as membrane constituents (Maurer et al. 1992).

In addition to being provided with shelter, and a means of dispersal to new colonies by foundress queens, as well as the vegetative substrate upon which to grow, the fungus receives care from leafcutting ants that involves specialized hygienic behaviour called fungus grooming, and weeding to remove both propagules of invasive microorganisms, as well as infected garden material (Currie and Stuart 2001). Further, waste-management tasks are partitioned to prevent the spread of potentially harmful microbes from the refuse into the garden (Hart and Ratnieks 2001). In addition to these farming strategies, leafcutter ants possess a variety of chemical defenses and secrete compounds with antibiotic properties. There is a specialized thoracic gland (metapleural gland) that has been implicated as a major source of general antibiotic defence substances and which produce antibiotics that prevent germination of spores of harmful fungi in their gardens (Ortius-Lechner et al. 2000). Besides all of these defenses, some species of leafcutter ants permit the growth of actinomycete bacteria on their bodies in order to derive antibiotic compounds from these microorganisms. These substances suppress the growth of specialized garden parasites such as fungi of the genus *Escovopsis* (Currie et al. 1999b, Currie and Stuart 2001).

The interaction between leafcutter ants and plants can be mediated by diverse factors in the plant's armoury, such as morphological defences (e.g., thick cuticle, trichomes, thorns and spines) (Rico-Gray and Oliveira 2007), chemical defences (e.g., toxins, digestibility-reducing compounds) (Heil et al. 2002), biotic defences (e.g., ants and wasps) (Cuatle and Rico-Gray 2003) and, potentially, microorganisms acting as bodyguards, such as endophytic fungi (Elliot et al. 2000).

Endophytic fungi appear to be ubiquitous, having been found in algae (Hawksworth 1988), mosses (Schulz et al. 1993), ferns (Fisher 1996), conifers (Legault et al. 1989) and angiosperms (Clay 1988, Faeth and Hammon 1997, Fröhlich and Hyde 1999, Arnold et al. 2000). Within hosts, fungal endophytes may inhabit all available tissues (Boddy et al. 1987, Chapela and Boddy 1988, Sieber 1989, Clay 1990, Bills and Polishook 1991, Lodge et al. 1996, Lupo et al. 2001, Meyer et al. 2001). The term endophyte is generally taken to describe a microorganism that for some or all of its life cycle lives internally within plant tissues without causing any sign of infection (Wilson 1995). Thus, an endophytic organism can be a pathogenic fungus with an extremely brief endophytic period; a latent pathogen that can have an asymptomatic period; a decomposer which emerges from senescing or decaying tissue; or obligates that are never seen outside their host (Saikkonen et al. 1998). In summary, their interactions with host plants occur along a continuum and range from parasitic to mutualistic (Schulz and Boyle 2005).

Endophytic fungi may belong to two groups: one that is intercellular symbionts that belong to the family Clavicipitaceae, which grow within above-ground tissues of many grasses in the temperate zone. Asexual clavicipitaceous endophytes are transmitted vertically through seeds and grow systemically (Clay and Schardl 2002). Fungi of other group are associated with woody plants (Arnold et al. 2001), which are transmitted horizontally and can benefit hosts by increasing the biomass, competitiveness abilities (Clay 1990, Malinowski et al. 1997, Clay and Holah 1999, Cheplick 2004), and improvements in host plant tolerance to environmental stresses such as drought (Cheplick 2004). Beside this, fungal endophytes can protect their host plants against insects (Clay 1990, Breen 1994, Arnold and Lewis 2005). There is mounting evidence that foliar endophytes can alter insect herbivore consumption, performance, and preference (Clay 1990, Meister et al. 2006, Hartley and Gange 2009).

Although many studies have focused on the role that these endophytic organisms play in increasing host resistance to herbivores (e.g.Clay 1988, Faeth 2002) and pathogens (e.g.Giménez et al. 2007), the view of endophytes as defensive mutualists has mainly stemmed from studies of seed-borne fungal endophytes benefitting their grass hosts as 'acquired plant defences' (Cheplick and Clay 1988). In contrast to this unique and less frequent group of clavicipitaceous endophytes (Cheplick and Clay 1988, Breen 1994), the non-clavicipitaceous endophytic fungi are much more diverse (Arnold et al. 2001, Schulz and Boyle 2005). The non-clavicipitaceous endophytic fungi associated with leaves of angiosperms in tropical forests are thought to represent an order of magnitude higher in diversity (Arnold and Engelbrecht 2007) but are poorly understood in terms of diversity and their interactions with their hosts, as well as with herbivores.

Within this context, the aim of this study was to investigate interactions between leafcutter ants, plants and endophytic fungi. It focuses on the ant species *Atta laevigata* and the subspecies *Atta sexdens rubropilosa*, based in fragments of Atlantic forest.

Chapter One is a multi-author review and synthesis of interactions (known or predicted) between the ant-fungus mutualism and the plant-endophyte mutualism.

In **Chapter Two,** we present initial results aimed at documenting the wider community of endophytic fungi being carried or rejected in leaf fragments. As this field is very new, we have few specific hypotheses, aiming principally to characterize the fungi that could affect and modulate leafcutter attack.

Chapter Three focuses specifically on our discovery of the mycoparasite fungus *Trichoderma* in fragments of leaves being carried by leafcutter ants. The results are directed at the hypothesis that plants use endophytic *Trichoderma* as a bodyguard defence against leafcutter attack.

In **Chapter Four**, the final and more applied part of this work, we propose to study the behaviour of the foraging workers that handle substrate containing

Trichoderma with the objective of assessing the potential of this fungus as a biological control agent of leafcutter ants.

Chapter Five offers a summary of the conclusions of this study and perspectives for future work.

Chapter 1
Symbiotic complexes in opposition: How do endophytic fungi affect leafcutter ant attack?

SIMON L. ELLIOT[1*], HARRY C. EVANS[2], SILMA L. ROCHA[1], OLINTO L. PEREIRA[3], ANDRÉA M. ALMEIDA[4], ROBERT W. BARRETO[3], TEREZINHA M. C. DELLA LUCIA[1], DAVID J. MURRELL[5] AND ADAM G. HART[6]

[1] *Department of Animal Biology, Federal University of Viçosa, Viçosa, Minas Gerais, CEP 36.570-000, Brazil.*

[2] *CAB International, E-UK Centre, Egham, Surrey TW20 9TY, UK*

[3] *Department of Plant Pathology, Federal University of Viçosa, Viçosa, Minas Gerais, CEP 36.570-000, Brazil.*

[4] *Center of Plant Biotechnology, Andrés Bello University, República 217, 8370146, Santiago, Chile.*

[5] *Department of Genetics, Evolution and Environment, University College London, Gower Street, London, WC1E 6BT, UK.*

[6] *Department of Natural and Social Sciences, University of Gloucestershire, Cheltenham, GL52 2AJ, UK.*

* Author for correspondence: selliot@ufv.br

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Running Head: Endophytic fungi and leafcutter ants

Symbiotic complexes in opposition: How do endophytic fungi affect leafcutter ant attack?

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[1] *Department of Animal Biology, Federal University of Viçosa, Viçosa, Minas Gerais, CEP 36.570-000, Brazil.*

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[3] *Department of Plant Pathology, Federal University of Viçosa, Viçosa, Minas Gerais, CEP 36.570-000, Brazil.*

[4] *Center of Plant Biotechnology, Andrés Bello University, República 217, 8370146, Santiago, Chile*

[5] *Department of Genetics, Evolution and Environment, University College London, Gower Street, London, WC1E 6BT, UK*

[6] *Department of Natural and Social Sciences, University of Gloucestershire, Cheltenham, GL52 2AJ, UK*

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Abstract

Leafcutter ants are considered the dominant herbivores in neotropical forest and grassland systems. They sit within a complex web of mutualistic and antagonistic interactions, chief of which is the mutualism with the fungus that comprises their fungus gardens. However, the plants they attack also have mutualistic and antagonistic interactions, notably with endophytic fungi, and it is well known that these fungi can affect insect herbivory and thereby the community based around plants and insect herbivores. Considering the importance of both sets of organisms, in particular their importance as examples of mutualistic interactions, we offer a synthetic review of the two areas. Many of the parameters affected by endophytic fungi, such as hydric status of leaf material and the presence of toxins, are also known to affect leafcutter ant foraging. Meanwhile, there is the potential for endophytic fungi, within leaf material, to be transported into nests where they could potentially act directly against the ants' symbiont. We offer a framework in which to consider these interactions, grounded in recent work on the phylogenetic origins of animal and plant pathogens, and offer new hypotheses to be tested. We propose that plants may be using endophytes directly as bodyguards, in particular the mycoparasite *Trichoderma*, which we have found being carried by ants in leaf fragments. We discuss the ants' possible responses to endophytic antagonists, for example the use of leaf caches as a 'quarantine post' to detect fungal antagonists. We consider how fungal endophytes and plants may provide honest signals betraying the presence of antagonists within leaves, leading to long-term avoidance. Finally, we consider how these interactions may structure neotropical forest communities.

275 words

Key words: *mutualism, parasitism, symbiosis, food webs, herbivory, foraging, Leucoagaricus, Hypocreales, Attini, Neotropical forests*

INTRODUCTION

Historically, symbiotic interactions have mostly been considered in isolation from the ecological communities in which they exist. Meanwhile, ecological communities have been considered principally in terms of trophic relationships, despite an abundance of important non-trophic interactions (e.g. mutualism and parasitism). Recently, a more integrated approach has considered discrete groups of symbiotic organisms acting in concert (as an *extended phenotype*) and interacting with other groups of organisms within an ecological community (Thomas *et al*. 2005, Thrall *et al.* 2007). Consequently, organisms such as endophytic fungi that live, for example, in intimate association with plants, can indirectly affect the quality and quantity of the resources available to herbivores (Finkes *et al.* 2006). Similarly, symbiotic organisms associated with herbivores may have an important role in releasing nutrients from consumed plant material.

Here, we consider the interaction between two pairs of mutualistic symbionts: (1) plants and endophytic fungi and (2) leafcutter ants (*Atta* and *Acromyrmex*; Formicidae: Attini) and their obligatory basidiomycete fungal mutualist (*Leucoagaricus gongylophorus*). We consider how leafcutter fungiculture may be affected by microbial symbionts of the substrate, i.e. how is the ant-fungus mutualism influenced by plant-endophytic fungi associations? Since the central interaction (ant 'herbivory') is affected by plant quality, which is itself affected by endophytic symbionts, interactions can occur via several routes, which we outline. We also consider the possible importance of abiotic factors, particularly water availability, on these interactions. Our goal is to use current knowledge to create a framework of testable hypotheses.

To understand this complex interaction network, we first consider the biology of leafcutter ants, their importance in neotropical ecosystems and their relationships with fungi and plants. We then shift our focus to endophytic fungi and their roles in affecting

herbivory and plant physiology. This provides the basis to consider the pattern of selective forces which we expect to act on plants and ants. Van Bael *et al.* (2009) recently proposed a sequential model of ant and fungus garden defenses against potentially antagonistic endophytes. We extend this model to provide a broader theoretical context and a number of testable hypotheses. Throughout, we consider recent advances in phylogenetics pertaining to the fungi involved; it is increasingly evident that this can guide an understanding of the various roles played by fungi in ecosystems, and particularly the possibilities of shifts between activities as insect, plant or fungal antagonists (Sung *et al.* 2007).

LEAFCUTTER ANT BIOLOGY AND ECOLOGICAL CONTEXT

Leafcutter ants are considered the dominant ant species and the most important primary consumers in the majority of natural or semi-natural habitats in the Neotropics (Beattie and Hughes 2002). Between 12 and 17% of the production of leaves in tropical forests is cut by *Atta* alone (Cherrett 1986); although, more recently, the importance of their overall impact has been questioned (Herz *et al.* 2007). Further effects are revealed by exclusion experiments in Amazonian forest that demonstrated a profound effect of ant activity on the establishment of tree seedlings, which would likely influence tree species composition (Vasconcelos and Cherrett 1997). Meanwhile, in areas of Brazil where Atlantic Forest has become fragmented, the density of leafcutter ant nests is increasing, possibly because of reduced parasite and predator pressure (Freitas *et al.* 2005), and it has been proposed that this could have an impact on fragment viability (Lewinsohn *et al.* 2005). A recent study from South America supports this idea – in the absence of predators, smaller islands (water-bound islands rather than forest fragments) are more vulnerable to increases in leafcutter ant populations, limiting the recruitment of tree seedlings (Terborgh *et al.* 2006). To date, the impact of this activity on forest structure

has yet to be given a detailed treatment, but we touch on this subject below, in the context of the specific interaction under consideration.

The association between leafcutter ants and *Leucoagaricus* is one of the classic examples of obligate mutualistic symbiosis. The ants provide vegetation (typically leaf fragments) as substrate for the fungus, while the fungus in turn provides nutrients for the ants in the form of gongylidia (swollen hyphal tips)[refs; see reviewer 2]. Over the past decade, investigation of the interaction has revealed further complexity. For example, the bacterium *Pseudonocardia* is carried on the cuticle of Attine ant workers and helps to defend the garden against parasitic microorganisms (Currie et al. 1999b, Poulsen et al. 2007). Similar, but apparently more facultative, interactions have been revealed involving other bacteria or actinomycetes sensu lato (e.g. *Burkholderia* and *Streptomyces*) associated with leafcutter colonies (Santos et al. 2004, Kost et al. 2007, Mueller et al. 2008, Haeder et al. 2009), underlining the complexity in this system.

Of the potential antagonists to this mutualism, the best known is the obligatory mycoparasitic fungus *Escovopsis*, which are typically associated with ant colonies in decline (Currie et al. 1999a). However, there is a range of fungi which can be associated with nests and which have the potential to harm fungus gardens (Fisher et al. 1996, Rodrigues et al. 2005, Lacerda et al. 2006), notably another mycoparasitic fungus, *Trichoderma harzianum* (Rodrigues *et al.* 2005) that can substantially inhibit the growth of *Leucoagaricus* . The threat from these invaders may be mitigated by ant defences, notably anti-fungal compounds produced by the metapleural glands (Fernandez-Marin *et al.* 2006). A recent study suggested that there was a risk to leafcutters of bringing endophytic fungi into their colonies and a set of responses (from both ants and the fungal mutualist) that may reduce this risk (Van Bael et al. 2009). Clearly, there is a complex web of interactions between micro-organisms concentrated in the fungus gardens. Meanwhile, endophytic fungi are being found at high prevalences and with

huge diversity in neotropical forests (Arnold et al. 2000, Evans et al. 2003, Arnold and Lutzoni 2007, Thomas et al. 2008), so it is to be expected that they have an important role in leafcutter interactions.

THE BENEFITS CONFERRED BY ENDOPHYTIC FUNGI UPON PLANTS

Fungal endophytes colonize healthy plant tissues inter- or intra-cellularly, forming relationships with the host plant along a continuum from parasitism to mutualism (Redman et al. 2001, Schulz and Boyle 2005). Some of the commonest endophytes in the foliage of dicotyledonous plants belong to the ascomycete genus *Glomerella* (Glomerellaceae, Hypocreales; see Kirk *et al.* 2008), and its anamorph *Colletotrichum*, which include a number of important plant pathogens – and, as will be shown below, can also be insect pathogens (Batista and Bezerra 1966, Marcelino et al. 2008a). Endophytes may be transmitted between plant hosts horizontally or vertically. Vertical transmission is characteristic of endophytes of monocotyledonous plants and is expected to lead to selection for low virulence (Lipsitch *et al.* 1996, Kover *et al.* 1997), while both transmission routes are expected to select for defensive mutualisms with the plant by some authors (Herre et al. 1999, Faeth 2002) considered that this would not apply to horizontally-transmitted endophytes . Endophytic fungi can produce biologically active molecules that can confer upon the host plant resistance to attack by insect herbivores (Azevedo et al. 2000) or microbial plant pathogens (Mucciarelli *et al.* 2002, Bandara *et al.* 2006). Molecules with toxicity to insects include alkaloids, terpenoids, flavonoids and chlorinated compounds (Clay and Cheplick 1989, Tan and Zou 2001, Zhang *et al.* 2006, Guo *et al.* 2008). Thus, infection with endophytic fungi may confer chemical defense against herbivory and invasion by pathogens (Clay 1988, Saikkonen et al. 2006) and have knock-on effects on higher trophic levels (Omacini et al. 2001, Härri et al. 2008).

Endophytic fungi may also act as a *biological* plant defense. These 'indirect' or 'third party' defenses are well known from tritrophic systems in which a predator or parasitoid is considered as a plant 'bodyguard' capable of reducing herbivory (Price *et al.* 1980). A similar scenario has been proposed for entomopathogens as plant bodyguards(Elliot et al. 2000) . This indirect form of defense can take various forms: bodyguards may be maintained on or within the plant; they may be activated as an induced defense following herbivory; or characteristics of the plant may simply promote the efficacy of natural enemies of the herbivores (Elliot et al. 2000). It is possible that endophytic fungi can protect a plant, most likely in the first of these three manners. Indeed, this has been proposed to explain the occurrence of the entomopathogen *Beauveria bassiana* within plant tissues (e.g. in the maize/European corn borer (Lepidoptera: *Ostrinia nubilalis*) system (Arnold and Lewis 2005). *Beauveria bassiana* has been shown to colonise hosts systemically (Wagner and Lewis 2000), and this association is widespread within the corn-growing regions of North America (Arnold and Lewis 2005), making this an interesting prospect for pest management. *Beauveria* species have also been recorded as endophytic in hosts as diverse as cotton, cocoa and balsam (Evans et al. 2003, Arnold and Lewis 2005). Meanwhile, in Australia, the entomopathogen *Lecanicillium* (*Verticillium*) *lecanii* has been isolated from cotton leaves and implicated in the reduced growth of the cotton bollworm (Lepidoptera: *Helicoverpa armigera*,*)* as well as a feeding deterrent (McGee 2002). Isolates have the ability to colonise cotton plants asymptomatically and also to infect the cotton aphid, *Aphis gossypii* (Anderson *et al.* 2007). Meanwhile, the ubiquitous entomopathogen *Metarhizium anisopliae,* has been recorded amongst a range of endophytes isolated from the tree *Taxus chinensis* (Liu et al. 2009a). In addition, this strain was found to produce significant amounts of taxol, which has fungicidal activity. Finally, confirmation that the plant pathogen and common endophyte *Glomerella*

(*Colletotrichum*) also contains strains that are pathogenic to hemipteran pests (Marcelino et al. 2008a), raises the intriguing possibility that other endophytes may have cryptic entomopathogenic properties. There are further indications that these are not isolated instances: a recent phylogenetic analysis of endophytic fungi across a range of ecosystems and hosts (Arnold *et al.* 2009) revealed previously unidentified taxa sitting within the *Cordyceps* clade of the Hypocreales – well known entomopathogens (these from neotropical trees). Thus, plants may well use endophytes as generalist 'bodyguards' – that is, a given endophyte could protect its host against both herbivores and pathogens.

Hosting endophytic fungi may also also confer more direct benefits. They can increase tolerance to drought (Malinowski and Belesky 2000), heavy metals (Monnet *et al.* 2001), viral infection and low pH (Lewis 2004), high temperatures (Redman *et al.* 2002) and high salinity (Waller *et al.* 2005). They may also improve growth through improved nitrogen fixation, solubilisation of phosphate and production of auxins (Peters *et al.* 1998, Bandara *et al.* 2006). It is evident that these factors and the conferred resistance to pathogens and herbivores will affect the quantity and quality of resources available to consumers on higher trophic levels.

Considering the complexity of plant-herbivore and plant-pathogen interactions, even the more direct benefits of harboring endophytes may not always translate into increased plant fitness. Endophytes require nutrients and water for growth, while the production of secondary metabolites, which are often rich in nitrogen, will also be costly (Faeth and Fagan 2002). A further cost is the risk of the mutualistic relationship becoming, on balance, parasitic, through pathogenicity or through an increased nutritional demand on the host (Cheplick *et al.* 1989, Thrall *et al.* 2007). Consequently, the propensity to harbor endophytes will vary according to the selective environment. This type of scenario has been explored theoretically in terms of the likelihood that

host-symbiont interactions are mutualistic or parasitic, with the prediction that mutualistic or parasitic interactions dominate in harsh or benign conditions respectively (Thrall *et al.* 2007). It will be interesting to see in the near future if this prediction gains support from endophytic fungus-plant associations.

In the case of defense against herbivory, fitness benefits to plants of hosting endophytic fungi have been demonstrated (Lewis 2004). However, studies have focused on a few, select organisms, so the generality of this phenomenon is still a matter for debate (Saikkonen *et al.* 2006). Considering the elevated prevalence of endophytic fungi in dicotyledonous species, their role in plant defense remains comparatively little explored. Neotropical forests show very high prevalences and a considerable diversity of endophytic fungi (Arnold et al. 2000, Arnold and Lutzoni 2007, Rodriguez et al. 2009, Van Bael et al. 2009), so there is every reason to expect endophytes to have an important role as modulators of herbivory in these forests.

MICROBIAL MEDIATION OF LEAFCUTTER ATTACK

Two empirical studies have considered the possible effect of endophytic fungi on leafcutter ants. The presence of endophytic fungi in grasses affected the amount of material harvested by, and the performance and survival of *Acromyrmex versicolor* in the south-western USA (Tibbets and Faeth 1999). Meanwhile, the amount of *Glomerella cingulata* (the teleomorph of *Colletotrichum gloeosporioides*) being carried in inoculated leaves was reduced by ant handling of the material in *Atta colombica* in Panama and it has been shown that the ants' fungal symbiont is able to inhibit growth of endophytes *in vitro*, presumably by producing antifungal or fungistatic secondary metabolites (Van Bael et al. 2009). These authors proposed a model of sequential, multi-modal defenses on the part of both symbionts (ant and fungus), combining ant hygienic behavior with fungal chemical defense.

We propose a more extensive framework for this interaction (Table) to describe the possible roles of endophytic fungi in modulating herbivory by leafcutter ants. Central to this framework is the plant-ant antagonism and the inclusion, when considering these two organisms, of their fungal symbionts (Fig. 1). Crucial to this antagonism is the choice exerted by ants regarding which plants to attack. Ant foraging will be affected by the usual trade-offs of resource quality and costs of foraging, but it is important to remember that the ants are attending to the needs not of individuals but of a complex society, as well as more than one species of microbial symbiont. Additionally, their interaction with the plants that they harvest is one which may last for years or even decades and which is spatially very limited: factors which may lead to a milking of their resource rather than over-exploitation, and even to collusion with their antagonists (van Baalen and Jansen 2001) and honest signalling on the part of those plants which are well-defended.

ENDOPHYTE EFFECTS ON THE ANT-FUNGUS MUTUALISM

Endophytes as plant bodyguards

Endophytic fungi may have negative effects on the ant-fungus mutualism, via toxins produced within the plant or by directly parasitizing the fungus garden. The effect of endophyte-produced toxins is well known, especially in the case of vertebrate herbivores such as cattle. It was the effect of endophytic fungi on herbivory (specifically the intoxication of grazing livestock due to fungal secondary metabolites produced in grasses) which led to their discovery and which drove early research (Bacon *et al.* 1977, Schardl and Phillips 1997). Given that these fungi can produce a range of secondary metabolites, leaf material carried into the nest by ants may well be unsuitable as a substrate or, worse, have fungicidal, fungistatic or insecticidal toxins present. Fungi present in leaf fragments may also grow within these fragments after they have been

excised, so competing for nutrients or producing further toxins. In conjunction with the plant's own chemistry, this forms, then, the most obvious means by which a fungal endophyte can defend its host and which will affect the ants' choice of which plants to attack.

As well as defending their hosts chemically, endophytes may have the capacity to attack foraging ants directly. It has been proposed that plants may use insect pathogens as bodyguards much as they do parasitoids or predators (Elliot et al. 2000) and it is now established that strains of the entomopathogenic fungi *Beauveria bassiana, Lecanicillium lecanii* and *Metarhizium anisopliae* are able to exist as endophytes and can therefore defend plants from herbivory (Azevedo et al. 2000).

Perhaps most intriguing, however, is the possibility that ants harbor endophytes which will attack, once taken into the ants' colonies, the ants' fungal garden. Our preliminary data support a similar scenario for Atlantic Forest plants and leafcutter ants – the mycoparasite *Trichoderma* has been isolated from leaf fragments being transported by *Atta sexdens rubropilosa* and *Acromyrmex* spp. (SLR *unpublished data*), or else found deposited in the ants' caches or in waste piles around nest entrances. If these ants were to transport leaf fragments containing this mycoparasite to their fungal garden, they would effectively be inoculating their fungus gardens with a parasite (Silva et al. 2006b).

We and other workers have found that almost all fragments of leaf material being transported by leafcutter ants have endophytic fungi inside them (Van Bael et al. 2009, SLR unpubl. data). We hypothesize, therefore, that some of the fungi found in these leaf fragments will be prejudicial to the fungus garden (as proposed by Van Bael et al. 2009), by one means or another, and that this may provide a fitness benefit to the plant host, favoring the association with endophytes. Of particular interest is *Trichoderma* as it is known for antagonistic interactions with other fungi, via the

production of toxins, parasitism or competition for space, nutrients and oxygen (Martins-Corder and de Melo 1998). Moreover, Samuels (2006) has highlighted the recent discovery and importance of new *Trichoderma* species isolated as endophytes from neotropical tree hosts. Additional species have been described since (e.g. Samuels and Ismaiel 2009) and there is now molecular data to show that these endophytes represent a unique clade within the genus, suggesting evolutionary separation from the free-living *Trichoderma* species (Chaverri et al. in press).

Endophytes may affect the suitability of plant material as a substrate

In general, the association with endophytic fungi tends to improve a plant's nutritional and hydric status (but seeArnold and Engelbrecht 2007, Rodriguez and Redman 2008). The latter may be critical in leafcutter herbivory given experimental evidence that *Atta colombica* foragers may prefer drought-stressed leaves (Meyer et al. 2006). If plant water relations shape population dynamics and community structure in tropical forests (Bunker and Carson 2005), then endophytes may be playing a crucial, if cryptic, role. For example, the presence of endophytes in leaves may improve drought-tolerance and thereby provide a secondary benefit of being less attractive to leafcutter ants. It will be important to determine empirically the pattern of such pleiotropies. Where they are negative, there is a mechanism for the generation of a diversity of strategies for plants based upon their selective environment. Where positive (and positive pleiotropies may also be expected with defenses against other herbivores or pathogens), they would contribute not only to the stability of the plant-endophyte symbioses over evolutionary timescales but also to the success of the association in competition with other plants over ecological timescales.

ANT SELECTION OF LEAF MATERIAL AND ITS FEEDBACK

Leafcutter ants live in an intimate association with their fungal mutualist and can recognize fungal parasites in their gardens (Currie and Stuart 2001). Additionally, negative effects on the fungus garden of toxins contained within leaf material can be detected, and ant foraging adjusted accordingly (Herz *et al.* 2008). Thus, it is perfectly feasible that they discriminate between leaf materials with or without endophytes, based upon (1) the identity of the endophyte involved, (2) the presence of endophyte-produced toxins within the leaves, or (3) effects upon the fungus garden. The first of these may initially seem unfeasible, given the quantities of mycelium involved, but we propose that ants could get around this by using caches. Many leafcutter species employ caches, temporary repositories for foraged material (Hart and Ratnieks 2000), in which leaf fragments dry out before being taken into the nest. This behavior has been explored in terms of optimization of foraging (Hart and Ratnieks 2000, 2001) but it could equally well be a defense mechanism: caching would reduce the risk posed by endophytes because of the drying and also because the endophytic mycelium would emerge from the cut leaf surfaces within a couple of days, thereby facilitating direct examination. Furthermore, leafcutters often reject some leaf fragments a few days after these are taken to the garden (TMCDL *pers. obs.*), a possible mechanism to reduce mycoparasites being brought into the garden, and potentially entomopathogenic fungi being introduced into the nest (see Table).

So, ant behaviors may limit the negative effects of endophytes, even over short time scales. There are other possibilities: a preference for plants suffering drought stress (Meyer et al. 2006), could be a means of avoiding these fungi if it indicates an absence of endophytic fungi. Also, the distribution of endophytes within a plant is unlikely to be uniform and it may take time for endophytes to grow into new tissue; they may also occur less in certain tissue types, such as petals, or be seasonal in their behavior within

their hosts. This could well explain some of the pattern of ant selection of plant material.

Over longer timescales too, ants may cope with the presence of antagonistic endophytes. Given that ant colonies may be present in a single site for years or even decades, they will have long associations with neighboring plants and will establish foraging patterns to avoid certain individuals and harvest from others. Plants which are well-protected then benefit from a certain amount of harvesting, as they can signal this fact to their antagonist in a form of collusion (van Baalen and Jansen 2001).

Plant competition as feedback

A key consideration in any assessment of plant fitness, and thus selection upon traits which might favor a symbiotic association, is competition. In the case of endophyte-infected versus endophyte-free plants, a competitive edge has been shown for the former, but this has principally been done in pot experiments with grasses (e.g. Malinowski and Belesky 2000, Lewis 2004). This approach, effectively a laboratory one, is of great value in initial determinations of the fitness benefits of symbioses, but has its limitations when it is extended to predictions of phenomena expected in the more complex context of the field (Hartley and Gange 2009).

For Neotropical tree species, and other plants subject to leafcutter attack, it is to be expected that even marginal differences in leafcutter attack could tip the competitive balance between neighboring trees. If ants choose to avoid some plants on the basis of the presence of endophytic fungi, then there is the potential for positive feedback as these plants will have a competitive edge over their neighbors. This will amplify the benefits of the endophytes and these may be further amplified given the likely pleiotropies involved, as discussed above. Considering all of this, it seems at first glance that selection on Neotropical tree species to associate with fungal endophytes would be

very strong. A recent meta-analysis of ontogenentic patterns in plant defense finds that there is an increase in toxicity of leaves of woody plants during the seedling phase (Barton and Koricheva 2010). This probably implies that herbivory is most dangerous early on in life, whereas later on it may be less important. In the case of leafcutter ants that are capable of killing seedlings (Vasconcelos and Cherrett 1997), this would lead to the expectation that beneficial mutualisms with endophytes should be formed early on in a tree's life, at least in situations where the ants exert a significant selection pressure.

The problems with endophytes

Problems may arise, however, with endophytes. As with any symbiosis, there will be direct costs for the host to maintain the symbiont, and it may cheat. Endophytic fungi such as *Colletotrichum* are traditionally considered plant pathogens so the mechanisms for cheating certainly exist. For endophytes residing in long-lived and large plants, the initial expectation would be that cheating is not a good strategy as the endophyte may end up reproducing less from a weakened host. However, trees are very apparent in the environment so an endophyte may find it is competing with a number of other endophytes, or traditional pathogens and parasites, so selection may increase its virulence.

Perhaps the greatest problem for the plants, however, arises from just the lengthy interaction with the ants which may afford it protection. In the vicinity of a nest, any tree species which is not harvestable represents a waste of space to the ants, or worse. These trees are effectively weeds. The ants could, of course, attack the tree for the sake of reducing its growth and so benefit other, more useful individuals. This could even explain apparent inefficiencies in ant foraging. Far worse for the well-defended tree, though, is that the ants' best strategy is to kill any of its offspring which might attempt to establish, if these have a propensity to establish similar interactions with endophytes.

Although this aspect of the interaction will require further investigation, an initial prediction is that trees which are well-defended will adopt dispersal strategies which take their seeds away from the local area. Conversely, however, given most infections with the endophytic symbionts are horizontal, reduced dispersal might benefit a tree, as it could serve as a source of inoculum for its offspring.

FINAL CONSIDERATIONS OF THE FUNGI INVOLVED

It is increasingly clear that those endophytes which impact on leafcutters derive predominantly from the order *Hypocreales*. These include the obligate grass endophytes, *Epichloe* (*Neotyphodium*), the majority of entomopathogenic ascomycetes (*Beauveria*, *Metarhizium* etc) and the mycoparasites exemplified by *Trichoderma* (and its teleomorph *Hypocrea*). Thus, as Rehner (2009) concludes: "The 'clavicipitalean' fungi are a remarkably diverse assemblage of biotrophs [sic] that form an array of pathogenic, parasitic and mutualistic associations with different kingdoms of life, including plants, fungi, insects and other microinvertebrates". Intriguingly, it has now been shown through multigene phylogeny that the clade of grass endophytes within the Clavicipitaceae are derived, via inter-kingdom host switching, from insect pathogens (Spatafora *et al.* 2007), and that the closest relatives include the genera *Hypocrella* (pathogens of hemipterans) and *Metarhizium* (a generalist entomopathogen) (Arnold *et al.* 2009). There is, therefore, a strong possibility that switches in the opposite direction apply for the other entomopathogens within the Hypocreales. Certainly, the endophytic habit has been around far longer than insects, since fungal endophytes appear to have been integral to the colonisation of land by plants (Blackwell 2000).

It is this ability to switch between kingdoms, as shown in the species pertaining to *Ophiocordyceps* which occur on both insects and macrofungi (Fukatsu and Nikoh 2003), that probably enables them to adopt an endophytic lifestyle. As discussed

previously, there are ever increasing examples where common entomopathogens are being recorded as endophytes. These must pose a potent threat to leafcutter ants. However, until recently, these ants seemed to be unusually immune to pathogenic fungi, especially compared to other Formicidae (Evans 2001). Recently, however, leafcutter queens and workers (both *Atta* and *Acromyrmex*) have been found infected with *Ophiocordyceps* in Panama and Brazil (Hughes *et al.* 2009). There are indications that '*Cordyceps'* morphospecies are endophytic in neotropical trees (Thomas *et al*. 2008, Arnold *et al.* 2009), suggesting a possible mechanism of how these fungi entered the nest to infect the queens.

Meanwhile, endophytes with mycoparasitic ability would be a constant danger to the fungus garden and, therefore, probably the biggest threat to the stability of leafcutter ant societies. The relatively recent finding of a novel clade of endophytic *Trichoderma* species from the Neotropics (Samuels et al. 2006, Chaverri et al. in press), is of great significance since this genus, along with *Escovopsis,* have been recorded from Attine fungus gardens. Although the first reports involved *Trichoderma* endophytes from woody tissues, there is increasing evidence that they also colonise leaves, both in tropical (SLR *unpublished data*) and temperate ecosystems (Vujanovic and Brisson 2002). It would not be surprising, given the common occurrence of the endophytic habit in the Hypocreales, if *Escovopsis* is found in future surveys for endophytic fungi in Neotropical tree hosts.

Summary

Although this system is potentially highly complex, it is perfectly possible to elucidate the nature of some of the key interactions. Considering the current direction of ecosystem ecology (the inclusion of symbionts), we suggest that not to consider endophytic fungi when looking at leafcutter ant herbivory would be to ignore a key

component of the system. We have proposed a framework of possible interactions based both upon mechanistic considerations and expectations of how natural selection may play out. The framework could be seen as over-elaborate, but evidence is mounting, as discussed in the preceding section, of diverse and mutable roles for these versatile micro-organisms. To aid further work in this area, we have provided a number of testable hypotheses, building upon empirical work by Van Bael *et al.* (2009), and these are summarised in the Table.

Undoubtedly, leafcutter ants play a key role in structuring natural communities in Neotropical systems. However, it should also be taken into account that they are among the most important agricultural and silvicultural pests on the continent. Current control is heavily reliant on a few synthetic insecticides and these are becoming less and less available as international bans on their use are imposed. Symbiotic microorganisms such as these hypocrealean fungi from tropical forests, offer an untapped resource for future control of leafcutter ants in agricultural ecosystems, although ant societies may adopt many means to defend themselves against such fungal attack. Thus, we hope that this review serves not just to assist in understanding these mutualistic associations, but also in developing strategies for sustainable management of these insects.

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A summary of hypotheses presented in the text

Antagonistic fungi
Endophytic fungi produce toxins which negatively affect leafcutter ants or their symbiotic fungus.
Endophytic fungi may act as insect pathogens, infecting leafcutters
Endophytic fungi may act as mycoparasites, attacking the fungus garden
The fungus *Escovopsis* has endophytic capacity

Ant foraging behavior
To avoid the negative effects of certain endophytes, leafcutter ants avoid collecting infected leaf material, to the point of avoiding entire plants
Leafcutters over-harvest trees which are well-defended by endophytes, and kill seedlings they identify as being related.

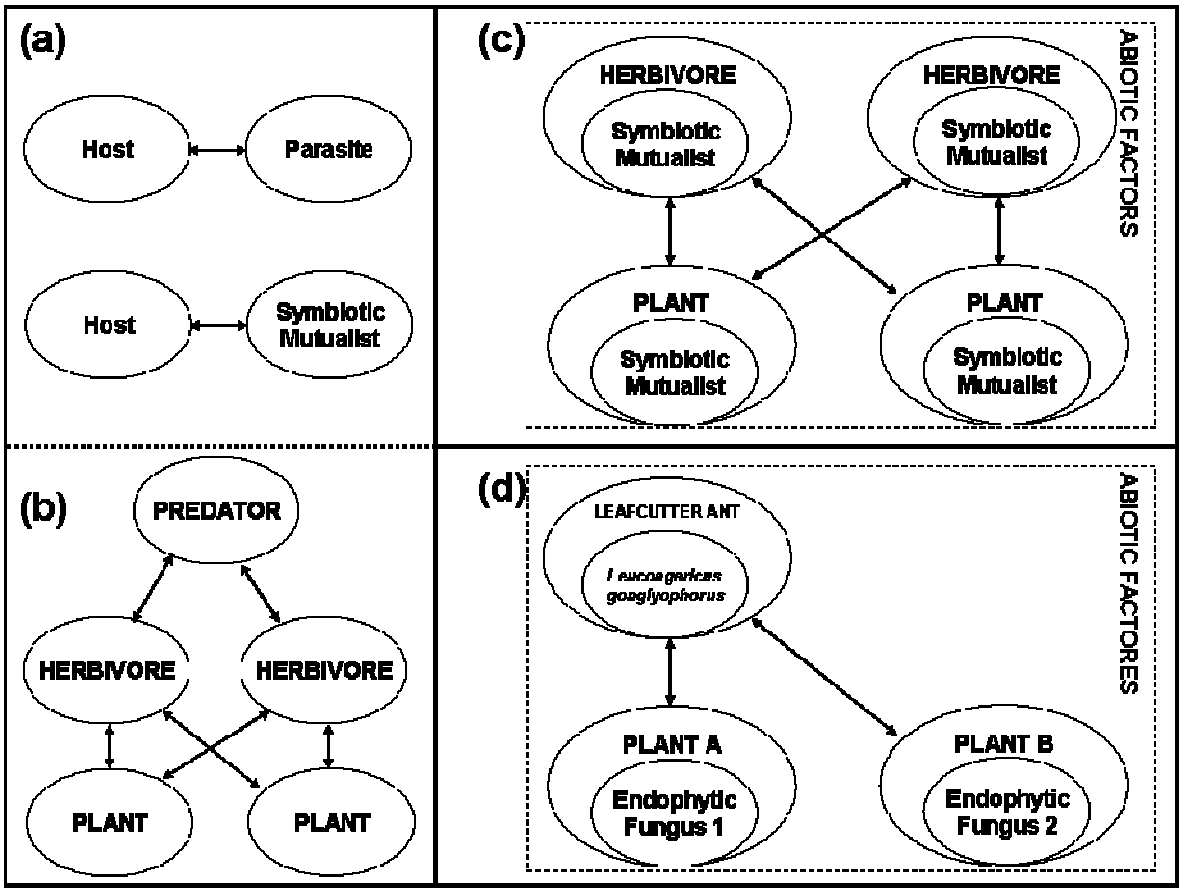
Ant hygienic behaviors
Leafcutters employ caches as a strategy to identify or weaken potentially harmful endophytes
Leafcutters remove leaf material containing endophytes a few days after this is placed inside the colony, once effects on the fungus garden begin to occur
Because of these strategies, harmful endophytes are actually rarely found contaminating leafcutter colonies

FIGURE LEGENDS

FIGURE 1

The changing view of symbionts' place in ecological food webs or communities, applied to webs in which leafcutter ants are to be found. (a) The original view of interactions between organisms and their symbionts, whether parasitic or mutualistic, these interactions occurring in isolation from the rest of the community. (b) Food webs constructed on the basis of trophic relations (therefore antagonisms), principally predation. (c) Communities (here represented by only a few elements) as groups of symbiotic species (hosts and their mutualists or parasites) interacting among themselves and being affected by abiotic factors. (d) The leafcutter ant and plant system considered in this paper – note that the mutualists here could as easily be parasites. [*Note that symbiosis is an intimate and long-lasting association between individuals of different species. Parasitic associations occur when one symbiont lives at the expense of the other and mutualisms occur when both interacting species benefit from the association.*]

FIGURE


(a)
Host
Parasite
Host
Symbiotic Mutualist
(b)
PREDATOR
HERBIVORE
HERBIVORE
PLANT
PLANT
(c)
HERBIVORE
Symbiotic Mutualist
HERBIVORE
Symbiotic Mutualist
PLANT
Symbiotic Mutualist
PLANT
Symbiotic Mutualist
ABIOTIC FACTORS
(d)
LEAFCUTTER ANT
Leucoagaricus gongylophorus
PLANT A
Endophytic Fungus 1
PLANT B
Endophytic Fungus 2
ABIOTIC FACTORES

Chapter 2

The community of endophytic fungi which leafcutter ants introduce into their colonies through foraging

Abstract

Endophytic fungi can be allies of plants in their defence against herbivores and plant pathogens. Leafcutter ants of the genera *Atta* and *Acromyrmex*, tribe Attini, exhibit criteria about the choice of material that will be cut and incorporated into the mutualistic fungal garden that comprises the main food resource of the colony. The presence of endophytic fungi in this material may affect the fungal symbiont and thus the choice of material by foragers. The collected material passes through two sorting phases: the first outside the colony and the second inside. The present study was conducted to document the endophytic fungi being carried in vegetative material and to determine whether there was a difference in material carried from that subsequently rejected by the ants. Collections were made of vegetative fragments which were being carried by foragers of the leafcutter ant *Atta laevigata* and of rejected material located at the entrance of these ants' nests. There was considerable diversity in fungi found, including several genera of mycoparasites such as *Trichoderma* (constituting the first report of this fungus as an endophyte in leaves). The specialised mycoparasite of ant fungal gardens, *Escovopsis*, was not found, providing evidence against endophytism as a route of infection of this fungus. Seven genera (*Cylindrocladium*, *Drechslera, Epicoccum, Fusarium, Mycelia sterilia* sp. 21, *Trichoderma* and *Ulocladium*) were found more in rejected material than expected, while five genera (*Glomerella, Mycelia sterilia* sp. 22, *Pestalotiopsis*, *Phomopsis* and *Xylaria*) were found less in rejected material than expected. The possibilities that ants are discriminating rejection of vegetative material based on the

presence of endophytes is discussed, as is the potential use of some of these fungi as possible biocontrol agents against leafcutter ants.

Key-words: *Atta laevigata*, biological control, endophytic fungi.

Introduction

Leafcutter ants (Formicidae: Attini) have been studied intensively for two main reasons. One is their status as major pests of agriculture and silviculture in the New World (Fowler et al. 1989, Zanetti et al. 2003) and the other is their multitrophic interactions with micro-organisms inside the colony (Currie 2001a, Currie et al. 2003, Little and Currie 2008, Caldera et al. 2009). In addition to the mutualist fungi, there are many other micro-organisms that can be found associated with colonies, such as filamentous fungi (Currie et al. 1999b, Lacerda et al. 2006, Lacerda et al. 2010), yeasts (Carreiro et al. 1997, Carreiro et al. 2004) and bacteria (Van Borm et al. 2002, Santos et al. 2004). More recently, endophytic fungi have been added to this list (Van Bael et al. 2009). These fungi live within plant tissues for all or part of their life cycle and cause no apparent harm to their plant host (Wilson 1995). These fungi can be important mediators of the interaction between plants and herbivores (Rajagopal and Suryanarayanan 2000, Omacini et al. 2001, Miller et al. 2002, Meister et al. 2006). Thus, it is of interest to document potential interactions between leafcutter ants and endophytic fungi, particularly as plants may be a vehicle for the entrance of damaging microorganisms into the nests.

In order to maintaining the cultivated fungus free of unwelcome microorganisms that might be brought in while foraging, leafcutter ants can use a range of strategies (Schmid-Hempel 1998, Andrade et al. 2002). Firstly, the workers exhibit choice of material, including leaf toughness and nutrient content, as well as the presence of attractant and deterrent secondary compounds (Nichols-Orians and Schultz 1989, Camargo et al. 2004, Meyer et al. 2006). According to Van Bael et al (2009), ants are able to remove spores when the plant contain spores of endophytic fungi. Then, workers of leafcutting ants can clean leaf fragments in order to remove fungal spores and

bacteria. Beyond these there is hygienic behaviour that occurs when there are individuals infected inside colonies (Ugelvig and Cremer 2007)

For plants attacked by leafcutter ants, endophytic fungi would be ideal mutualist partners, in particular if they are entomopathogenic or are antagonistic to the cultivated fungi. Endophytic fungi began to be explored in the last eleven years but there was previous speculation about high diversity (Lodge et al. 1996). Recent studies have provided evidence of the high diversity of endophytic fungi (Fröhlich and Hyde 1999, Arnold et al. 2000, Arnold et al. 2001, Gamboa and Bayman 2001).

The aim of this study was to verify which endophytic fungi would be found being carried into leafcutter nests and which are then being rejected. This serves two purposes: one is ecological, to begin to investigate how endophytic fungi may affect the interaction between leafcutter ants and plants; the other has the more applied goal of finding entomopathogenic, mycoparasitic or other fungi to use as biological control agents. The motivation for the second aim is that the control of leafcutter ants is principally based upon synthetic chemical insecticides, with serious negative effects on the environment. Because of this, their use will be limited from the year 2015 (established by the Forest Stewardship Council, an international entity that accredits certifying organizations; Forest Stewardship Council, 2002). Given this, alternative means to control of leafcutter ant will soon become a necessity. It is possible that leafcutter ants have evolved mechanisms to keep harmful fungi from gaining entry to the nest, including endophytic fungi. Thus, rejected material in the waste piles may be a rich source of such fungi, in addition to revealing potential preference behaviours; thus, we focus our study on vegetative material being carried and also material in the waste piles or middens. We focus on the ant *Atta laevigata*, a common species in fragments of Atlantic Forest in the study region.

Materials and methods

Sampling was conducted in a privately owned fragment of Atlantic Forest (Mata Atlântica) – seasonal, subtropical, semi-deciduous, montane forest ("Mata do Seu Nico" owned by Antônio Manoel de Freitas, Sítio BonSucesso, Viçosa, Minas Gerais State 20º45'23''S and 42º 52'23''W, Brazil, 750m altitude). Three nests of the leafcutter ant *Atta laevigata* were used, each measuring approximately 25 m^2 at the surface and separated from one other by approximately 250 m. The vegetation surrounding the nests was dense, composed of high and medium-sized trees.

Five collections were undertaken fortnightly from each nest in the period from April to June 2010. Each collection consisted of 50 leaf fragments that were being carried by foraging workers and 50 rejected fragments that were found on refuse piles (or middens) at the nest entrances. Thus, a total of 1,500 leaf fragments were collected: 750 were being carried and 750 had been rejected by workers. All collected material was taken to the laboratory and surface-sterilized through sequential immersion in 70% ethanol (1 min), 5% sodium hypochlorite (4 min) and three washes in sterile distilled water. Sterilized leaf pieces were placed on potato-carrot agar (PCA) and were incubated at room temperature (25±2 °C). The fungi were identified morphologically - following sporulation - to genera where possible, while sterile isolates ("*Mycelia sterilia*") were described as morphotypes being differentiated by cultural characteristics.

To verify if material containing specific endophytic fungi was more or less likely to be discarded, tests of independence of frequency were conducted incorporating *G*-tests (Sokal and Rohlf 1995), comparing frequencies of different genera fungi that were in the carried material versus material from middens.

Results

Diversity of endophytic fungi associated with *Atta laevigata*

Of the 1,500 plant fragments collected from three *A. laevigata* colonies, we isolated 1,466 fungal endophytes, representing 56 morphotypes. Of these, 1,159 (79.06%) isolates produced spores and were identified to 33 fungal genera, most of these belonging to the phylum Ascomycota; the other 307 (20.94%) isolates remained *Mycelia sterilia* and were grouped in 22 morphospecies. Within the taxa distinguished, the following fungi were found at frequencies of <1% of fragments (carried plus rejected) and are not reported further. These were: *Chaetospermum carneum; Chaetospermum* sp.1; *Chaetospermum* sp.2; *Clonostachys* sp.; *Codinaea* sp.; *Cylindrocarpon* sp.; *Mycelia Sterilia* spp.1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15, 17; *Mycosphaerella* sp.; *Ophionectria* sp.; *Scytalidium* sp.; *Thozetella* sp.; *Thysanophora* sp. Of the fungal taxa found in ≥ 1% of fragments (carried plus rejected), the following showed no significant difference in frequencies between material carried and rejected, and are shown in Figure 1: *Acremonium* spp., *Colletotrichum* spp., *Mycelia sterilia* sp. 14, *Mycelia sterilia* sp. 16, *Mycelia sterilia* sp. 20, and *Phomopsis* sp.2 (Values of *G*: 1.56, 3.14, 0.28, 3.13, 3.13 and 3.30 all smaller than $\chi^2_{[d.f.=1]}$=3,84, p=0.05).

Apparent preference

For five genera of endophytic fungi, there was apparent rejection or preferential acceptance of vegetative material by *A. laevigata*, as indicated by *G*-tests. Thus, *Cylindrocladium*, *Drechslera, Epicoccum, Fusarium*, *Mycelia sterilia* sp. 21, *Trichoderma* and *Ulocladium* were more common in rejected material than in material

that was being transported to the nest (Fig. 2 a-g; values of *G*: 28.61,12.20, 3.95, 22.46 14.84, 12.77 and 28.03, respectively, all greater than $\chi^2_{[d.f.=1]}$=3,84, p=0.05.

For the genera *Glomerella*, *Mycelia sterilia* sp. 22, *Pestalotiopsis*, *Phomopsis* sp. 1 and *Xylaria*, occurrence was greater in material that was being transported to the nest than in rejected material (Fig. 2 h-l; values of *G*: 27.01, 8.17, 16.24, 7.51 and 65.82, respectively, all greater than $\chi^2_{[d.f.=1]}$=3,84, p=0.05.

For *Mycelia sterilia* sp. 22, *Pestalotiopsis* spp. and *Xylaria* spp. the pattern was consistent in the three nests and over the five samples dates (Figure 2 (i, j and l). For *Glomerella* spp. and *Phomopsis* spp., however, there was no pattern (Figure 2(h and k).

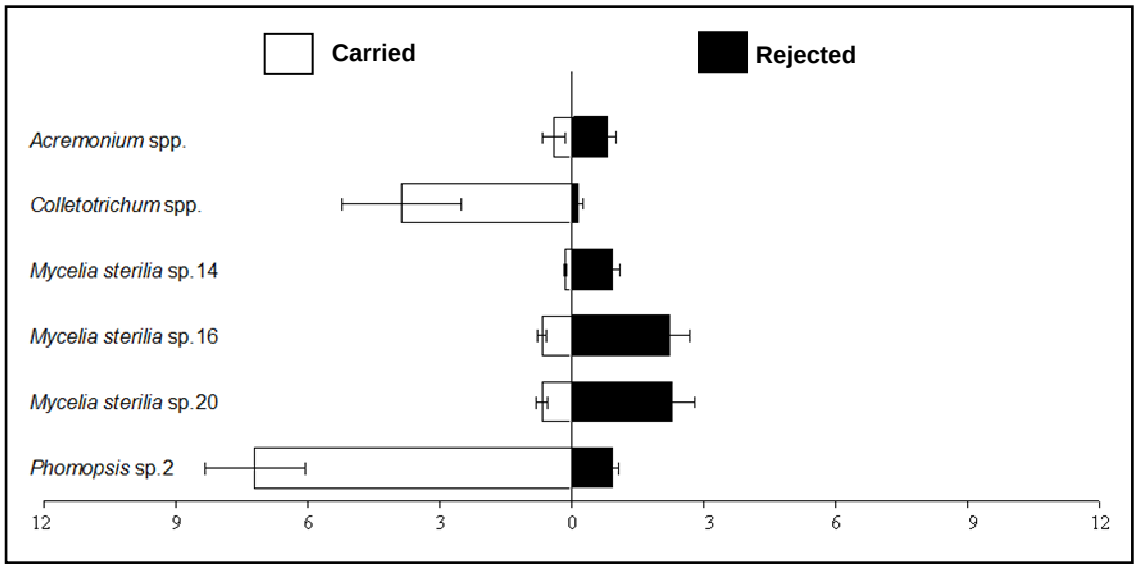


Figure 1 – Frequencies of occurrence of endophytic fungi in vegetative fragments that were being carried towards the nest by *Atta laevigata* workers ('carried' on the left) or that had been rejected and were found on middens outside entrances ('rejected' on the right). Shown are means (±SEM) of percentages of fragments bearing each taxon of fungi found in five fortnightly collections. Only fungi found in >= 1% of fragments (carried plus rejected) are shown. Fungi that were found significantly more in one sample or the other (*Cylindrocladium* spp., *Drechslera* spp., *Epicoccum* spp., *Fusarium* spp., *Glomerela* spp., *Mycelia sterilia* sp.21, *Mycelia sterilia* sp.22, *Pestalotiopsis* spp., *Phomopsis* spp., *Trichoderma* spp., *Ulocladium* spp. and *Xylaria* spp.) are shown in Figure 2, in greater detail.

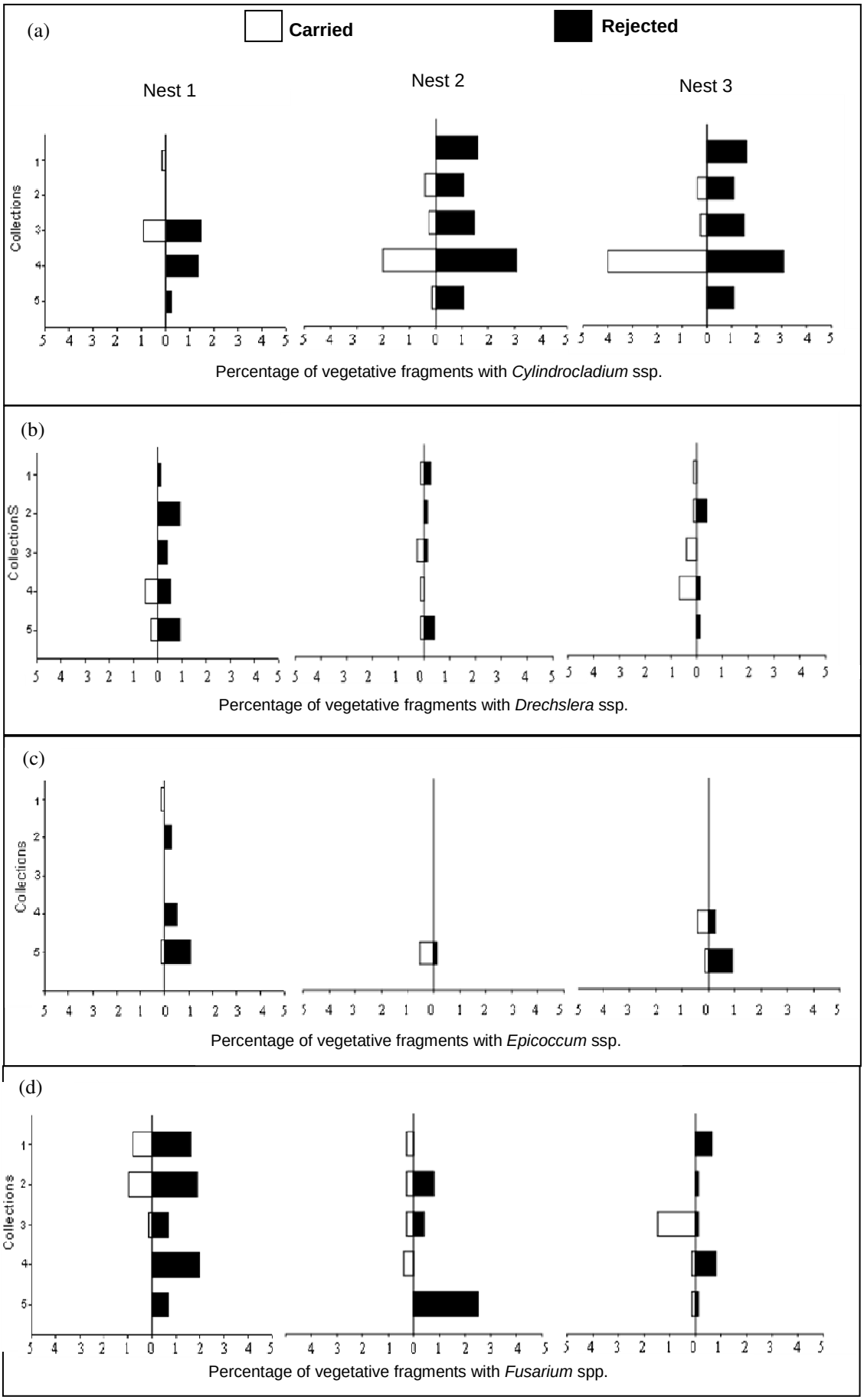


Figure 2 – Frequencies of occurrence of endophytic fungi in vegetative fragments that were being carried towards the nest by *Atta laevigata* workers ('carried' on the left) or

that had been rejected and were found on middens outside entrances ('rejected' on the right) that had significant difference in *G*-test.

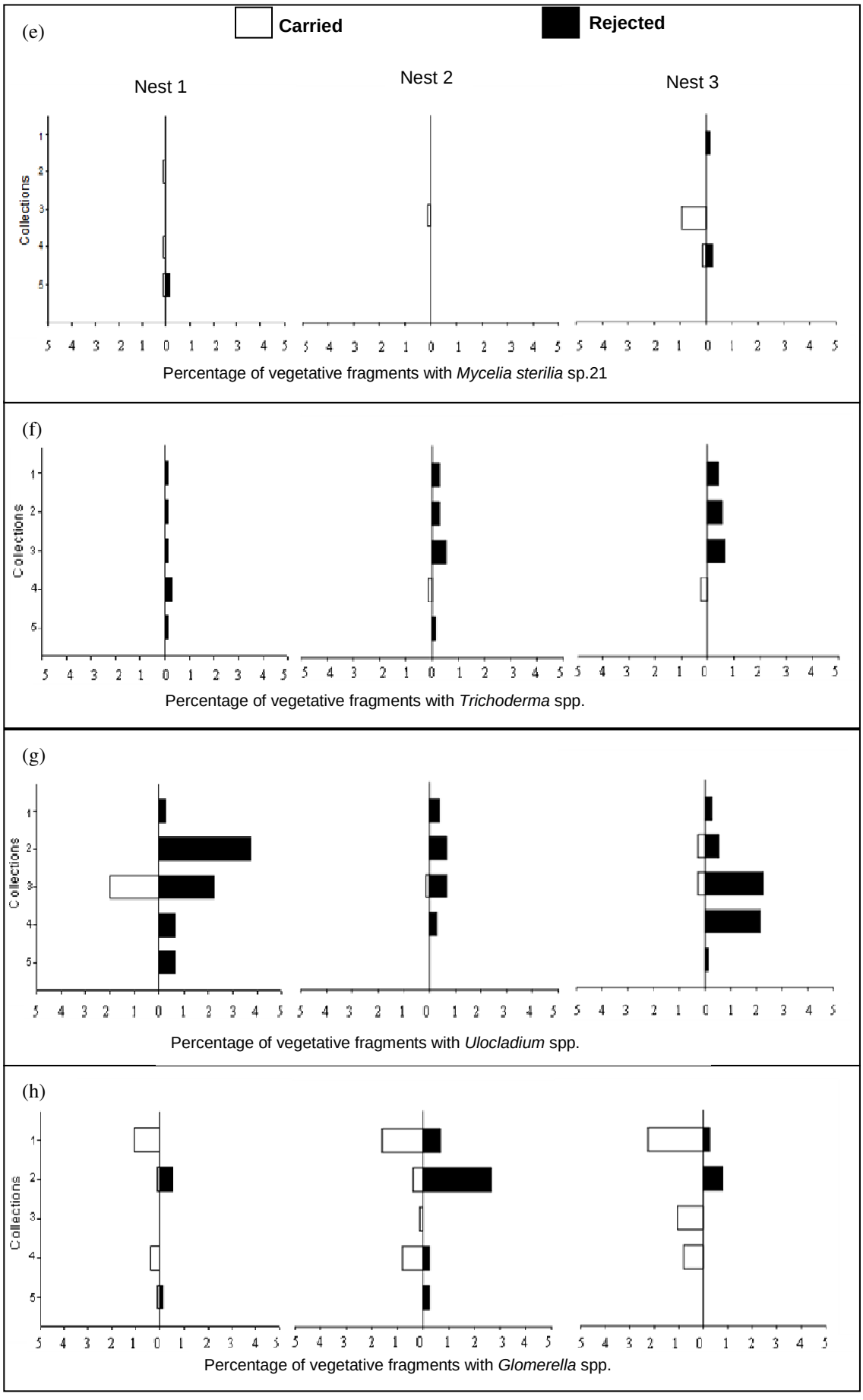
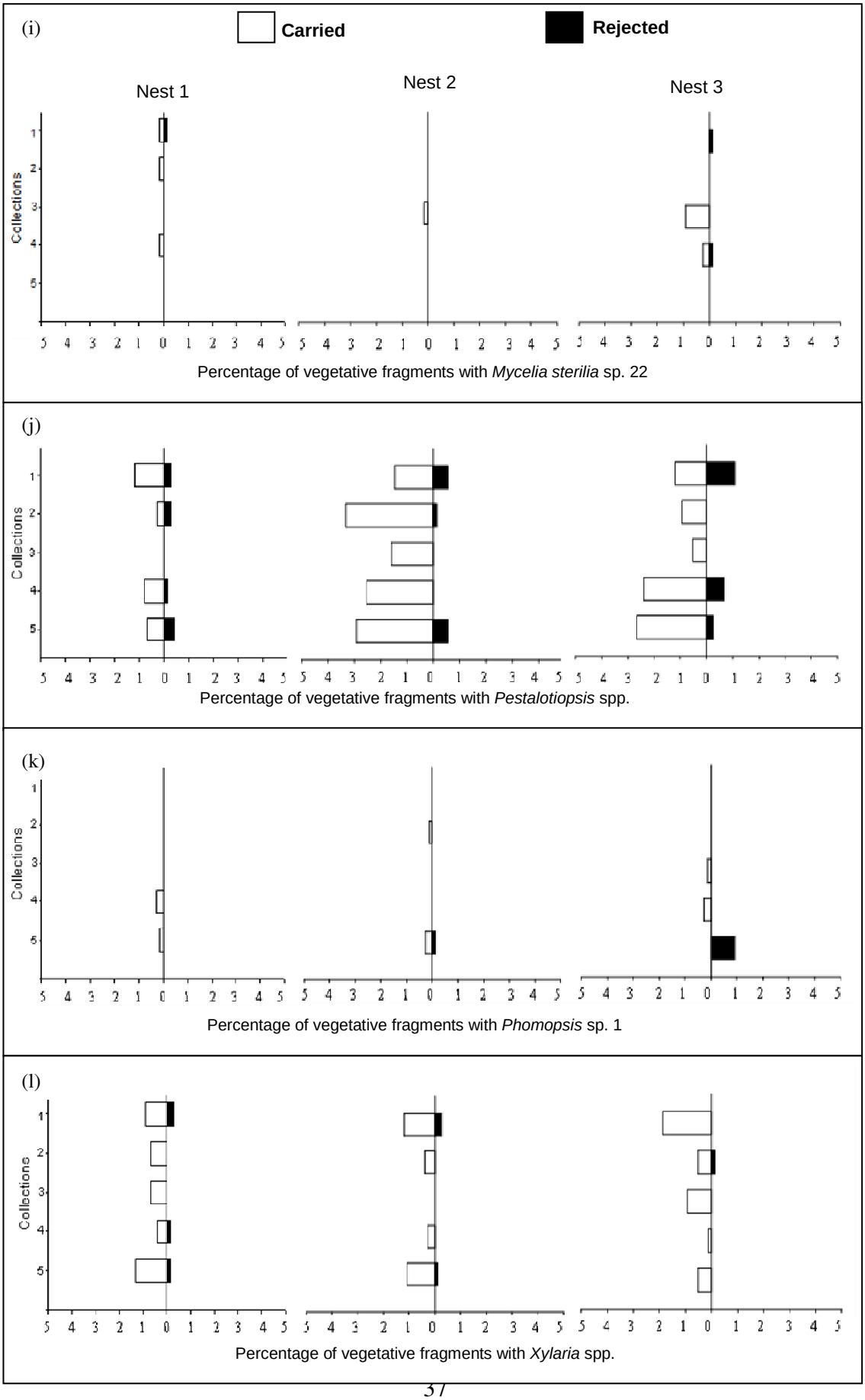


Figure 2 continued



Discussion

We conducted a survey of endophytic fungi associated with the leafcutter ant *A. laevigata* and found a high diversity; we found 33 genera, a result that corroborates other surveys of endophytic fungi from tropical areas (Arnold et al. 2000, Schulz and Boyle 2005, Arnold and Lutzoni 2007). From the 1,500 vegetative fragments analyzed, a total of 1,466 fungi were isolated, consisting of 1,159 isolates that sporulated and 307 that were *Mycelia sterilia.* Gamboa et al. (2002) found 11,7 ± 3,4 morphospecies of endophytic fungi per leaf in a survey of five tropical plant species, and Arnold & Herre (2003) isolated 13 morphospecies from 32 mm^2. If we do a calculation based on the total of fragments, the number of endophytic fungi per fragment was 1.95 in 1 mm^2, which would be low compared with the examples above. It is possible that the leafcutter ants are choosing leaves or cutting parts of leaves with less infection by endophytic fungi. So the diversity will be the same but the total of morphospecies was not. Of the genera found there were two of unusual occurrences these were the genera *Ulocladium* and *Trichoderma*. The genus *Ulocladium* is associated with roots and leaves of plants from desert and temperate areas (Suryanarayanan et al. 2005, Abdel-Motaal et al. 2010) and the genus *Trichoderma* has been studied with species inhabiting the soil (Weindling 1932, Brozová 2004) and more recently as endophytic in stems of *Theobroma cacao* and *T. gileri* (Evans et al. 2003, Bailey et al. 2006). Here, though, the isolates were associated with vegetative fragments cut by leafcutter ants and we cannot say if they were associated exclusively with the lamina or with the midrib that is an extension of the stem.

Associating with endophytic microorganisms the fitness of the plants can be affected positively or negatively. Positive effects include protection against pathogens, herbivores, and salt or water stress (Miller et al. 2002, Arnold and Herre 2003, Obledo

et al. 2003, Donoso et al. 2008). The negative effects may involve decreased photosynthetic efficiency and increased water loss from leaves under drought conditions (Pinto et al. 2000, Arnold and Engelbrecht 2007). Considering the possible roles of endophytic fungi when they are carried within vegetative fragments into the nest, it is possible that they have positive, neutral or negative effects. In the first case this would be associated with species that have action against bacteria and in this case the ants can use them against potentially harmful bacteria. The negative effect for the colonies would be with species that produce antifungal substances that would harm the mutualist fungi. Finally the neutral effect is when species do not present any action, being neutral, which would be exemplified by those species that belong to the genera that did not show a significant difference. It is important to note that identification of the isolates was done to genus level, so we do not know exactly the species involved in our survey. Further more is impossible to say if there was rejection or acceptance because of the presence of endophytic fungi because we did not do a choice test what would be possible if we inoculated fungi in plants and offered plants with and without inoculation for the ants.

Because the presence of endophytic fungi in vegetative fragments that have been carried is an indicator of acceptance, we can suppose that those genera may be helpful to the colonies, for example the genera *Glomerella, Mycelia sterilia* sp. 22, *Pestalotiopsis*, *Phomopsis* and *Xylaria.* Some species of *Xylaria* are producers of toxins that affect insects negatively (Azevedo et al. 2000) but here it seems that they do not affect leafcutters because the isolates of *Xylaria* were found more in carried than in rejected material. There is a theory that *L. gongylophorus* is capable of detoxifying vegetative material (Cherrett and Powell 1989, Nichols-Orians 1991) and probably the mutualistic fungus acts in the detoxification. Beyond producing some metabolites that are toxic to insects, some *Xylaria* produce substances with antifungal action (Hu et al.

2008) which would be harmful to the colony or which could be managed by the colony and used against invading fungi. And stated above, this could be used against bacteria as a tool to maintain the fungus garden free of enemies. Moreover, some genera of *Attini* have an association with a filamentous bacterium of the genus *Streptomyces* that produce substance that act against *Escovopsis* spp (Currie et al. 1999b, Currie 2001a). The other genus found that was more common in material that was carried than rejected was *Glomerella*. This has already been found in colonies of *Atta colombica* by Van Bael et al (2009). These authors noticed that these ants did not reject plants inoculated with *Glomerella cingulata* but just spent more time processing material that was carried more of the fungus and this could be interpreted as a lack of conflict between ants and endophytes. These authors suggested furthermore that the more time processing material with high density could be related to increased leaf toughness.

There is a different situation which is indicative of rejection because of the presence of some genera that possibly were causing damage inside the nests of leafcutter ants. This is the case of the genus *Cylindrocladium* which species has been studied because they are disease-causing (Coelho Neto et al. 2003, Ferreira et al. 2006). But there are also some endophytic species that benefit the host plant (Evans et al. 2003). This genus was also found in the waste of *A. cephalotes* (Fisher et al. 1996). In the present study, as we did not know the species involved we supposed that the rejection of vegetative material containing *Cylindrocladium* would be because of the competition for substrate if the species involved were diseasing-cause or because of some direct damage because it has a mycoparasitic nature.

The genus *Fusarium* was also found more in rejected material than expected. This fungus is reported causing disease in crops (Jeffries et al. 1990, Goswami and Kistler 2004, Silva and Bettiol 2005), but some species have been reported as endophtytic acting against nematodes (Altman et al. 2006, Mwaura et al. 2010), and

phytopathogenic fungi (Mandeel and Baker 1991, Fuchs et al. 1999, Silva and Bettiol 2005). So the indication of rejection could be associated with competition for nutrients or because of production of antifungal substances. Interestingly, the species *Fusarium pallidoroseum* was found in the waste of *Atta cephalotes* (Fisher et al. 1996).

The occurrence of the genus *Ulocladium* is a novelty in tropical areas. This fungus seems not be welcome in colonies of *A. laevigata*, maybe causing some damage to the mutualist fungus. Some species of *Ulocladium* have been shown to be antagonistic to fungi that cause disease in onion leaves such as *Botrytis* and the authors suggest that this antagonistic action can be associated with competition (Kohl et al. 1997). In other work, *Ulocladium chartarum* showed antagonism to some plant-pathogenic fungi (Abdel-Motaal et al. 2010). So, based on this information and on the fact that we did not identifiy the fungus to species level, we can suppose that the negative effects can be associated with competition or antagonism that might decrease the fitness of the mutualist fungus.

Like we expected in rejected material were found two genera with a history of mycoparasitism, *Epicoccum* and *Trichoderma*. So the "ability" to distinguish leaves containing endophytic fungus mycoparasite there seems to be. Beyond the capability of recognize the presence of endofictic fungi with negative effects, there are other defence strategies of leafcutter ants that can mitigate the negative effects of these fungi such as the removal of the mycelium or spores (weeding) if contaminated substrate has been incorporated into the fungal garden. Another is the cleaning of vegetative material before incorporation, since we found these fungi in both carried and rejected vegetative material, so we can hypothesize that these fungi cannot be detected or they were eliminated later.

Another genus that was found more in rejected vegetative material was *Drechslera*. Normally, this is associated with plant disease, especially in Gramineae

(Hodges 1980, Mathur and Bunker 2002, Mohan et al. 2009, Mohan et al. 2010), but it has also been found to be endophytic in medicinal plants (Deshmukh et al. 2010), producing antifungal substances (Abdel-Motaal et al. 2010), what can be the cause of the rejection by the ants The other genus that is in the same situation as *Drechslera* is *Pestalotiopsis* which hav been reported as prolific producers of bioactive substances, many of them showing antifungal activity against plant pathogens (Tejesvi et al. 2005, Inacio et al. 2006, Verma et al. 2007, Ding et al. 2008), and here this seems to be accepted the leafcutter colonies.

Pestalotiopsis species are broadly distributed in the world and show different lifestyles (Wei et al. 2007): as saprobes (Ho and Hyde 2002), as degraders of plant material (Osono and Takeda 2000, Tokumasu and Aoiki 2002), as plant pathogens (Zhang et al. 2003, Serra and Coelho 2007, Keith 2009) or as endophytes (Ding et al. 2009, Liu et al. 2009b, Liu et al. 2009c, Liu et al. 2009d). They have been reported producing secondary metabolites with antimicrobial action (Li et al. 2001, Strobel 2002, Harper et al. 2003, Li et al. 2008, Xu et al. 2010). Based on this information we can hypothesize that some of these metabolites may affect the mutualistic fungi because but the occurrence here of *Pestalotiopsis* was more in material that was being foraging. So either there is no negative effect or if there is this is negated by the ants that have a powerful mechanical defence. Another hypothesis is that the ants would be using *Pestalotiopsis* against other microorganismos.

Some species of *Phomopsis* are reported to negatively affect insects, for example, against the beetle *Physocnemum brevilineum*, through the production of a toxic compound (Webber 1981), or to have moderate antifungal activity against *Cladosporium* (Corrado and Rodrigues 2004, Silva et al. 2006b, Wijeratne et al. 2006, Huang et al. 2008, Borges et al. 2009). Here were found two species of *Phomopsis*, one of them (sp. 2), was found in both kinds of vegetative fragments and seemingly, not

appear to affect the activities of the leafcutter ants (*G*-test= 1.55), perhaps the compounds of this specie are species-specific, not affecting the ants or the fungal gardens. But the other specie (sp. 1) was in more material foraging (Fig 2-k), maybe the compounds of this be used to control inavaders like *Cladosporium* sp.

Other fungus that had presence in vegetative fragments rejected and that were foraging was *Acremonium* spp., that are among the unspecialized, widespread soil-borne fungi that are horizontally transmitted. This genus is predominantly restricted to the root systems of host plants (Raps & Vidal 1998; Jallow, Dugassa-Gobena & Vidal 2004), but here it as isolated from leaves and stmens. Some species of *Acremonium* have previously been reported to have antifungal (Mmbaga et al 2008) and antibacteria activity (Xu et al 2008). Here species of the genus *Acremonium* were present in material rejected and foraged (Fig 1) we can hypothesized that there is no harm for the colony, although we did not the identification of the species.

The genus *Colletotrichum* is found frequently as an endophyte in tropical plants (Cannon and Simmons 2002, Suryanarayanan et al. 2002). Members of the genus are producers of antifungal metabolites (Inacio et al. 2006), as well as having entomopathogenic properties causing disease in insects of the order Hemiptera (Marcelino et al. 2008b, Marcelino et al. 2009). Here it cannot be noticed or the ants can negate its action, because it was found in vegetative material reject and foraging. *Colletotrichum* which has been reported as prolific producers of bioactive substances, many of them showing antifungal activity against plant pathogens (Tejesvi et al. 2005, Inacio et al. 2006, Verma et al. 2007, Ding et al. 2008). Considering colonies about the presence of *Colletotrichum*

It is possible to analyze the situation from another perspective, wherin the main factors that are involved in the relationship between the choices of leafcutter ants based on the presence of endophytic fungi can be the changes in morpho-physiology of the

plants that harbour them. In the case of *Trichoderma*, for example, the endophyte can protect its host directly through parasitism, antibiosis or nutrient competition, and may also benefit the host plant by enhancing growth or through induced resistance (Howell 2003, Harman et al. 2004, Holmes et al. 2004, Khan et al. 2004, Bailey et al. 2008). Based on the fact that *Trichoderma* can promote plant growth, there is the possibility that the leafcutter ants harvest the material "considering" the plant's vigour. Or if some genera found here were phytopathogenic and cause wilt. In a study by Vasconcelos & Cherrett (1996) it was noticed that leafcutter ants *Atta laevigata* preferred wilted leaves not caused by fungi but naturally. But considering the presence of *Trichoderma* spp. Seems that the characteristics of the plants were not more important for the colony than the healthy because the were more *Trichoderma* in rejected material.

Other important point to discuss here is the absence of the genus *Escovopsis* (Ascomycota: anamorphic Hypocreales) as this is considered to be a major pathogen of the fungal mutualist (Currie 2001b), being commonly found associated with fungus gardens in decline (Currie et al. 1999a, Currie et al. 2003). The action of these fungi against the colony can be direct when it consumes the mutualist, or indirect, by decreasing colony survival and reproduction (Currie et al. 1999a, Currie 2001b, Reynolds and Currie 2004). The transmission of this parasite to the colony was verified for Currie et al (1999a) in laboratory colonies of *Atta colombica.* They concluded that the inoculum of the mutualistic fungus and the founding fungal gardens were free of *Escovopsis* and, based on this observation, they argued that the fungus is horizontally transmitted. There is preliminary work by Pagnocca et al. (2008) that supports the theory of horizontal transmission but this has not been confirmed. An alternative hypothesis is that transmission of *Escovopsis*, as well as other mycoparasites such as *Trichoderma*, could be via the cut plant material as endophytes. Although we did not find endophytic *Escovopsis* this possibility cannot be discarded and if the species of this

genus has a slow growth and may not compete with others fungi when in rich media so the endophytism of *Escovopsis* must be investigated with other tools such as genetics, for example.

It is important to note that the data showed here are results from collections, not from choice tests, because of this it is necessary to do tests of choice in future work in order to know the role of endophytic fungi in choices of leafcutter ants. Moreover the data were collected fortnightly and this can cause some interference and because of this the data are presented in graphics by nest and by collection (Figure 2) in order to verify the collections over the week.

The conclusion is that through this research there is a diversity of genera of endophytic fungi associated with leafcutter ants including mycoparasite genera as *Trichoderma* and *Epicoccum.* Some genera were associated more with fragments that were in middens and others with carried material and that this can be an indication that endophytic fungi may affect the choice of leafcutter ants. Although we know which genera are associated with vegetative fragments that were being carried or in middens, we do not know the real role of these fungi in the choice of leaves by the ants and to be certain if the presence of fungi affect the choice would be necessary to do tests of choice. Besides what we observed was outside the nests we do not know exactly the role of each endophytic fungi inside the colonies. This research paves the way for further study (1) verifying the endophytic fungi associated with other species of leafcutter ants (2) testing the endophytic fungi found, *in vitro* with *L. gongylophorus* and (3) testing choice using plants inoculated with endophytic fungi. This research may allow one to verify possible biocontrol agents of leafcutter ants that can be not exactly killing the ants but perhaps protecting the plants by making it unwelcome inside the nest.

Chapter 3

Do plants emplodophytes as bodyguards against herbivory? Leafcutter ants may carry endophytic mycoparasites towards their fungus gardens

Abstract

There is growing evidence of the importance of symbiotic micro-organisms to their hosts. Symbiotic fungi can have an important role in plant communities and in leafcutter ant survival. Here we consider endophytic fungi, micro-organisms which for all or part of their life cycle live in plant tissues and cause unapparent and asymptomatic infections and leafcutter ants and their mutualist fungi. The plants that are cut by the ants can harbour a diversity of endophytic fungi that can be mycoparasite or producers of antifungal or anti-herbivore products, for example fungi of the genus *Trichoderma* whose endophytic capacity has recently been demonstrated. This genus is studied because of the capacity of some species to act as a mycoparasite against phytopathogens, being used as a biocontrol agent in a diversity of crops. Considering the mycoparasitism and endophytism of *Trichoderma*, this study was conducted in order to investigate its interaction with leafcutter ants and their mutualist fungus. A further objective was to investigate *Trichoderma*'s potential as a biocontrol agent against leafcutter ants. This research has three parts: (1) collections of vegetative fragments which were carried by foragers in order to know about the presence of *Trichoderma;* (2) *in vitro* experiments to determine the behaviour of isolates of endophytic *Trichoderma* found associated with *A. sexdens rubropilosa* when in paired culture with *Leucoagaricus gongylophorus* in different days of incubation (3) the endophytic capability of *Trichoderma* isolates in *Eucalyptus grandis* and *Ligustrum japonicum.* The results showed that there were more isolates of *Trichoderma* rejected than in carried material indicating that the presence of *Trichoderma* may affect the choice of leafcutter

ants about what to forage. Some isolates of endophytic *Trichoderma* have the growth affected by *Leucoagaricus gongylophorus* in different time of incubation showed that the fungi cultivated by the ants has some defence when in the presence of harmful fungi. Some *Trichoderma* isolates showed a weak endophytism in *Ligustrum japonicum* when they were re-isolated from leaves (isolate 5 and 33) or from the bud (isolate 7, 26, 28 and 49) and four isolates were shown to be not endophytic (1, 9 25 and 71). In *Eucalyptus grandis* no isolats were present in the bud, but four isolates (1, 5, 26 and 28) were present in the inoculated area (0), in leaves below (-1) and in leaves above (+2). Isolate 7 was the only that was not present in inoculated area but was re-isolated in leaves below (-1) and above (+2). Three isolates (9, 25 and 33) were re-isolated from the inoculated area and from the leaves below, and only isolate 71 showed no endophytism. This work showed three important points (1) there is a possibility of the presence of *Trichoderma* affecting the choice of the ants, (2) isolates of *Trichoderma* has their growth inhibited by *L. gongylophorus* and (3) some isolates have mycoparasitic endophytic fungi.

Key words: Atta sexdens rubropilosa, endophytism, Leucoagaricus gongylophorus, Trichoderma.

Introduction

There is growing evidence of the importance of symbiotic microorganisms to their hosts (Nee 2004). Prominent examples are microbial pathogens affecting plant and animal communities (Grenfell and Dobson 1995) and symbiotic fungi that have an important role in plant communities (van der Heijden et al. 1998, Hedlund et al. 2004) and insects leafcutting ant survival (Quinlan and Cherrett 1979, Mueller et al. 2001, Mueller et al. 2005, Caldera et al. 2009). Here we consider endophytic fungi, which appear to be ubiquitous associates of all species of plants (Faeth and Hammon 1997, Arnold et al. 2000, Rajagopal and Suryanarayanan 2000, Clay 2004). The term endophyte refers to an organism which for all or part of their life cycle lives in plant tissues and cause unapparent and asymptomatic infections (Wilson 1995).

Plants serve as substrate for the mutualist fungus *Leucoagaricus gongylophorus* of leafcutter ants (Basidiomycota: Agaricales: Lepiotaceae, Leucocoprinae) (Chapela et al. 1994, Mueller et al. 1998) and leafcutter ants can choose what to harvest to incorporate in their fungus garden. Although leafcutter ants are extremely polyphagous (Cherrett 1968), they show marked preferences for leaves of certain plant species, individuals and even within individual plants (Meyer et al. 2006). The causes of host plant selection in leaf-cutting ants can be related to leaf toughness and nutrient content, as well as the presence of attractive and deterrant secondary compounds (Nichols-Orians and Schultz 1989, Camargo et al. 2004, Meyer et al. 2006). According to Knapp et al.(1990) leafcutter ants appear to have two kinds of selection: one that may occur when some leaf compounds are harmful to the ants and the other when the substrate is deleterious to the cultivated fungus. The plants can be a route for micro-organisms that are harmful to the mutualist fungi to enter the fungus garden, potentially including

mycoparasitic endophytic fungi. Studies of the interaction between leafcutter ants and plants harboring endophytic fungi are at an early stage. (Van Bael et al. 2009)

Endophytic, mycoparasitic fungi could be used by plants against leafcutter ants, acting indirectly against the ants' mutualist fungus. A candidate for this is *Trichoderma*. This mycoparasitic fungus has been studied extensively because of its potential as a biological control agent against plant disease (Martins-Corder and Melo 1998, Howell 2003, Samuels et al. 2006, Almeida et al. 2007). This potential is due to competition for nutrients, the production of antifungal metabolites and cell wall degrading enzymes (Lorito et al. 1996, Sivasithamparam and Ghisalberti 1998). Furthermore, *Trichoderma* can induce systemic acquired resistance (Enkerly *et al.*, 1999), solubilization of inorganic plant nutrients (Altomore et al. 1999) and growth promotion (Harman et al. 2004).

In this context, and considering the interaction between two pairs of organisms (leafcutter ants and their cultivated fungus on one hand, and trees of the Atlantic Forest and endophytic fungi on the other), one objective of this study was to evaluate the presence of endophytic *Trichoderma* fungi that was being carried into the nest within vegetative material or that was in middens constituting rejected material. Another objective was to use the isolates of *Trichoderma* found in paired culture with *Leucoagaricus gongylophorus* in order to verify how would be this interaction, expecting that as a mycopasite, *Trichoderma* isolates would affect negatively *L. gongylophorus.* The third objective was to verify the endophytic capacity of these *Trichoderma* isolates in the plants *Ligustrum japonicum* and *Eucalyptus grandis*.

Materials and methods

Trichoderma in vegetative fragments from *Atta sexdens rubropilosa* nests

Field work was conducted in two fragments of Atlantic Forest (Mata Atlântica) – seasonal, subtropical, semi-deciduous, montane forest. One was "Mata do Seu Nico", owned by Antônio Manoel de Freitas, Sítio BonSucesso, Viçosa, Minas Gerais State 20º45'23''Suth and 42º 52'23" West, Brazil, 750m altitude and the other was located at the Federal University of Viçosa, Mata do Paraíso, 20°48'07"South and 42°51'31"West, mean altitude 690m. Samples of leaf material were conducted at six colonies of *Atta sexdens rubropilosa*, three from each site. Ten night-time collections were undertaken from each nest in the period from January to February 2010. Each collection consisted of 50 leaf fragments that were being carried towards the nest by foraging workers and 50 rejected fragments that were found on refuse piles (or middens) at the nest entrances. A total of 3,000 vegetative fragments were collected, 1,500 being carried and 1,500 that were in middens at the nest entrance. The collected material was kept overnight in a refrigerator to be processed the following morning.

All material was surface-sterilized as follows: fragments were washed twice in sterile/ tap water then immersed for 1 min in 70% (v/v) ethanol, 4 min in sodium hypochlorite (5% v/v) and then washed three times in sterile distilled water. After surface-sterilization, the samples were aseptically transferred to plates containing V8 vegetable broth agar (VBA) (Pereira et al. 2003) containing the antibiotic terramycin (Pfizer). Sealed plates were incubated at room temperature (25±2 °C). An initial screening for *Trichoderma* isolates was conducted macroscopically, based on a green colony colouration typical of the genus. This preliminary identification was subsequently confirmed microscopically based on the characteristic sporulation of *Trichoderma*.

The presence of *Trichoderma* was also verified in the leaves of plants from the foraging area of each nest that had been examined. The collections were made randomly from plants around each of the six marked ant colonies, in the period from February to March 2010. Two old and two new leaves of 20 plants from each area were collected (for a total of 120 plants or 480 leaves). Four segments (each 5 mm^2) of each part (new and old) were cut from the middle lamina of each leaf and surface-sterilized through sequential immersion in 70% ethanol (1 min), 5% sodium hypochlorite (4 min). Sterilized leaf pieces were placed on VBA and were incubated at room temperature (25±2 °C) for detection of *Trichoderma* isolates as described above.

Experiments in vitro: *Trichoderma* isolates versus *Leucoagaricus gongylophorus*

The fungus *L. gongylophorus* was isolated from a nest of *A. sexdens rubropilosa* originally taken from the field near to the study sites and maintained in the laboratory. For this, pieces of the fungal garden were taken and aseptically transferred to plates containing culture media composed of peptone, sodium chloride, malt extract, glucose, oat flakes, agar, distilled water up to 1 L and chloranphenicol, autoclaved at 120ºC and 1.1 atm for 30 min. Cultures were maintained in an incubator (25±2°C) for one month, due to the slow growth. Plugs (1 cm diameter) were taken with a cork borer from the colonies and aseptically transferred to the edge of fresh VBA (one per plate). Half of these were incubated for 15 days and the other half for 30 days (at 25±2°C).

Five isolates of *Trichoderma* (isolates 5, 9, 26, 32 and 65) were used in the *in vitro* experiments to assess the interactions with *L. gongylophorus*. These isolates were found as endophytes in the leaves (in the sampling described above) and were grown on potato carrot agar for seven days. A plug of each isolate (1 cm diameter) was placed on the opposite edge of the plates colonized by *L. gongylophorus* (both 15- and 30-day old plates were used) while control plates were set up by placing a plug containing only

media (1 cm diameter) on the opposite edge of the *Trichoderma* isolate. After that, measurements of radial growth of *Trichoderma* were taken every 24 hours. Each combination of *Trichoderma* isolate with 15- or 30-day old *L. gongylophorus* was replicated 5 times, as were controls.

Testing the endophytism of *Trichoderma* isolates in *Ligustrum japonicum* and in *Eucalyptus grandis*

Ten isolates of *Trichoderma* that were found associated with *Atta sexdens rubropilosa'* nests were tested for endophytism in two plant species. Two plants were used: *Ligustrum japonicum* (Oleaceae) and *Eucalyptus grandis* (Myrtaceae). For the experiments with *L. japonicum*, seeds were collected in August 2010. They were washed, the pulp was removed and the seeds were then left to dry in the shade for two days and scarified to facilitate germination. The seeds were sterilized in sodium hypochlorite (5% v/v) for one minute and washed in sterile water to remove residues of hypochlorite. After surface sterilization, the seeds were placed in plastic boxes (gerbox), previously sterilized with hypochlorite and containing filter paper sterilized and moistened with stile water. These were checked daily. Following germination (radicles ca. 1 cm in length), the geminated seeds were immersed in a solution of spores of *Trichoderma* that were prepared with sterile distilled water and Tween 80. For the control, the germinated seeds were suspended in sterile distilled water containing Tween 80 only. After inoculation, the germinated seeds were placed in a humid chamber for 48h to provide optimal conditions for spore germination and penetration of the seed tissues. The treated seeds were then individually planted in pots and transferred to a greenhouse with overhead irrigation (10 min two times a day). The seedlings were kept in the greenhouse for 30 days, after which five seedlings from each treatment (plus control) were analyzed for the presence of *Trichoderma* as follows. The bud and the last

pair of the youngest leaves (Figure 1) were removed and taken to the laboratory. Four segments (each 5 mm^2) of each part were cut from the middle lamina of each leaf and surface-sterilized through sequential immersion in 70% ethanol (1 min), 5% sodium hypochlorite (4 min) and sterile water. Sterilized leaf pieces were placed on VBA, and were incubated at 25±2°C.

The inoculation protocol with *Trichoderma* in *E. grandis* differed from that for *L. japonicum* because seedlings were used. The same isolates of *Trichoderma* were used however. A total of 150 seedlings, of approximately 15cm height, were acquired from a forestry nursery (Viveiro de Mudas do IEF, Viçosa-MG). These were inoculated by brushing spore suspensions (see above) on the shoot (apical bud and first two leaves). Control shoots were brushed with SDA containing Tween 80. All treated shoots were covered with plastic bags for 48 h. After that, the plastic bags were removed and plants were placed in ar irrigated greenhouse. After one month, five seedlings of each treatment were analyzed for *Trichoderma.* For this, four leaf pairs of each seedling were removed. These leaf pairs were: -1 (below the point of initial inoculation, 0 (point of inoculation) +2 (second pair of leaves above the point of inoculation and the apical bud) (Figure 3 B). As above, four segments (each 5 mm^2) were cut from the middle lamina of each leaf and were surface-sterilized through sequential immersion in 70% ethanol (1 min), 5% sodium hypochlorite (4 min) and sterile water. Sterilized leaf pieces were placed on VBA and were incubated at 25±2 °C.

Statistical analyses

In order to verify if the frequency of endophytic *Trichoderma* differed between the vegetative material that was been carried or that had been rejected by *A. sexdens rubropilosa*, tests of independence were conducted, incorporating *G*-tests (Sokal and Rohlf 1995). For the *in vitro* experiment, comparisons were made of mean radial growth

of *Trichoderma* isolates in the presence vs. absence of *L. gongylophorus* using two-tailed *t*-tests. Data from the third day of growth were used as these presented the largest differences. Finally, in the experiments of the endophytic capability of *Trichoderma*, only the presence or absence of the isolates in each selected leaf part of *L. japonicum* and *E. grandis* was recorded.

Results

Trichoderma in vegetative fragments from *Atta sexdens rubropilosa* nests

Of a total of 3,000 vegetative fragments collected from six nests of *A. sexdens rubropilosa*, we found sixty-six *Trichoderma* isolates. Of these isolates, 8 (12.12%) were in material that was being carried in to the nest and 58 (87.88%) were in the middens consisting of rejected material (Figure 2), this representing a significant difference (Value of G = 73.02567 greater than $\chi^2_{[d.f.=1]}$=3,84, p=0.05. In the collections of leaves from plants around the foraging areas of the six nests, *Trichoderma* was isolated only from old leaves of two plants in one of the forest fragments (Mata do Paraíso).

Experiments in vitro: *Trichoderma* isolates versus *Leucoagaricus gongylophorus*

In all interactions between the ants' mutualist fungus *L. gongylophorus* and isolates of *Trichoderma*, the growth of *Trichoderma* isolates was reduced in the presence of *L. gongylophorus* (Figure 3). This result was consistent when the 15-day old *L. gongylophorus* was used (comparisons with controls at day 3; isolate 5: $t_{1,8}$ = -19.29, p<0.0001; isolate 9: $t_{1,8}$ = -4.42, p=0.0011; isolate 26: $t_{1,6}$ = -2.89, p=0.0314; isolate 32: $t_{1,8}$ = -5.39 p=0.0008 and isolate 65: $t_{1,8}$ =-15.03, p<0.0001). The same pattern was observed when *Trichoderma* was paired with 30-day old *L. gongylophorus*

(comparisons with controls at day 3: isolate 5: $t_{1,8} = -26.80$, $p<0.0001$; isolate 9: $t_{1,8} = -19.92$, $p<0.0001$; isolate 26: $t_{1,8} = -31.39$ $p<0.0001$, isolate 32: and $t_{1,8} = -2.65$, $p=0.0164$ and isolate 65: $t_{1,8} = -4.11$, $p=0.0017$).

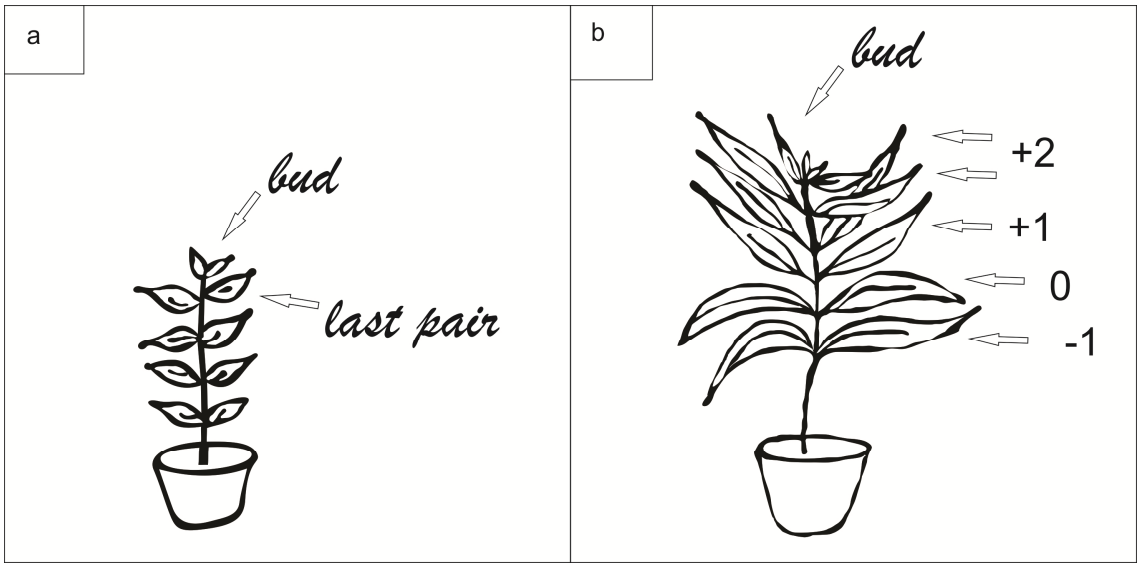
In some of the interactions between *L. gongylophorus* and *Trichoderma*, an inhibition halo was observed, probably indicating that *L. gongylophorus* has some defence against some *Trichoderma* (Figure 3 a, b, d, f, g, h, and j).

Testing the endophytism of *Trichoderma* isolates in *Ligustrum japonicum* and in *Eucalyptus grandis*

Ten isolates of *Trichoderma* were tested for endophytism. In *L. japonicum*, we re-isolated six isolates (5, 7, 26, 28, 33 and 49) (Table1). Of these, isolates 5 and 33 were isolated from the last youngest leaves in only one repetition; isolates 7, 26, 28 and 49 were re-isolated from the bud in only one repetition except for the last isolate when it was re-isolated three times. The isolates were not found in control plants (Table 1).

In eucalyptus seedlings none of the isolates of *Trichoderma* were re-isolated from the bud (Table 1). Five isolates (1, 5, 7, 26 and 28) were re-isolated from the area of inoculation (0) and above (+2) and below it (-1) (Table 1). For three isolates (9, 25 and 33) the re-isolation occurred in inoculated area (0) and below it (-1) (Table 1). The isolate 49 was not re-isolated from inoculated area but was re-isolated from the leaves below it (-1). Only isolate 71 was not re-isolated at all (Table 1).

Figure 1 – Scheme showing the parts of the plants that were used to do the re isolation of *Trichoderma* isolates in (a) *Ligustrum japonicum* and (b) *Eucaliptus grandis*.



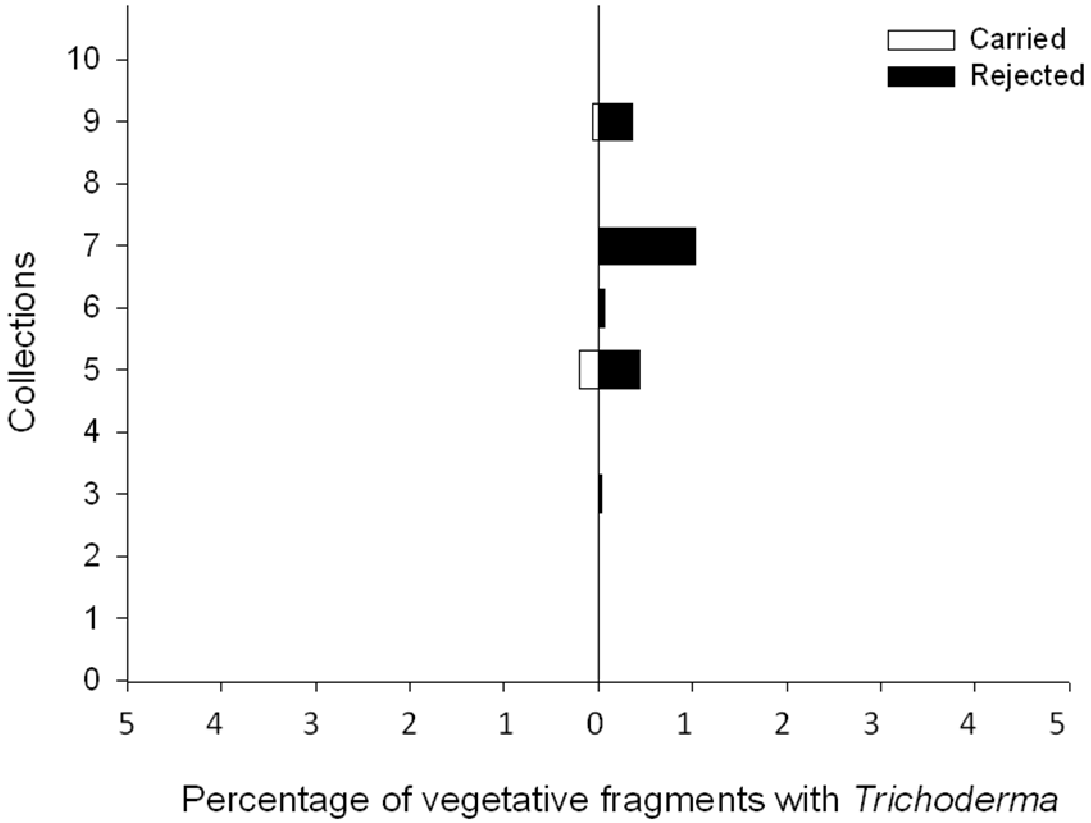
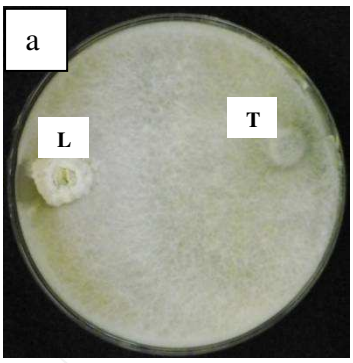
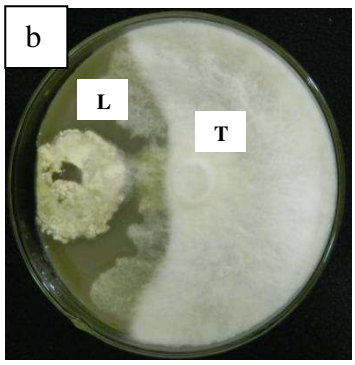
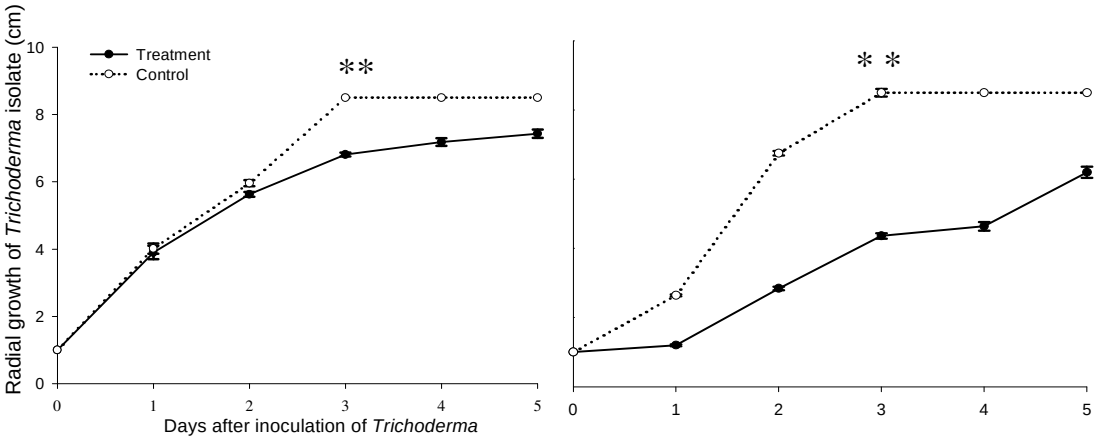


Figure 2 – Frequencies of occurrence of *Trichoderma* in vegetative fragments that were being carried towards the nest by *Atta sexdens rubropilosa* workers ('carried' on the left) or that had been rejected and were found on middens outside entrances ('rejected' on the right). Shown are percentages of fragments bearing the genus *Trichoderma* found in ten weekly collections.

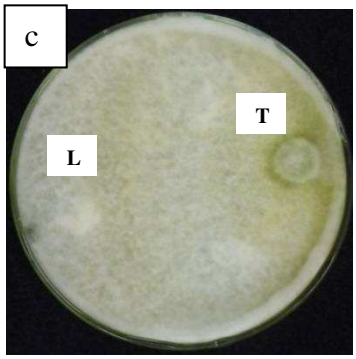
Isolate 5

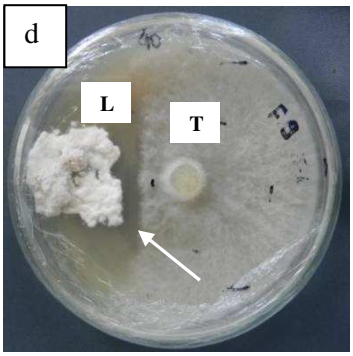


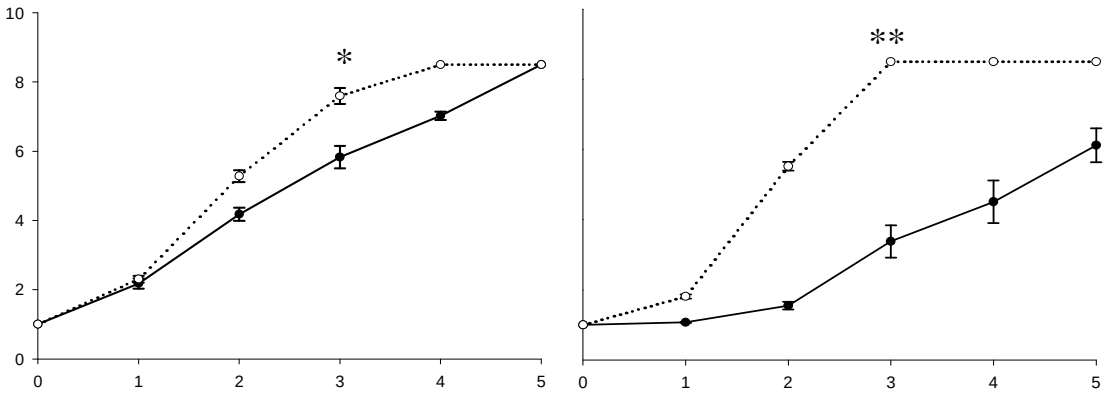




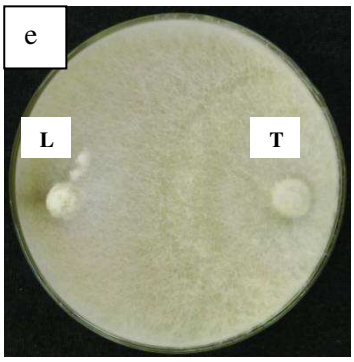
Isolate 9

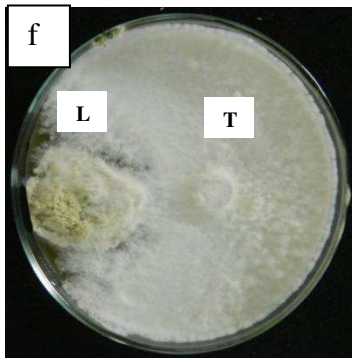


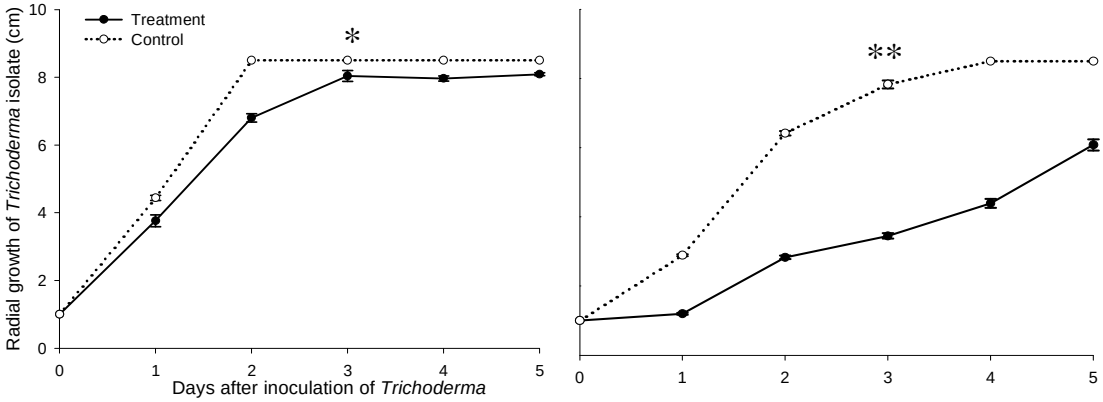




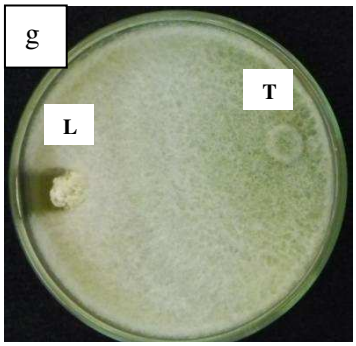
Isolate 26

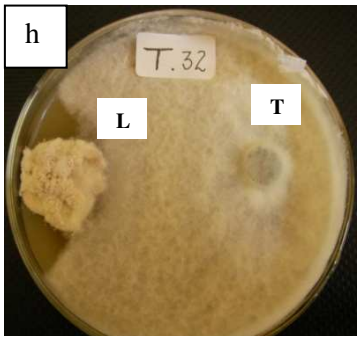


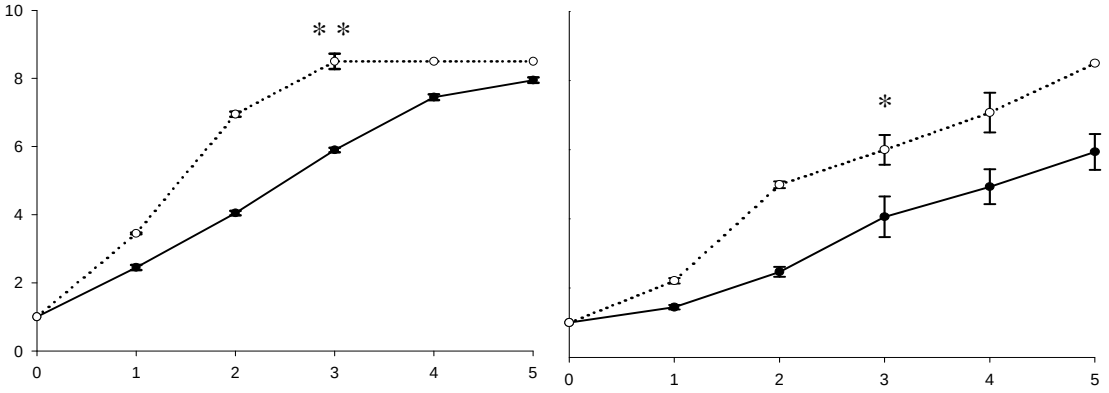




Isolate 32







Continuation

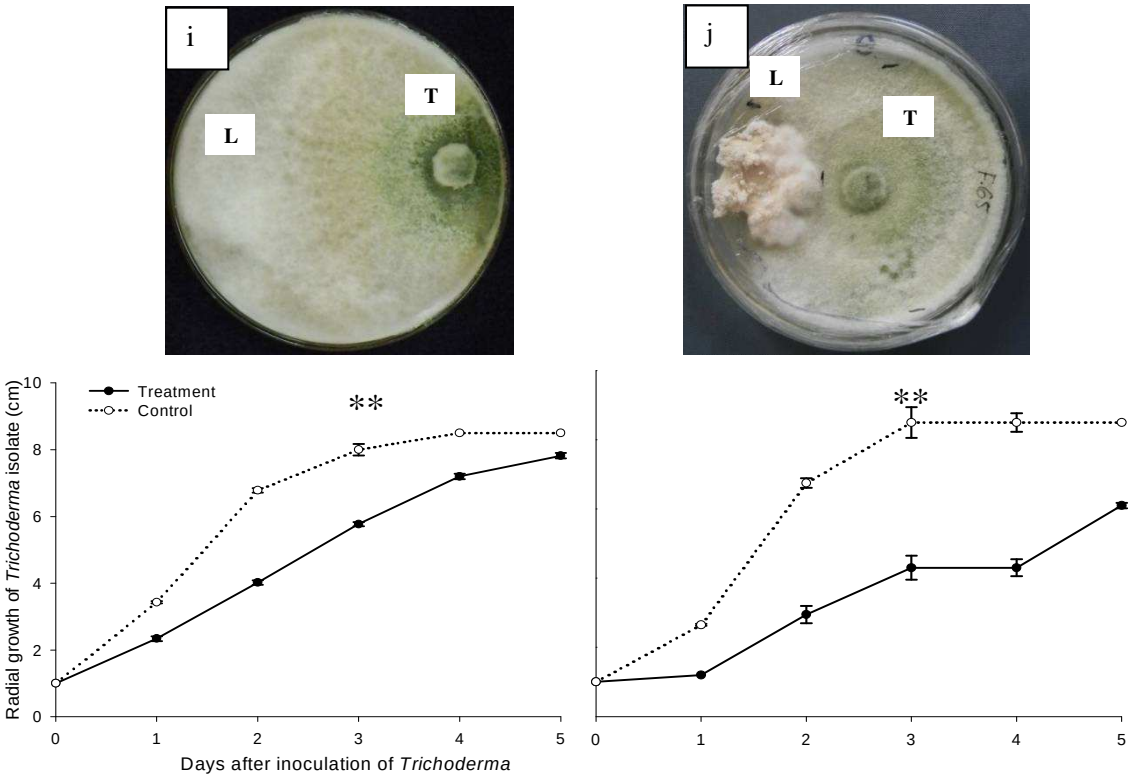


Figure 3 – Mean radial growth of *Trichoderma* isolates in paired cultures with *Leucoagaricus gongylophorus* of 30 days of incubation. **Anova** was done comparing the growth in the third day between treatment and control. The error bars are of the growth. L = *L. gongylophorus*; T = *Trichoderma.* Arrows indicate zones of inhibition. Means with (*) and (**) are significantly difference based on t test ($p \leq 0,05$ and $p \leq 0,001$ respectively).

Table 1 – *Trichoderma* isolates that were re re-isolated from seedlings of *Ligustrum japonicum* and *Eucalyptus grandis* inoculated with different isolates of *Trichoderma* and without *Trichoderma* (control). The parts of plants in *L. japonicum* were the bud and the pair of leaves below the bud. In *E. grandis* four parts was analyzed the pair of leaves that was inoculated (0), the pair above the leaves that were inoculated (-1), the pair of leaves above those that were inoculated (+1), the pair above the pair +1 (+2) and the bud

	Ligustrum japonicum		*Eucalyptus grandis*			
			Leaves			
Trichoderma Isolates	Bud	First pair of leaves	-1	0	+2	Bud
1	0/5	0/5	4/5	2/5	2/5	0/5
5	0/5	1/5	1/5	4/5	1/5	0/5
7	1/5	0/5	2/5	0/5	1/5	0/5
9	0/5	0/5	2/5	5/5	0/5	0/5
25	0/5	0/5	4/5	3/5	0/5	0/5
26	1/5	0/5	3/5	3/5	2/5	0/5
28	1/5	0/5	3/5	5/5	5/5	0/5
33	0/5	1/5	4/5	4/5	0/5	0/5
49	1/5	0/5	4/5	0/5	0/5	0/5
71	0/5	0/5	0/5	0/5	0/5	0/5
C	0/5	0/5	0/5	0/5	0/5	0/5

Discussion

Isolates of endophytic *Trichoderma* were found more in material rejected than in that carried by *A. sexdens rubropilosa* (Figure 2). It is possible to consider that (1) *Trichoderma*. can be causing damage to the *L. gongylophorus* and because of this the fragments that contained it were discarded and (2) plants are using *Trichoderma* as a bodyguard. Is important to note that this is a survey and we did not do a choice test so the results are indicative that the fragments were rejected because of the presence of *Trichoderma*. Ortiz and Orduz (2000) noticed that some isolates of *Trichoderma* inhibited the mycelia growth of *Attamyces* sp. = *Leucoagaricus gongylophorus.* Furthermore *Trichoderma* affects negatively commercial crops of the mushroom *Agaricus bisporus* (Agaricaceae) through competitiveness for substrate (Chen et al. 1999, Hatvani et al. 2007), attack via antibiotic secretions that inhibit growth (Krupke et al. 2003), production of hydrolytic enzymes such as chitinases, glucanases, and proteases that degraded fungal cell walls.

Observations of leafcutter ants making choices among available leaves showed that these insects have strategies in the process of collection (Hubbell et al. 1980, Hölldobler and Wilson 1990). In general, they choose leaves based on leaf age (Rockwood 1976), moisture content (Cherrett 1972), leaf toughness (Nichols-Orians and Schultz 1989), secondary toxic components (Barrer and Cherrett 1972), nutrient content (Berish 1986), vegetative material that not contain materials unsuitable for the mutualistic fungus (Knapp et al. 1990, Verza et al. 2007). More recently, it has been suggested that endophytic fungi cause damage as the leafcutters spend more time preparing leaves (Van Bael et al. 2009). Leaves that were infiltrated with the fungicide

cycloheximide were avoided by the leafcutter ants *Acromyrmex lundi* after 10 hours that the plants were offered (Herz et al. 2008).

According to Knapp et al (1990) and North et al (1997) leafcutter ants maybe are capable beyond to detect directs changes in fungus garden may to respond a semiochemicals produced by it. Moreover, the rejection by some material seems to be more related to alterations in *L. gongylophorus* than changes in the quantity and/or quality of the nutritious gongylidia (Herz et al. 2008). Based on this information, our results may indicate rejection of leaves because of the presence of *Trichoderma*, due to negative effects on *L. gongylophorus*. To investigate this possibility further would require for example offering plants inoculated with *Trichoderma* as substrate and observing the behavior of the colony manly the mutualist behavior.

The protection against herbivorous insects that may be provided to plants by endophytic fungi (Howell 2003) has been studied principally for grasses (Cheplick and Clay 1988, Clay 1990, Clement et al. 2005, Schardl et al. 2007, Zhang et al. 2009). According to these authors, this protection is related to the mode of transmission of the endophyte that in grasses is is almost exclusively vertically. This is considered an important requisite for mutualism although there are some situations of mutualism without vertical transmission as in mycorrhyza (Herre 1999). Because of this, there are doubts that plants may be protected from herbivores by endophytic fungi that are horizontally transmitted, but examples have been found (Webber 1981, Bing and Lewis 1991, Azevedo et al. 2000, Hartley and Gange 2009), including endophytic fungi producing metabolites that are toxic to insects (Johnson and Whitney 1994). Thus, the possibility of plants using *Trichoderma* as a bodyguard against leafcutter ants would be associated with indirect effects.

Following the survey of *Trichoderma* in vegetative fragments of *A. sexdens rubropilosa* colonies, we conducted *in vitro* experiments in order to see how these

isolates interact with *L. gongylophorus*. From our results it was possible to see that *L. gongylophorus* has some from of defence against *Trichoderma* that was independent of the time of incubation (15 or 30 days) (Figure 2). According to Silva et al. (2006b) the growth of *L. gongylophorus* was negatively affected by *Trichoderma harzianum* but these authors concentrated on the initial and final colony diameters without considering the possible defence of *L. gongylophorus* in the days before the final day of the experiment. Van Bael (2009) showed that the mutualist fungus of leafcutter ants inhibited the growth of the endophytic fungus *Glomerella cingulata*, 48 hours after the start of the experiment via diffusible antibiotics and not through hyphal contact. Here we found that independent of time of incubation, *L. gongylophorus* has defence against all isolates of *Trichoderma* tested, so it is not totally dependent of protection of leafcutter ants. In experiments where plant material was infiltrated with fungicide, ants incorporated the material into the fungus gardens and rejection behaviours were only observed hours later (Herz et al. 2008). We therefore suppose that in the time it takes for ants to perceive the damage to their mutualist fungus, this fungus can protect itself.

Because of the possibility that *Trichoderma* has negative effects on *L. gongylophorus*, we decided to test the endophytism of our isolates, with the ultimate goal of developing a biological control product that could be inoculated into plants. Results of greenhouse tests showed that the endophytism of *Trichoderma* is different according to isolate and according to the plant (Table 1). In *Ligustrum japonicum*, using the inoculation in germinated seeds, endophytism of the *Trichoderma* isolates seems weak as it only occurred in the buds in single replicates for five isolates and in the first pair of leaves after the bud, again in single replicates, for two isolates. Maybe in this part of the plant it is probable that the plant did not recognize the presence of *Trichoderma* and the cascade of defence against invaders was not triggered. According to Rodriguez and Redman (1997), although they do not cause disease, endophytic fungi

keep the same genetic and biochemical mechanisms required for infection and colonization of host plants as plant-pathogenic fungi. Therefore, plants may recognize endophytic fungi as phytopathogenic and defend themselves.

The apparent weak endophytism of the *Trichoderma* isolates may be because of a failure of the inoculation method, germinated seeds. We used a different method, inoculation in seedlings of *E. grandis.* Another possible justification for the weak endophytism would be the specificity of *Trichoderma*. However, the relation between plants and endophytic fungi is considered to be to be less specialized than other symbioses (Yedidia et al. 1999).

When we inoculated *Trichoderma* in seedlings of *E. grandis*, unlike the case of *L. japonicum* plants, *Trichoderma* were not re-isolated from the bud. This is similar to the finding that endophytic fungi are most prevalent in old leaves in tropical plants (Lee and Collins 2001, Arnold and Herre 2003). For five isolates (1, 5, 7, 26, and 28) re-isolation was possible in leaves below and above the point of inoculation, leading us to suspect that these isolates are able to infect the plants systemically and travel up and down within them. In *Theobroma cacao*, isolates of *Trichoderma* colonized the tissues in the area of inoculation and distant tissues including root tips, roots, stems, plumules, and leaves (Bailey et al. 2008). Meanwhile, one isolate (71) seems not to be endophytic because it was not re-isolated from any part of the plant. But to confirm of non-endophytism in *E. grandis*, other methods of inoculation must be tested.

Considering the possibility of systemic infection of plants by *Trichoderma*, an important point to note is that the leaf fragments taken from the plants in the endophytism studies contained leaf midrib. Thus, it is not possible to say whether the re-isolated *Trichoderma* isolates were associated with the lamina or with the midrib. We also do not know if they were taken from leaf fragments containing midrib or not in the initial isolations from field material. A study has shown no significant difference of the

colonization rates of different species of endophytic fungi between lamina (9%) and midrib (14%) tissues (Sun et al. 2011). It is worth considering that leafcutter ants could easily choose to cut just the lamina, excluding the midrib.

In conclusion, we found *Trichoderma* as an endophyte in both carried and rejected material associated with *A. sexdens rubropilosa.* This work opens the possibility to use these fungi as bodyguards to protect crop plants against leafcutter ants, however, further experiments are necessary to confirm this relationship and to investigate further the processing of material by leafcutter ants below ground. Regarding the *in vitro* experiments, *L. gongylophorus* was able to inhibit the negative effect of some of the *Trichoderma* isolates for some days, possibly sufficient to communicate to its ant mutualists that the material is potentially harmful. Finally, endophytism of *Trichoderma* seems weak in *Ligustrum japonicum* and was moderate in seedlings of *Eucalyptus grandis* but other methodologies must be tested to be certain of endophytism and to manipulate this for plant protection. The broader implication of this work is the future possibility of using some of these endophytic fungal isolates as biological control agents of leafcutter ants through disruption of their fungal gardens.

Chapter 4

Infection of leafcutter ant colonies by the mycoparasitic fungus *Trichoderma* reduces worker survival

Abstract

Fungus-growing ants (Hymenoptera: Formicidae: tribe Attini) have a mutually beneficial symbiosis with a basidiomycete fungus that serves as their main food source. The success of this partnership depends on both partners. An important behaviour of the leafcutters ants (genera *Acromyrmex* and *Atta*) is to maintain the cultivated fungus free of natural enemies through behavioral defences. Meanwhile, there is an urgent need for new means of control of these ants, one possibility being the use of microbial pathogens and parasites. Here, we investigate the response of leafcutter ants when their harvested material is contaminated with the mycoparasitic fungus *Trichoderma*. The treatments were sterilized rice with and without spores and mycelium of *Trichoderma* and a citrus bait. This material was offered to queenless colonies of *Atta sexdens rubropilosa*. The rice with *Trichoderma* and bait was incorporated into the fungus gardens and decreased the survival of workers, although no changes in behaviour were observed. This suggests that *Trichoderma* can impose a cost on colonies. It also indicates that *Trichoderma* may have potential as a tool in the integrated pest management of leafcutter ants, perhaps by weakening the colony and increasing its susceptibility to the action of chemical insecticides.

Key-words: Biological control, leafcutting ants, mycoparasitism, *Trichoderma***.**

Introduction

Leafcutting ants (Hymenoptera: Formicidae: tribe Attini, genera *Atta* and *Acromyrmex*) have an agricultural symbiosis with fungi that originated 50-60 million years ago (Mueller et al. 2001), considerably older than human agriculture which is believed to have originated some 10,000 years ago (Kumar et al. 2006). The symbiosis between leafcutter ants and the cultivated fungus is an obligate mutualism and shows a net benefit to both partners. In this symbiosis, the role of the cultivated fungal symbiont, belonging to the family Agaricaceae (Agaricales: Basidiomycota) (Chapela et al. 1994, Schultz and Meier 1995, Mueller et al. 1998), is to act as food for the ants. In return, the ants provide the fungus with shelter, a means of dispersal to new locations, substrate for growth, and protection from competitors and parasites (Mackintosh et al. 1995, Ortius-Lechner et al. 2000, Lopez and Orduz 2003). The fungus cultivated by leafcutter ants is continuously at risk from antagonistic fungi that could be imported into the nest by the ants with the plant material that they collect or that can be acquired from the soil (Rodrigues et al. 2008a), especially by specialized or coevolved garden pathogens, such as the fungus *Escovopsis* (Currie et al. 1999a) . Despite the continuous presence of pathogenic and parasitic microorganisms in soil or in the foraged material, leafcutter ants are apparently capable to deal with these natural enemies (Hölldobler and Wilson 1990, Mueller et al. 1998).

The strategies adopted by fungus-growing ants to remove or suppress the growth of harmful microbes are a combination of actions: weeding and grooming nest parts (Currie and Stuart 2001), antimicrobial secretions of the ants (Bot et al. 2002, Rodrigues et al. 2008a) and the application of antibiotics derived from symbiotic bacteria (Currie et al. 1999b, Santos et al. 2004, Mueller et al. 2005).

Leafcutting ants are polyphagous herbivores and are considered important agricultural pests in Brazil. Routine control of leafcutter ants is conducted using chemical insecticides (Della Lucia and Araújo 2000). In general the use of chemical insecticide can cause serious problems as resistance, resurgence of primary pests, upsurges of secondary pests, and overall and environmental contamination (Kogan 1998). This has led some companies to restrict their use in order to obtain a green seal (Forest Stewardship Council, 2002). In the case of leafcutter ants, biological control could be used in three ways: one is direct, affecting the ants through insecticidal action; another is indirect, affecting the ants' mutualistic fungus, and a third is to protect plants from attack. The use of fungi as biocontrol agents of pest insects was first carried out in Russia in 1888 and since then this discovery has became as a source of inspiration for control of pests in others countries (Lord 2005). The control of leafcutter ants using entomopathogenic fungi has been studied using isolates of *Metarhizium* and *Beauveria* (Lima et al., 1986; Alves & Sosa Gómez, 1983; Silva & Diehl-Fleig, 1988; Blowers, 1994; Jaccoud, 1996) and using some fungi that are harmful to the mutualist fungi (Lopez and Orduz 2003).

Plants can harbor fungi that can be harmful to leafcutter ants such as endophytic fungi that live within plant tissues for all or part of their life cycle and cause no apparent harm to their plant host (Wilson 1995). A genus that can be danger for leafcutter ants with a history of mycoparasitism and has been found as endophytic is *Trichoderma* (Samuels et al. 2006). These fungi have been considered for the biological control of plant pathogenic fungi (Faeth 2002, Evans et al. 2003, Wicklow et al. 2005, Bailey et al. 2008). Meanwhile, *Trichoderma* has been extensively studied for control of plant diseases, using various mechanisms against the target organism, including mycoparasitism, antibiosis, competition and enzyme production (Howell 2003). Some

studies have been made using *Trichoderma* against leafcutter ants, but in these cases, the isolates were from soil (Lopez and Orduz 2003, Rodrigues et al. 2005).

Thus, the objective of the present study is to observe the action of *Trichoderma* in the behavior and survival of the mini-colonies. The behavioral traits executed by workers of *Atta sexdens rubropilosa* during the collection and preparation of substrate as well as the survival of colonies were determined We hypothesize that colonies challenged with substrate containing *Trichoderma* will change the behavior as increasing the foraging activity, increasing the quantity of harvest leaves and weight of waste and decreasing the time of survival of the colonies.

Materials and methods

Preparation of mini-colonies of *Atta sexdens rubropilosa*

Ten queenless mini-colonies of *A. sexdens rubropilosa* were used following a methodology adapted from Loeck et al. (1994). Before preparing mini-nests, the health of the colonies was analyzed; for this, samples were aseptically transferred to potato-carrot-agar (PCA) plates and incubated (25±1 °C). This was done in order to know if there was *Trichoderma*. in the colonies of origin. To prepare the mini-colonies, samples of approximately 350 ml of fresh fungal garden, containing ants and brood, were taken from 10 different colonies of *A. sexdens rubropilosa* and placed in plastic pots (350 ml volume), and covered with ventilated lids. Next, 2-cm diameter exit holes were made in the base to allow for the passage of worker ants for foraging. The pots were placed along the edges of trays (30 x 25 x 5 cm), whose sides were treated with talcum powder in order to prevent the escape of ants. These colonies were maintained at 25 ± 2°C, 75 ± 3% r.h. with a L12:D12 photoperiod, in the laboratory, following Della Lucia et al.

(1993). One week before starting the experiment, the colonies were fed *ad libitum* with leaves of *Acalipha wilkesiana.*

Inoculation with *Trichoderma* on rice substrate

The isolate of *Trichoderma* used was found as an endophyte in a leaf fragment in an *A. sexdens rubropilosa* midden consisting of harvested but subsequently rejected material (see Chapter 4). It was maintained in the culture collection of the Laboratory of Insect-Microbe Interactions (Federal University of Viçosa). It was grown on potato-carrot-agar (PCA), and incubated at room temperature (25±2 °C) for 5 days until sporulation.

There were two treatments: sterilized rice as control and sterilized rice plus *Trichoderma*, each replicated five times. The choice of the rice as substrate is based on (1) the fact that it is used for the mass production of *Trichoderma* so we already know that this micro-organism would develop well on this substrate, and (2) a previous experiment in which ants collected sterilized cooked rice and incorporated it into their fungus garden. The inoculation of substrate followed Jenkins et al (1998) with adaptations. For this, 200g of rice and 80ml of distilled water were placed in polypropylene bags (2 Kg capacity). The bags were left for 30 min for the rice to absorb water, with frequent shaking to maintain the homogeneity of the substrate. After that, the bags were autoclaved at 120ºC and 1.1 atm for 30 min. Once cool, the bags were transferred to a laminar flow cabinet and each one was inoculated with five mycelial plugs (5 mm diameter) of *Trichoderma*. The control consisted of bags inoculated with plugs of PCA only. The bags were sealed and transferred to a bench at room temperature at 25±2°C and turned every two days to promote gaseous exchange, increase surface contact between fungus and substrate, and to break up the mycelium and thus ensure homogenization. Preliminary tests were done and it took 8 days until

the treatment was totally full of spores and mycelium, and so ready to be offered to the leafcutter ants. Before this, all bags were opened and the treatment and control were mixed separately in a pot in order to obtain an homogenous mixture. A sample (1 g) was taken to count the number of spores using a haemocytometer. This process was conducted twice as the substrate with or without *Trichoderma* was offered twice (see below); on the second occasion, however, when the substrate was ready to be offered it was mixed with commercial citrus powder (1% of natural pulp) at a ratio of 1:1 (w: w), in order to disguise the strong smell of *Trichoderma*. This was done because in a preliminary experiment, colonies had been offered rice containing *Trichoderma* but rejected totally this material, and we supposed this could be due to the smell.

Experimental set-up and data collection

For eleven days prior to inoculation, ants were offered only leaves of *A. wilkesiana* (5g/colony) daily. Then, following a period of 12h without food, specific substrate was offered for treatment and control colonies for three days (rice (5 g/colony) with *Trichoderma* and rice free of the fungus, respectively; see above). For the following 14 days, ants were offered only *A. wilkesiana*, as above. After a further 12h without food, colonies were again offered rice with and without *Trichoderma* for three days, this time with the citrus additive. Finally, colonies were returned to a diet of *A. wilkesiana* as above for the remainder of the experiment.

Over the experimental period, a number of records were made. Foraging activity was measured by counting workers for one minute five times per day as they passed a specific point carrying pieces of leaves in the direction of the fungus garden. The weight of rice harvested was estimated (both times) by weighing the rice before offering it to the colonies and three days later. The consumption of leaves was measured daily. The

weight of fresh waste was taken every three days. Finally, the survival times of the colonies were based on the death of the mutualist fungus, visible when it turned black, and of the workers of each colony, measured by counting the number of dead ants every three days. All parameters excepted for survival times were analyzed in three periods: before the first offer of rice (BF), after the first offer of rice (AF) and after the second offer of rice (AS).

When the death of the fungus garden was observed, samples were taken and placed aseptically in plates that were sealed and incubated in chamber with controlled temperature (25±2 °C) in order to verify the presence of *Trichoderma* spp.

Differences between control and treatments were tested using t-tests where data inspection allowed, or Mann-Whitney U-tests. Survival analyses were performed using Kaplan-Meier survival curves in SPSS v.8. Comparisons of survival times were made using log-rank tests.

Results

Once mini-colonies had been prepared, sampling revealed no *Trichoderma* in the fungus gardens. However, three genera of alien fungi were found: *Penicillium*, *Aspergillus* and *Escovopsis*, but all mini-colonies appeared healthy.

In comparing control and treatment colonies, there were no differences in foraging activity (AF: t = 0.59, df = 1, p= 0.29; BF: t = 1.28, df = 1, p= 0.21and AS: t = 0.33, df = 1, p= 0.38; Figure 1a). Similarly, there was no difference in the amount of rice taken (First feeding event: t = 0.2190, df = 1, p= 0.42; Figure 1b. Second feeding event: U=11, df=1, p= 0.421; Figure 1b). There was also no difference in weights of leaves taken (period BF: t = 0.71, df = 1, p= 0.25; BF: U = 8, df=1, p= 0.37 and AS: U = 9 df = 1, p= 0.45, Figure 1c). Finally, there was no difference in the weights of waste

(BF: t = 0.1681,df = 1, p= 0.44; AF: t = 0.56, df = 1, p=0.17; AS t = 0.82 df = 1 p= 0.22; Figure 1d).

The survival times of the colonies was analyzed and there was no statistically significant difference between control and treated colonies (*log-rank* statistic =0.33, p= 0.5638). Comparisons among means of survival time of treatments and control showed that ants from the control survived longer than those submitted of *Trichoderma* treatment (means of 52.62 ± 0.36 and 43.72 ± 0.32 days *log-rank* statistic =282, 14.19, P < 0.001).

The samples that were taken from dead fungus gardens showed that *Trichoderma* was absent in all control colonies and was present in four of the five treatments colonies.

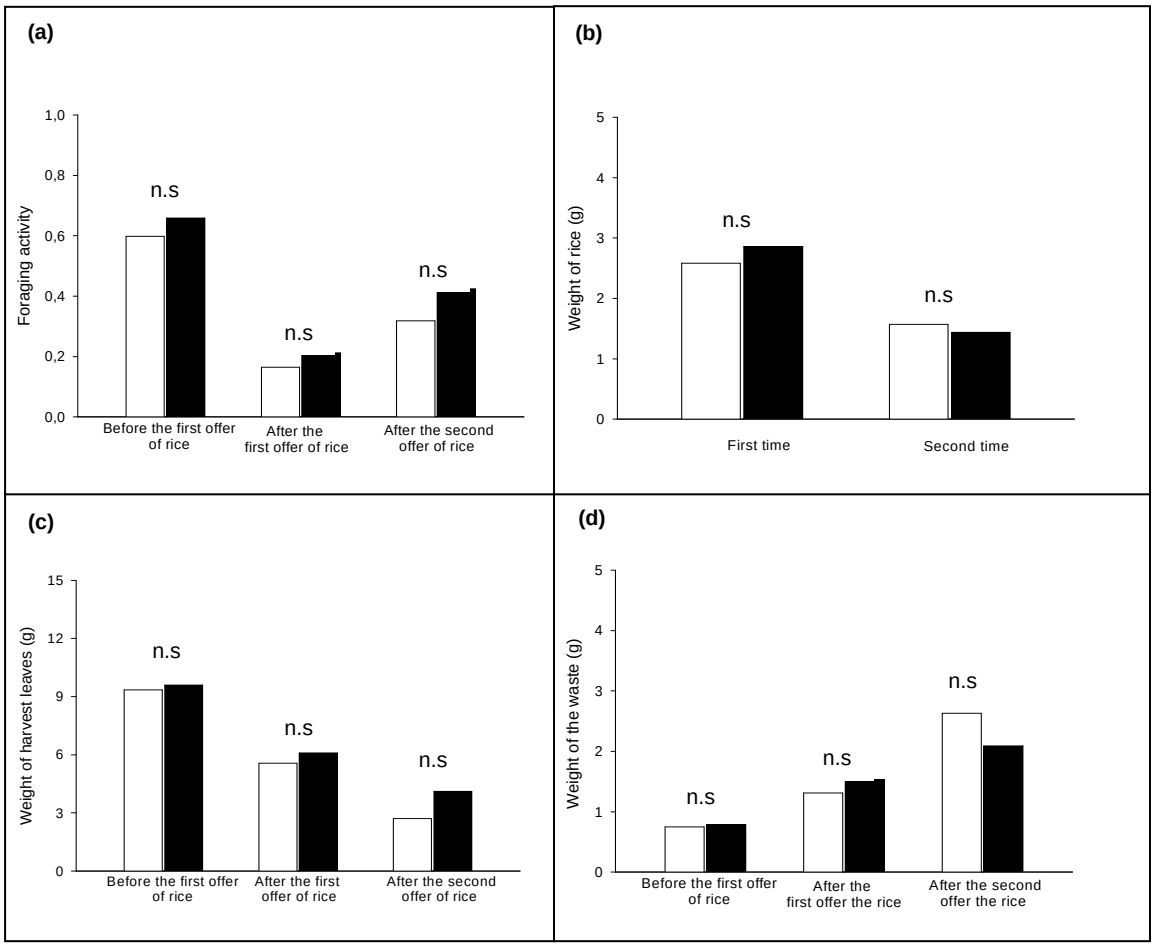


Figure 1 – Colonies behavioral response before the first offer of rice (BF), after the first offer rice (AF) and finally after the second offer of rice (AS) each period of seven days, being the rice with *Trichodema* spp and without (control). (a) The average of foraging activity in three periods already described above there was no significant difference between control and treatment (t = 0,59; t=1,28 and t = 0.33); (b) There was no difference in mean of weight harvest rice with *Trichoderma* (treatment) and without (control) in two times (first and second) (t = 0.22 and U=11, df=1, p= 0.421 respectively); (c) The average of weight harvest leaves in three periods that are the same described above there was no significant difference between control and treatment (BF – t = 0.71; AF - U=8, df=1, p= 0.37 and AS - U=9 df=1, p= 0.45) and finally (d) The mean fresh waste between control and treatment was not different in three periods (BF – t = 0.168; AF – t = 0.56; AS – t = 0.82).

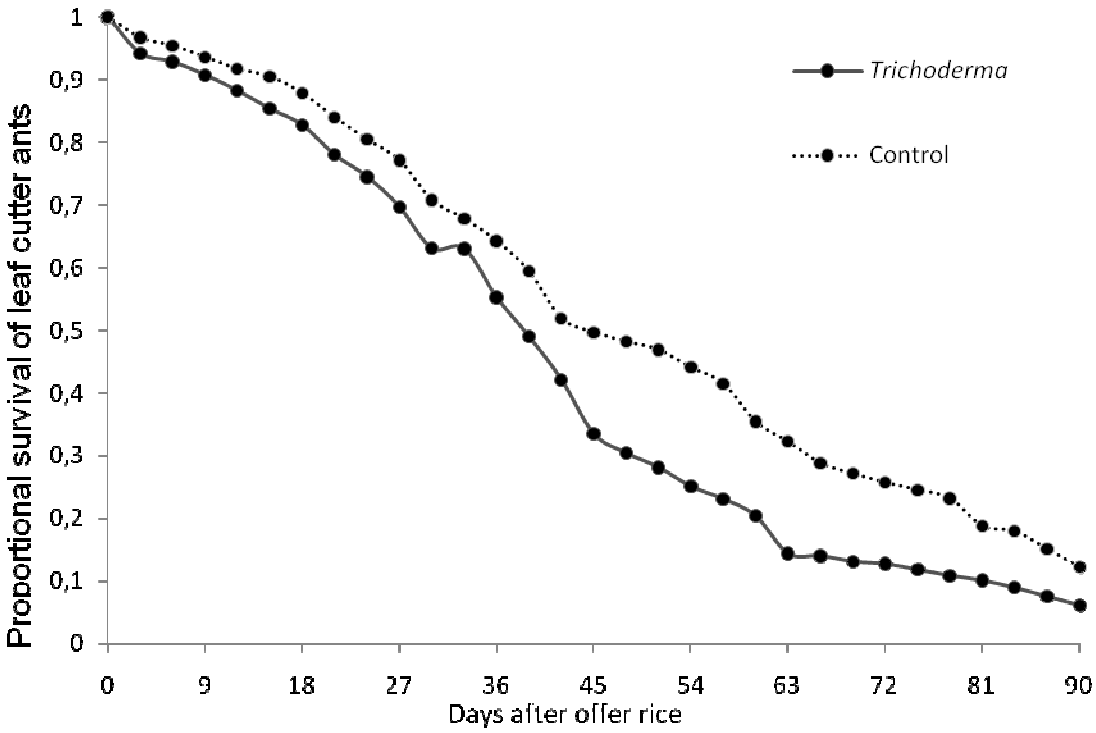


Figure 2 – Survival curves of workers of *Atta sexdens rubropilosa* of queenless colonies that were fed for twice with rice containing *Trichoderma* and without (control). The leafcutter ants' mortality was assessed every three days and the leafcutter ants that survived were not count here. The death of the colony was recorded when there was the death of the mutualist fungi *Leucoagaricus gongylophorus*. Significant differences were found in survival rates.

Discussion

Our study indicated that the mycoparasitic fungus *Trichoderma* did not affect the behavior of the ant colonies (Figure 1. We found no effect of *Trichoderma* on foraging activity. We had expected that, once material containing *Trichoderma* had been incorporated in the fungus, foraging activity would increase in response to declining of the mutualist fungus. In a previous experiment in which baits containing *Trichoderma viride* were offered, the behavior of *Atta cephalotes* colonies changed after two weeks (Lopez and Orduz 2003). In that study, however, foraging activity was measured as the number of ants entering and leaving the nest in each one of the entrances in field nests (Lopez and Orduz 2003), while here we counted the numbers of ants carrying pieces of leaves and entering the nest. Although an explanation for the lack of a difference in foraging activity between the treatments could be based on non-establishment of the inoculum in the fungus garden (through efficient protection by the ant, say), *Trichoderma* was successfully re-isolated from the majority of the treated nests. According to Della Lucia et al. (2003) there can be a reduction in the leaf cutting and transportation in queenless colonies. In contrast, Sousa-Souto and Souza (2006) found no changes in the average time of cutting and transporting the first fragment to the nest of *A. sexdens rubropilosa,* but observed a decrease in the time of transporting the tenth fragment to the nest. In sum many it seems that there are behavioral changes when the queen is not present that can influence the results when the colonies were under stress of the mycoparasite fungus presence.

There was also no difference in the quantity of harvested rice between treatments (Figure 1b).One parameter that could change would be the time taken to process the material containing *Trichoderma* but we did not record this behavior. In our preliminary experiment, the rice with *Trichoderma* was totally rejected by the ants,

hence the subsequent addition of powdered orange juice. We suspected that this rejection could be due to the strong smell of the fungus or some other form of recognition. In one study, it was shown that workers of *Atta colombica* did not show a preference towards leaves with high or low densities of conidia of the endophytic fungus *Glomerella cingulata* but that they spent significantly more time in the process of cutting the leaves with high density (Van Bael et al. 2009). Another explanation for the equal acceptance of the rice with or without *Trichoderma* is that the ants were not offered a choice (except to delay harvesting substrate). Perhaps if we had given the opportunity to choose the ants would have opted for the rice without *Trichoderma.* Although *A. sexdens rubropilosa* accepted the rice containing *Trichoderma* in an experiment where leaf substrate contained a fungicide, this was avoided by leafcutter ants *Acromyrmex lundi* 10 hours after it was first offered (Herz et al. 2008). Is important to note that although we mentioned that there is a work showing that leafcutter ants avoided plants contained fungicide the action of *Trichoderma* is slower than fungicide. The acceptance of rice containing *Trichoderma* may have been influenced by the absence of the queen, according to Sousa-Souto and Souza (2006) the absence of the queen decreased the average weight of the refuse accumulated and the authors suggested that this can be associated with the tendency of disruption of colony internal tasks, such as fungus garden maintenance.

The other parameter observed was the weight of harvested leaves, showing no difference between treatments (Figure 1c). We expected that the presence of *Trichoderma* would cause colonies to harvest more material but this did not happen. The lack of a difference could be attributed to the ants not perceiving the presence of the mycoparasite or to communication between the ants and the *L. gongylophorus* being affected in the absence of the queen.

The lack of a difference in the weight of waste (Figure 1d) could be ascribed to an absence of infection in the ants' mutualist fungus because of hygienic behaviors such as cleaning the material or/and stocking the spores in the infrabuccal pocket. However, four of the *Trichoderma*-inoculated colonies were found to be contaminated with the fungus at the end of the experiment. We therefore suggest that the ants did not perceive the presence of the fungus, so did not discard any fungus material over and above that normally discarded.

There was no difference found in the colony survival times based on the death of the mutualist fungus gardens. This could represent that leafcutter ants protected their cultivated fungus, perhaps cleaning the material removing, or if this was incorporated with substrate the ants might be able to remove infected material. However, we reran the survival analyses, artificially decreasing the survival times of the inoculated colonies and it was necessary to reduce survival times by 21 days (to a difference of 39 days between treatments) to obtain a significant difference. In other words, with only five colonies in each treatment, survival analyses are insensitive. There was a reduction in ant survival time with *Trichoderma* inoculation. It is possible that the mortality is associated with insecticidal activity, as has been reported for *Trichoderma harzianum* (Ganassi et al. 2000). These authors also suggested that the presence of degrading enzymes (chitinases, proteases), might also help to facilitate the entry of insect toxins. Other point that must be considered here is about the cost of survival when in contact with a mycoparasite but we did not measure here although is difficult measure the costs in social insects.

Interpretation of our results is naturally limited by considerations of experimental design. Thus, the colonies used here were queenless and the absence of the queen may have increased worker mortality. It could also decrease refuse accumulation (against a possible increase due to *Trichoderma* contamination) as the lack of a queen

can disrupt internal colony tasks such as fungus garden maintenance (Sousa-Souto and Souza 2006). There was no reposition of the workers because of the absence of queen; with new workers, perception of the presence of a mycoparasite fungus might be more evident. The experiments were naturally simplified in the laboratory, There was no choice of substrate, and this would be an important factor to include in future studies – it could well have led to avoidance of rice with *Trichoderma* the second time it was offered, for example. Finally, the colonies were small and were in the laboratory so this work must be repeated in the field.

The main idea of this work was try to work at a control method utilizing an endophytic mycoparasite fungus. We utilized an isolate that was found as an endophyte in vegetative material that was rejected by *A. sexdens rubropilosa* in the field. Given the negative effect of this isolate on leafcutter survival, it might have potential as a control tool, perhaps inoculating it in plants of interest such as eucalyptus. Because of the problems in methodology would be necessary to repeat this experiment to know exactly the results in a "real" colony. It will also be important to investigate if this fungus produces some metabolite that is toxic to the ants. An important realization from this experiment is that it is necessary to add something to mask the smell of the fungus.

The control of leafcutter ants using chemical insecticides will soon be limited through legislation, so the search for alternative control strategies for these agriculturally-important pests is urgent. Mycoparasitic micro-organisms associated with plants and hidden within them as endophytes seem to be a good option for the future management of these ants. We have shown that this endophytic fungus can affect the leafcutter ants, although the effect is far from devastating. The next steps will be to test others isolates of *Trichoderma*, to investigate the potential endophytism and to verify if the behaviour of other species of leafcutter ants is the same when in contact with mycoparasitic and endophytic fungi.

Chapter 5
Conclusions and perspectives

- There is a high diversity of endophytic fungi associated with *Atta laevigata* and for some of those fungal genera there were significant difference in carried vegetative material compared to rejected, and vice versa. These results can be indicative of acceptance or rejection of plants because of the presence of endophytic fungi.
- *Trichoderma* isolates were more commom in rejected vegetative fragments than in those carried by *Atta sexdens rubropilosa.*
- *In vitro* experiments showed that *Leucoagaricus gongylophorus* has some defence against *Trichoderma.*
- *Trichoderma* isolates showed a weak endophytism in *Ligustrum japonicum*
- Some isolates of *Trichoderma* showed endophytism in *Eucalyptus grandis*
- *Trichoderma* did not affect the foraging activity, a quantity of harvested rice and leaves, nor quantity of waste.
- *Trichoderma* had a negative affect on the survival of the workers

This work is the first involving leafcutter ants and the endophytic fungi associated with vegetative fragments carried by them. The fungi associated with leafcutting ants have been identified to genus level. Some isolates of the mycoparasitic fungus *Trichoderma* were studied in others aspects, involving their interactions with the mutualist fungus, endophytism and experiments *in vivo* in order to find a potential biocontrol agent of leafcutter ants. Future work should be done in order to establish a biocontrol mechanism for leafcutter ants using endophytic fungi as bodyguards that can be associated either with direct action (toxic to the ants),

indirectly by killing the mutualist fungus. These future studies should be based on (1) search for new isolates, (2) to test their endophytism, (3) choice testes, offering plants with or without endophytic fungi for leafcutter ants and through this know if the plant would be rejected because of the presence of a antagonistic, (4) to do tests *in vitro* in order to verify the action of endophytic fungi on the mutualist fungus *Leucoagaricus gongylophorus* and (5) to do tests *in vivo* to know the behavior of the ant colonies when in contact with a harmful fungus.

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