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Pathogenic interaction between *Escovopsis weberi* and *Leucoagaricus* sp.: mechanisms involved and virulence levels

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ABSTRACT

Attini are the only ants that use fresh plant material to cultivate species of *Leucoagaricus*, which are their source of nutrition. *Escovopsis* species are specialized mycoparasites of *Leucoagaricus* sp. and *Escovopsis* parasitism has a negative impact on the health of the ants' colonies. The goals of this work were: to test if the virulence of different isolates of *Escovopsis weberi* were the same across *Leucoagaricus* sp. and to analyze if structural mechanisms were related to variation in the virulence of *E. weberi* isolates. All *E. weberi* isolates were able to parasitize isolates of *Leucoagaricus* spp. but with striking differences in virulence, and it was shown that the contact between hyphae of both fungi was the main process that generates the degradation of *Leucoagaricus* isolates. Additionally, the two most virulent isolates produced hook-like protuberances, increasing the damage caused to its target. Finally, *E. weberi* was re-classified as a destructive biotrophic parasite.

Introduction

The leaf-cutting ants are a group within the Attini (Hymenoptera, Formicidae) and the only ants that use fresh plant material to cultivate a fungus belonging to the family Agaricaceae (Basidiomycota: Agaricales) (Weber, 1972). Leaf-cutting ants include two genera, *Acromyrmex* and *Atta* (Hymenoptyera: Formicidae) (Schultz and Brady, 2008). These ants are characterized by an obligate symbiotic relationship with species of *Leucoagaricus* such as *Leucoagaricus weberi* (Muchovej et al., 1991) and *Leucoagaricus gongylophorus* (Bononi et al., 1981). These *Leucoagaricus* species form specialized hyphae called gongylidia that are considered the main source of nutrition for the brood and queen (Quinlan and Cherrett, 1979; Bass and Cherret, 2008). In return, the ants provide substrata for the fungus and protection from competitors and parasites (Chapela et al., 1994; Currie et al., 1999b; Currie and Stuart, 2001; Haeder et al., 2009).

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The gardens where *Leucoagaricus* spp. are cultivated in nature are not pure cultures. These gardens are continuously colonized by several microorganisms such as anamorphic fungi (Currie et al., 1999a; Rodrigues et al., 2005; Pinto-Tomás et al., 2009; Ribeiro et al., 2012), yeasts (Little and Currie, 2008; Pagnocca et al., 2008) and bacteria (Currie et al., 1999b; Haeder et al., 2009), some of them being pathogens of the *Leucoagaricus* sp. cultivated.

Among these pathogens, species included in the genus *Escovopsis* (Ascomycota: Hypocreales) (Muchovej and Della Lucia, 1990) are considered specialized, and the most frequent, mycoparasites of the fungus gardens (Currie et al., 1999a). It is well known that parasitism by *Escovopsis* of the Attini fungus garden had a single evolutionary origin, and that the evolution of the *Escovopsis* parasites is congruent with the evolution of the ants and their fungal cultivars (Currie et al., 2003; Taerum et al., 2007).

Sub-colonies treated with *Escovopsis* sp. have a reduced garden mass compared with uninfected colonies (Currie, 2001). Several researchers have shown that different isolates of *Escovopsis*, from different leaf cutting ant species, can overgrow the cultivar of the leaf-cutting ants (Silva et al., 2006; Taerum et al., 2007; Folgarait et al., 2011b). These studies led to the conclusion that the different isolates of *Escovopsis* could infect different isolates of *Leucoagaricus* sp. without apparent specificity. Nevertheless, it has been proposed that the extent of the reduction in *Leucoagaricus* sp. growth rate caused by *Escovopsis* sp. could depend on the isolate, suggesting that *Escovopsis* isolates may have different levels of virulence on different hosts (Currie, 2001).

Direct consumption of the hyphae of *Leucoagaricus* sp. through mycoparasitism by *Escovopsis weberi* has been shown for *Atta colombica* and *Acromyrmex octospinosus* (Reynolds and Currie, 2004). However, the exact physiological and structural mechanism involved in the degradation of the fungal cultivar still remains unknown. Mycoparasitism generally involves four sequential steps: chemotropism, recognition, attachment and cell wall penetration, and digestion of host cell content (Harman and Kubicek, 1998). Chemotropism is the directed growth of an antagonist towards the host in response to a chemical stimulus, usually elicited by a gradient of soluble compounds (Gerardo et al., 2006; Folgarait et al., 2011b). Chemotropism usually precedes recognition, and probably the two processes are related. Recognition is mediated by lectin-carbohydrate binding between host and parasite (Elad et al., 1983a; Barak et al., 1985; Kolattukudy et al., 1995). Immediately after recognition, the mycoparasite attaches and, in some cases, coils around the host hyphae. This process can lead to host hyphal penetration and collapse (Shigo, 1960; Elad et al., 1983b). Mycoparasites can attach to their host by specialized structural mechanisms that include short branches that vary in shape, including curved hooks (Elad et al., 1983a), wedges (hyphal invagination from the apex of a single hook) (Rakvidhyasastra and Butler, 1973), finger-like appendages (Whaley and Barnett, 1963), clamps (Rakvidhyasastra and Butler, 1973), holdfasts (Shigo, 1960), buffer cells (Barnett and Lylly, 1958) and elongated absorptive hyphae with flattened points of contact (Rakvidhyasastra and Butler, 1973). In most cases a portion of the trapped wall is dissolved, creating a pore. Through this pore, nutrients are taken from the host by the parasite, ending the mycoparasitic process (Elad et al., 1983a).

The knowledge about the mechanism involved in the *Escovopsis*' parasitism is poor. While it is true that *Escovopsis* spp. isolates can overgrow different isolates of *Leucoagaricus* spp., the virulence level of different *Escovopsis* isolates is poorly understood. We have observed different structural mechanisms involved during parasitism, but we do not know if these mechanisms are related to a difference in the virulence of the isolates of *E. weberi*.

Based on the lack of information mentioned above, the goals of this work were: first, (i) to elucidate the structural mechanism involved in the parasitism of *Leucoagaricus* species by *E. weberi*; (ii), to test if the virulence of different isolates of *Escovopsis* is the same over three different isolates of *Leucoagaricus* sp.; (iii), to define the intrinsic factors correlated with *E. weberi* virulence; and (iv), to analyze the relationship between different structural mechanisms and the virulence levels of the *Escovopsis* isolates.

Materials and methods

Fungal isolation

Field work was performed between 2008 and 2009 (Table 1). Six isolates of *E. weberi* were obtained from three different sites and ant species (Table 1) and identified according to the description given by Muchovej and Della Lucia (1990). Collection sites were located in Corrientes (29°12′25.3″S

Table 1 – *Escovopsis* and *Leucoagaricus* strains used in this study

Species	Culture No	Origin	Collection year	Host ant species
E. weberi	E12	Mercedes, Corrientes.	2008	*Acromyrmex heyeri*
E. weberi	E11	Mercedes, Corrientes.	2008	*Acromyrmex lobicornis*
E. weberi	E20	San Cristóbal, Santa Fe.	2008	*Acromyrmex lobicornis*
E. weberi	E10	Mercedes, Buenos Aires	2009	*Acromyrmex lundii*
E. weberi	E13	Mercedes, Corrientes.	2008	*Acromyrmex lundii*
E. weberi	E16	San Cristóbal, Santa Fe.	2008	*Acromyrmex lundii*
Leucoagaricus sp.	02	Hudson, Buenos Aires	2008	*Acromyrmex lundii*
Leucoagaricus sp.	209	Hudson, Buenos Aires	2009	*Acromyrmex lundii*
Leucoagaricus sp.	05	Hudson, Buenos Aires	2008	*Acromyrmex lundii*

058°04′36.0″W), Santa Fe (30°13′33.1″S 060°35′13.4″W) and Buenos Aires provinces (34°39′40.5″S 059°27′07.6″W), Argentina.

The three isolates of *Leucoagaricus* sp. used were obtained from different *Acromyrmex lundii* nests from Hudson, Buenos Aires (34°46′32.7″S 058°09′21.5″W), in Argentina (Table 1).

Microscopic interactions between E. weberi *and* Leucoagaricus *sp. isolates*

To determine if all the *E. weberi* isolates had the same ability to parasitize and consume *Leucoagaricus* sp., and to describe the structural mechanism involved in this parasitism, microcultures were prepared using a fine layer of PDA (potato dextrose agar) over the slides prepared using 39 g l^{-1} of PDA pre mix (Britania) following the protocol established by Reynolds and Currie (2004). On one edge of the slide we inoculated a *Leucoagaricus* isolate, and one isolate of *Escovopsis* was placed on the opposite edge. Inoculations were made at the same time for both fungi. The slides were then covered with a sterile coverslip (Reynolds and Currie, 2004). This experiment was carried out with all the isolates of *E. weberi* (Table 1) and the *Leucoagaricus* isolate sp. L0209. The slides with fungi were placed over sterile wet cotton within Petri dishes in a room with controlled temperature (25° ± 1 °C). The interactions between the fungi were observed daily using an optic microscope (Nikon, Eclipse E200) for 5 d.

In vitro *bioassays between isolates of* Leucoagaricus *sp. and* E. weberi

The effect of the six different isolates of *E. weberi* on the three isolates of *Leucoagaricus* sp. was analyzed *in vitro*. The bioassay was carried out in Petri dishes (9.5 cm in diameter) with PDA (39 g l^{-1}, Britania). A small piece of *Leucoagaricus* culture (≈1 mm^2) was placed at the edge of each dish. Since *Leucoagaricus* sp. grows slowly it was allowed to reach an area equivalent to 6.5 cm^2, according to previously established methods (Folgarait et al., 2011a,b). Then, an agar disc of 0.22 cm^2, with conidiated *E. weberi*, was placed at the other edge of the plate.

All the possible combinations between the six isolates of *Escovopsis* species and the three *Leucoagaricus* isolates were made (10 replicate plates for each combination tested). In control groups, each isolate of *E. weberi* and each isolate of *Leucoagaricus* sp. was plated individually (six replicate plates for each control). This experiment was carried out under controlled conditions of temperature (25° ± 1 °C) and humidity (80 % ± 5 % RH).

To measure the initial area of each fungus, a photograph was taken on the first day of the experiment. Colony areas were measured using ImageJ 1.4 software (Wayne Rasband, National Institutes of Health, Bethesda, MD). The same was done on the last day of the experiment, which allowed calculation of the growth rate for both fungi. Growth rate was calculated as the ratio between total growth and the time taken to completely cover each Petri dish (cm^2 d^{-1}). Whenever *E. weberi* did not cover all of the Petri dish by the 19th day, the trial was terminated because parasitism had been confirmed in all the cases.

Additionally, the time (d) that it took each *E. weberi* isolate to generate aerial mycelium, immature conidia, mature conidia, and to completely cover the surface of the medium were recorded.

Marfetán (2011) reported that *Leucoagaricus* sp. colonies cease being efflorescent and become appressed after being parasitized by *Escovopsis* isolates. In the present study the time (d) it took each *Escovopsis* isolate to alter the morphology of the *Leucoagaricus* colony was recorded.

Statistical analysis

Principal Component Analysis (PCA) was used to characterize the virulence of each *Escovopsis* isolate on three *Leucoagaricus* sp. isolates. For the PCA the following variables (measured from each combination of *E. weberi* and *Leucoagaricus* sp. isolates) were used: initial and final area of mycelial growth, the difference (Δ) between final and initial area, the growth rate of both fungi (cm^2 d^{-1}), the time (d) it took each *E. weberi* isolate to come into contact with the *Leucoagaricus* colony and the time (d) to generate aerial mycelium, immature conidia, mature conidia, and to completely cover the Petri dish. The number of days it took each *E. weberi* isolate to alter the morphology of the *Leucoagaricus* colony (degradation) and the numbers of days that *Escovopsis* colonies remained with aerial mycelium were also recorded. Each variable was measured in each replicate and the six replicates per combination were averaged. Then, these means were averaged for the three *Leucoagaricus* isolates and the grand means were used to run the PCA. Additionally, categorical data for the presence or absence of hooks, traps, and tropism in the microculture assays was added. These variables behaved equally across replicates and in the presence of different *Leucoagaricus* isolates.

The analysis was performed with the software PcOrd 4.01 (McCune and Mefford, 1999. MjM Software, Gleneden Beach, Oregon, U.S.A.).

Results

Microscopic interactions between E. weberi *and* Leucoagaricus *sp. isolates*

The mycelium of both fungi were differentiated by the characteristics of their mycelia. The hyphae of *E. weberi* had more septa and a smaller diameter than the hyphae of *Leucoagaricus* sp., and the *Leucoagaricus* sp. hyphae had a slower growth rate and fewer branches than those of *E. weberi* isolates (Fig. 1A and B).

As expected, for all combinations tested, the *E. weberi* isolates degraded the *Leucoagaricus* sp. mycelium (Table 2). The controls of *Leucoagaricus* sp. showed a normal and abundant growth, a thick cell wall, and typical production of gongylidia (Fig 1C). By contrast, in microculture, where *E. weberi* isolates and *Leucoagaricus* sp. were placed together, *Leucoagaricus* sp. mycelium was consumed by the mycoparasite before the production of gongylidia (Fig. 1D and E).

The hyphae of *Leucoagaricus* sp. were mostly degraded by all isolates of *E. weberi* in the contact areas between the

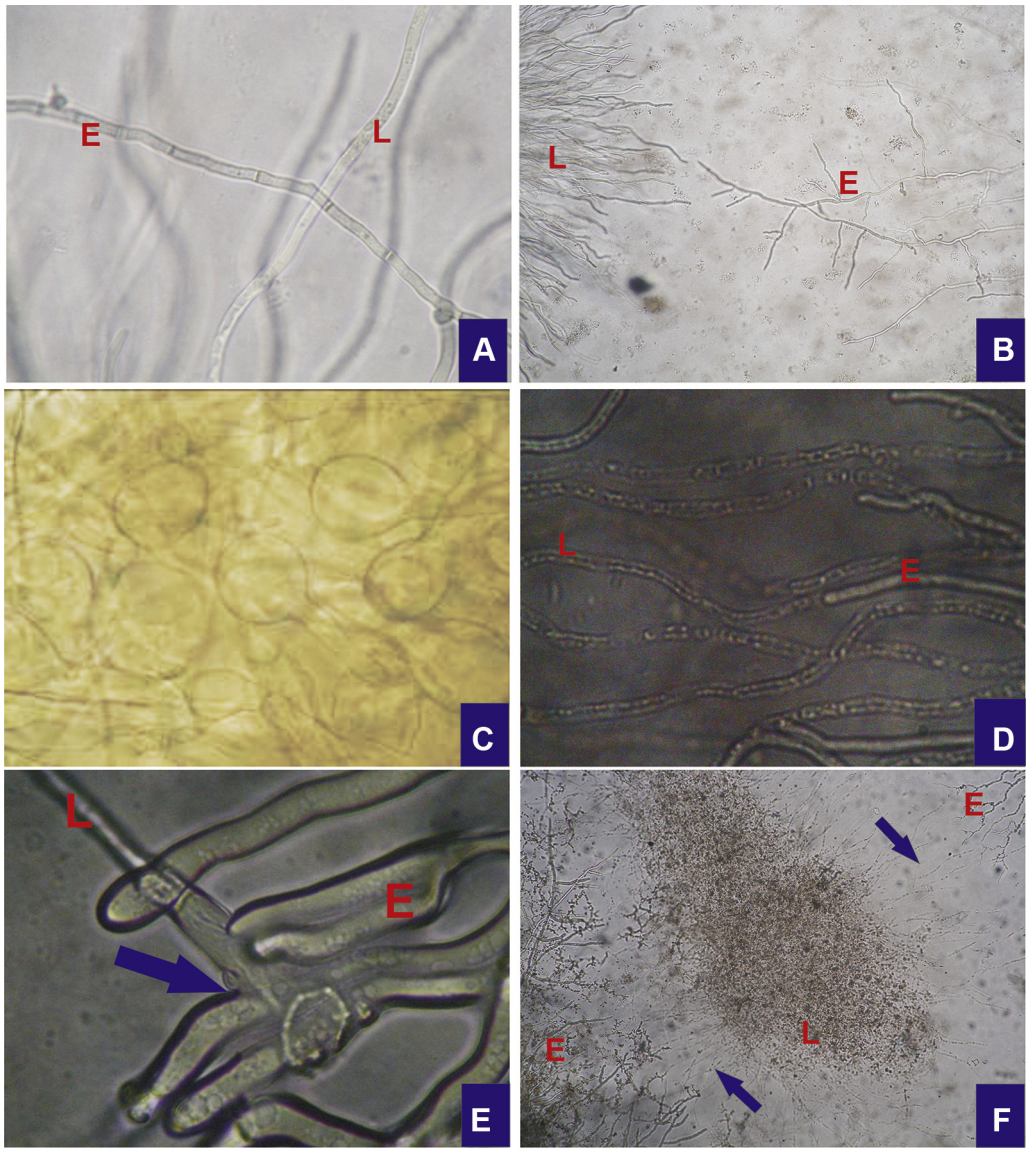

Fig 1 – Microscopic interactions between *E. weberi* (E) and *Leucoagaricus* sp. (L). (A)- *E. weberi* hyphae have more septa and are thinner than those of *Leucoagaricus* sp. (40×). (B)- *E. weberi* showed greater amount of lateral branches than *Leucoagaricus* sp. hyphae (10×). (C)- Mycelial growth of *Leucoagaricus* sp. with gongylidia present in control (40×). (D)- *Leucoagaricus* sp. hyphae show loss of cytoplasm and turgor during parasitism whereas the hyphae of *E. weberi* show a thick cell wall (40×). (E)- Hyphae of *E. weberi* degrading the hyphae of *Leucoagaricus* sp. through direct contact (arrow) (40×). (F)- *Leucoagaricus* sp. degradation (arrows) without direct contact (10×).

hyphae of the two fungus species (Fig. 1D and E). Additionally, all *E. weberi* isolates were able, in one or two replicates out of six to cause degradation also in areas without contact between the fungi, when hyphae of both fungi were in close contact but not touching each other (Fig 1F).

In two isolates of *E. weberi* (E13 and E16) the pathogen caught the *Leucoagaricus* sp. hyphae through the formation of hook-like protuberances (Figs 2A, B and 3). These structures were generated from ramifications, generally perpendicular to the parental hyphae (Fig 3). Initially at the beginning, hooks were formed by dichotomic ramifications, rounded in the apex. In most cases, after the cultivar was caught, a prolongation with a spiky tip was formed to penetrate the hyphae of *Leucoagaricus* sp. (Figs 2B and 3C and D).

E. weberi isolate E16 was the only one that showed an additional structure very similar to the trap formed by nematophagous fungi. These traps were generated through two ramifications perpendicular to the parental hyphae, and parallel to each other. Then, these ramifications were interwoven and formed anastomoses (Fig. 2C and D). These structures grew around *Leucoagaricus* hyphae (Figs 2C and 4), but no damage to the *Leucoagaricus* sp. cell wall was visible nor pressure over the hypha of *Leucoagaricus* sp., which remained inside this kind of trap (Fig 2C).

Table 2 – Mechanisms involved in the parasitism between *E. weberi* and *Leucoagaricus* sp.

	E. weberi E12	*E. weberi* E11	*E. weberi* E20	*E. weberi* E10	*E. weberi* E13	*E. weberi* E16
Contact between hyphae	+	+	+	+	+	+
Degraded mycelium of *Leucoagaricus* strains	+	+	+	+	+	+
Hooks	–	–	–	–	+	+
Coiling	–	–	–	–	–	+
Anastomosis	–	–	+	+	–	–
Tropism of the hyphae of *Escovopsis* species towards *Leucoagaricus* strain	–	+	+	–	+	–
Conidiation near to the mycelium of *Leucoagaricus* sp	+	–	–	–	–	–
Torulose mycelium with gutules	+	+	+	–	+	–
Hyaline torulose mycelium	–	–	–	+	+	+

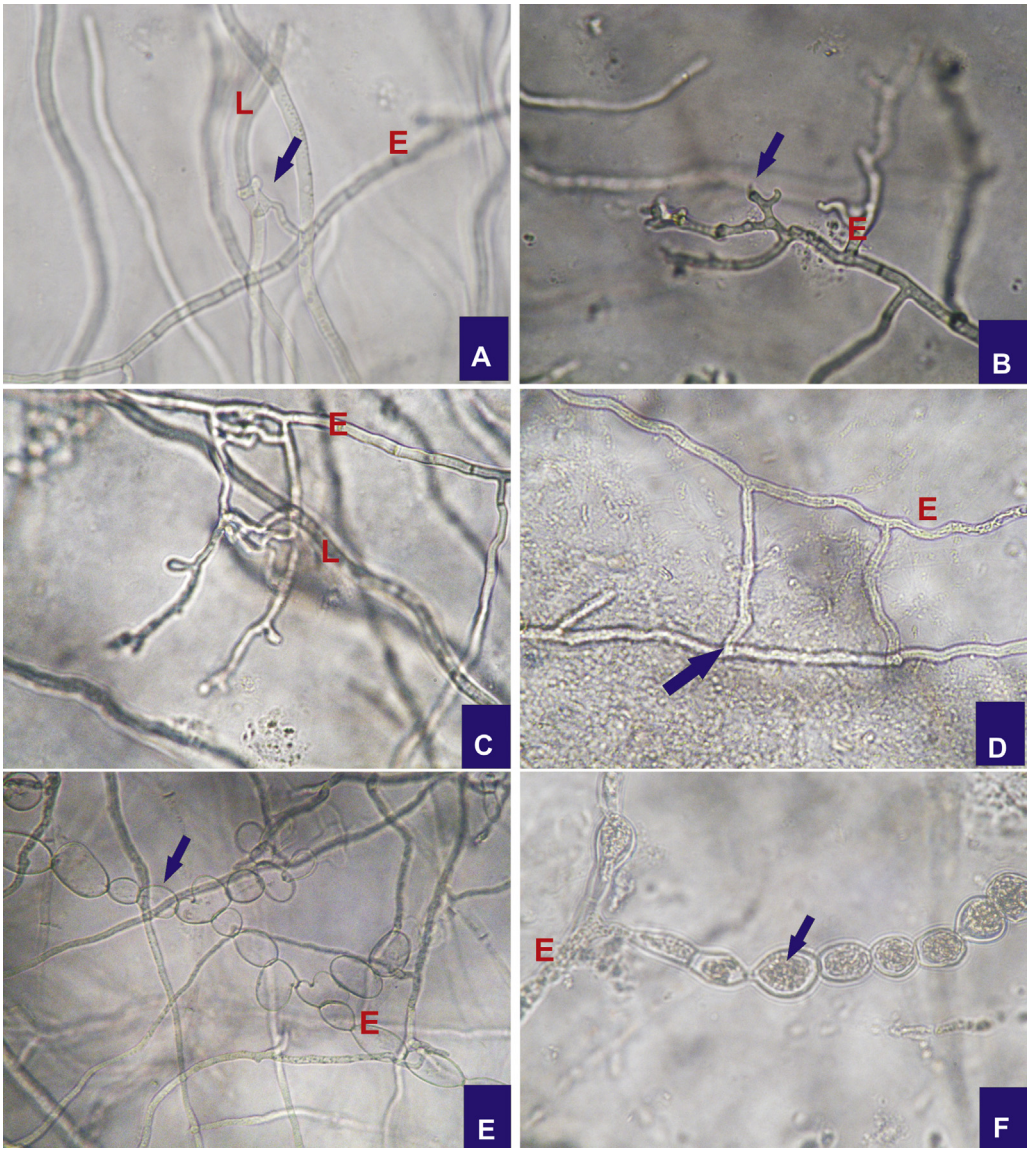

Fig 2 – Microscopic interactions between *E. weberi* (E) and *Leucoagaricus* sp. (L). (A)- Hook (arrowed) generated by *E. weberi* E13 and E16 in contact with hyphae of *Leucoagaricus* sp. (40×). (B)- Hook with a pointed appendix (arrowed) acting as a penetration peg (10×). (C)- Trap formed by anastomosis and coiling surrounding a hypha of *Leucoagaricus* sp. but without affecting *Leucoagaricus* sp. hyphae (40×). (D)- Anastomosis (arrowed) between hyphae of *E. weberi* E16 (40×). (E)- Torulose mycelium formed by short, hyaline, catenate and intercalary cells. (F)- Torulose and catenate mycelium with thick cell walls. Note oil drops (gutules) in the cytoplasm (arrowed).

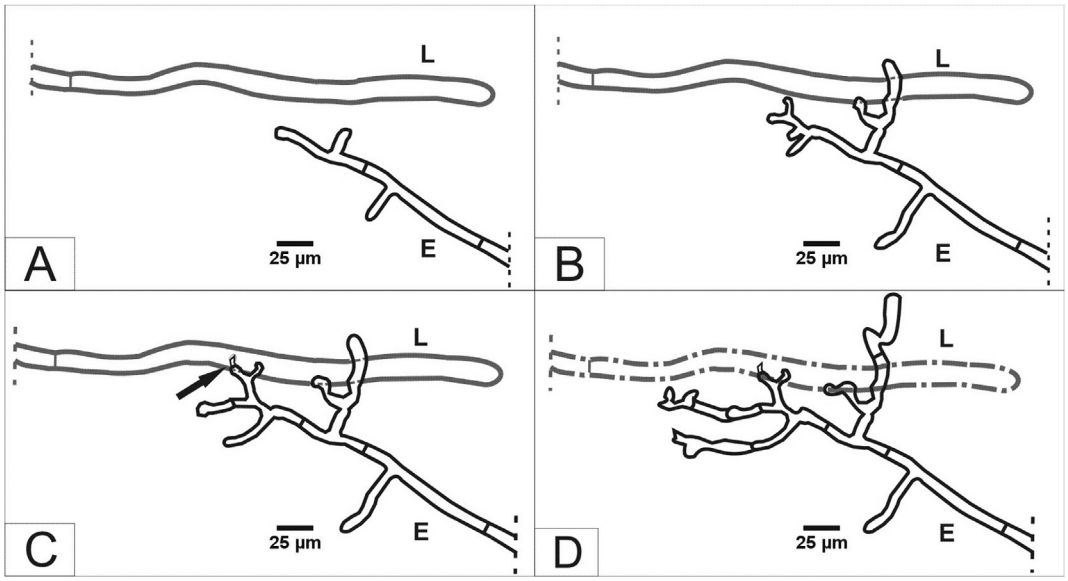

Fig 3 – Formation of hooks. (A, B)- Hyphae of *E. weberi* (E) and *Leucoagaricus* sp (L) before contact. (C)- The hook establishes contact with the hypha of *Leucoagaricus* sp. and forms a penetration peg (arrow). (D)- Degradation of the host.

Additionally, *E. weberi* isolates E11, E12, E13 and E20 exhibited a positive tropism towards the host. Furthermore, hyphae of *E. weberi* increased in density of mycelium in the areas closest to the *Leucoagaricus* sp. hyphae.

All isolates of *Escovopsis* sp. showed morphological changes in their hyphae that had not previously been described before for this genus. These changes were mainly the generation of two types of torulose mycelium (Table 2). The first type was composed of intercalary chains of cells which remained hyaline. This torulose mycelium was observed in *E. weberi* isolates E10 and E16 (Fig 2E; Table 2). In the second morphology, the cells of the torulose mycelium were intercalary and were arranged catenulately and terminally as well as verticillately. The cells had thick walls and brown cytoplasm with many gutules (Fig 2F). This second type of torulose mycelium was produced by isolates E12, E11 and E20. *E. weberi* E13 was the

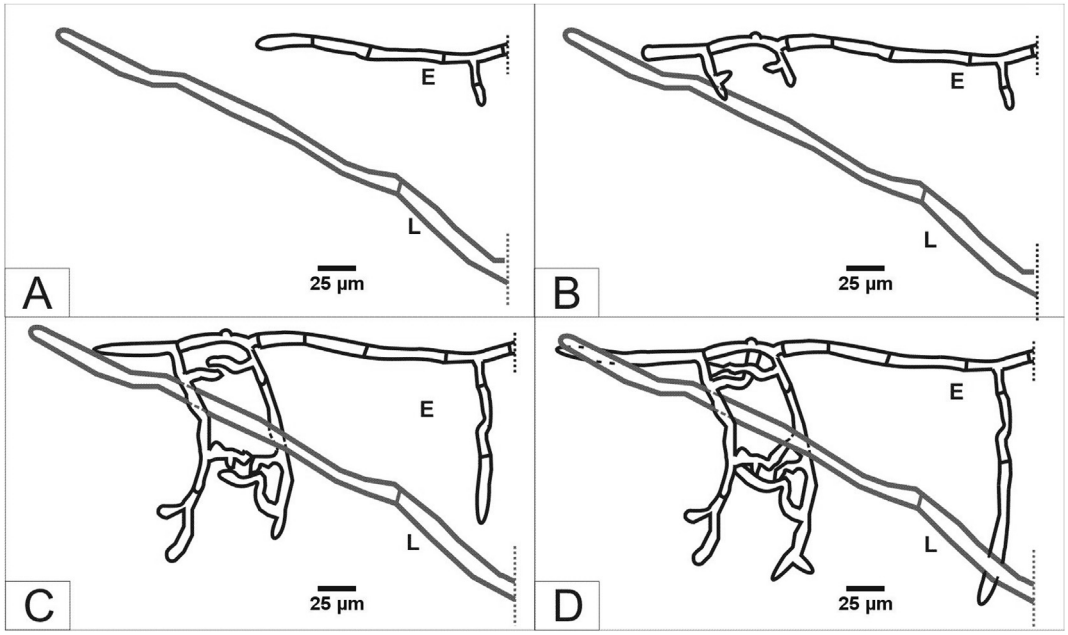

Fig 4 – Formation of traps. (A)- Hyphae of *E. weberi* (E) and *Leucoagaricus* sp. before contact (L). (B, C)- Initial lateral hyphal growth. (D)- Trap formed by interwoven and anastomosing hyphae. Note *Leucoagaricus* sp. hypha passing through the trap without showing any degradation.

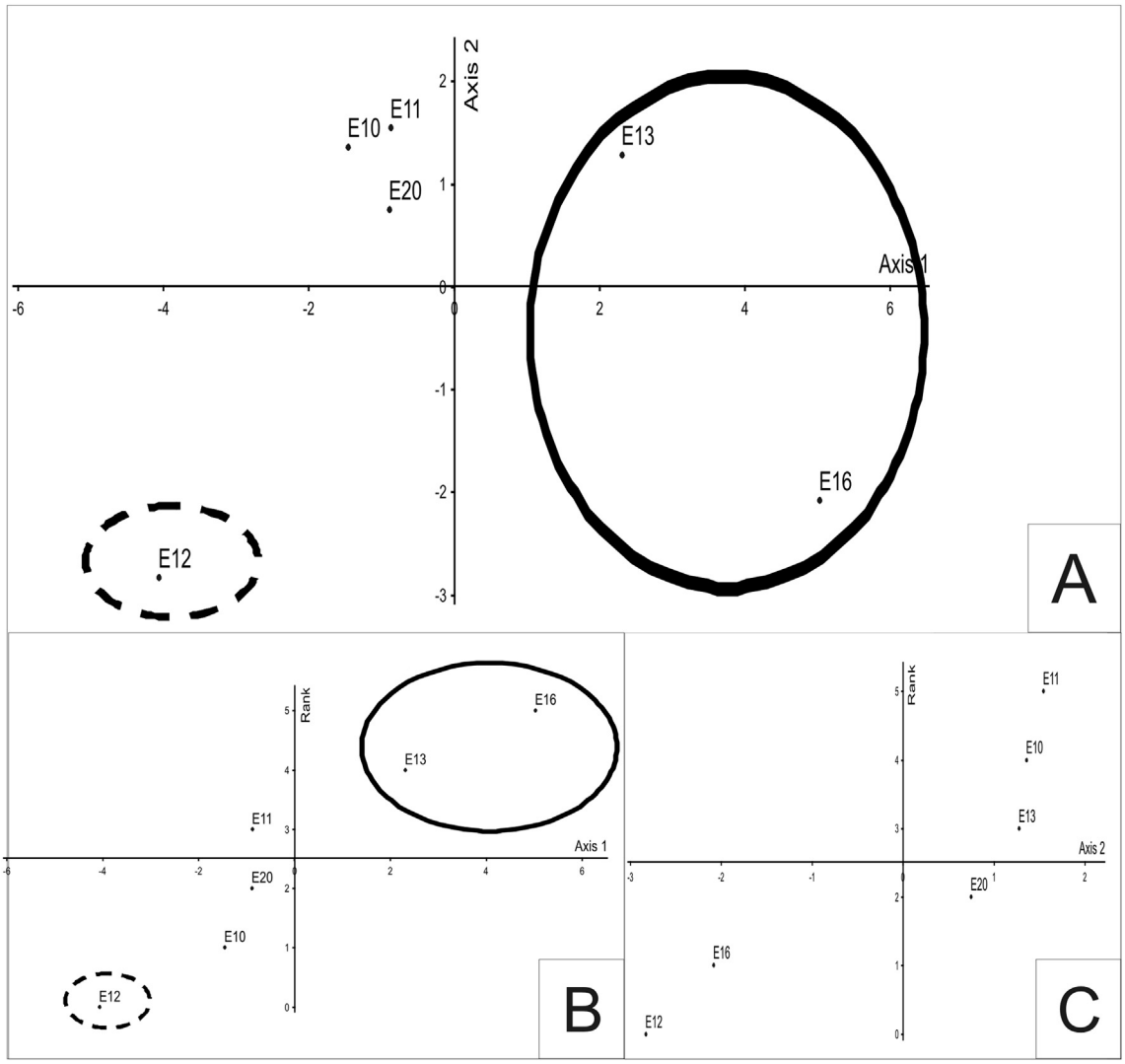

Fig 5 – PCA analyzing the effect of *Escovopsis weberi* isolates over the average of three *Leucoagaricus* sp. Ellipses with full lines show the most virulent isolates. Circle with dashed line shows the least virulent isolates. (A)- PCA showing the first two axes. (B)- PCA showing the axis 1 vs ranks. The isolates with a higher virulence were grouped towards the higher rank. (C)- PCA showing axis 2 vs ranks. This axis grouped the *Escovopsis* isolates with a higher final area towards the greatest rank. This axis was not related to virulence.

only isolate that showed both types of torulose mycelia (Table 2).

Multivariate analysis of the virulence level of E. weberi

The first two axes of the PCA were significant and explained 77.35 % of the variation in the data. The first axis explained 56.49 % of the variance whereas the second axis explained 20.86 % of the variation.

In the first axis the isolates of *E. weberi* that generated hooks and traps, and exhibited greater growth rate, were grouped towards the positive values. At the opposite end of this axis, *E. weberi* isolates that allowed the higher growth of *Leucoagaricus* isolates and needed a greater number of days to degrade *Leucoagaricus* sp. (i.e., caused less damage) were clustered together (Fig 5). In fact, the PCA showed that the growth rate of *E. weberi* isolates was negatively correlated with the number of days it took the pathogen to convert the colony morphology of *Leucoagaricus* sp. into appressed mycelium ($r = -0.96$) and with the total growth of *Leucoagaricus* sp. ($r = -0.78$).

Furthermore, the growth rate of *E. weberi* was positively correlated with the presence of hooks ($r = 0.94$) and traps ($r = 0.74$). Moreover, a higher total growth of *Leucoagaricus* sp. was negatively correlated with the presence of hooks ($r = -0.83$) and traps ($r = -0.78$). Finally, the isolates of *E. weberi* that took more days to degrade the *Leucoagaricus* sp. colony also needed more time to generate immature and mature conidia ($r = 0.75$ and 0.85, respectively).

On the second axis, the *E. weberi* isolates with higher total growth (Δ area) were grouped towards the positive values (Fig 5).

Based on the interpretation of the axes, the first axis can be considered as a virulence axis, due to the fact that all variables involved in parasitism (including *Leucoagaricus* sp. damage)

were present on this axis. The ranking of the isolates on the first axis showed that *E weberi* isolates E13 and E16 were the most virulent, whereas *E. weberi* isolate E12 was the least harmful towards *Leucoagaricus* sp. (Fig 5B). The second axis seemed to be related to the intrinsic growth of *Escovopsis* isolates.

Discussion

According to our *in vitro* bioassay and to microscopic observations, all isolates of *E. weberi* used were able to parasitize and degrade the hyphae of *Leucoagaricus* isolates, mainly by direct contact, but with different levels of virulence. We also showed for the first time that the most virulent *E. weberi* isolates were those which developed hooks involved in capturing *Leucoagaricus* sp., and that this type of structure, as well as a greater growth rate, were correlated positively with virulence.

This work supports the proposal that *Escovopsis* spp. are not saprotrophic, occasional contaminants, nor a harmless transient part of the mycobiota in the garden (Rodrigues et al., 2005). As shown by our experiment, *E. weberi* parasitized *Leucoagaricus* sp. hyphae so quickly that *Leucoagaricus* sp. was degraded before the production of gongylidia. Considering that gongylidia are the main source of nutrition for the brood and the queen (Quinlan and Cherrett, 1979; Bass and Cherret, 2008), the destruction of *Leucoagaricus* hyphae and of these special structures by *E. weberi* affect the survivorship of the ant colony. Considering that previous experiments have shown that sub-colonies treated with three isolates of an unidentified species of *Escovopsis* exhibited a reduction in the garden mass compared with uninfected colonies (Currie et al., 1999a; Currie, 2001; Reynolds and Currie, 2004). *Escovopsis* spp. clearly represents a threat to the health of the colony and can potentially kill the ant nest.

Our *in vitro* results suggest that the isolates of *Leucoagaricus* sp. did not have the ability to avoid parasitism by *E. weberi*. *Leucoagaricus* spp. may have missed the ability to protect themselves because of the strong mutualism with the leaf-cutting ants. The cleaning behaviour of ants (Currie and Stuart, 2001), their metapleural gland secretions (Fernández Marin et al., 2009), and the Actinomycetes present in the ants (Cafaro and Currie, 2005) are the main ways that ants protect their fungal cultivar, but the present study did not examine these behaviours.

When we analyzed the performance of the different *E. weberi* isolates with the PCA, differences in virulence were found, agreeing with previous work showing that different isolates of *Escovopsis* isolated from *Cyphomyrmex* nests, a lower Attini that does not cut leaves, also had different levels of virulence (Gerardo et al., 2004). Our work showed that the growth rate and presence of hooks in *E. weberi* isolates were the most important parameter to define which isolate was the most virulent.

The most virulent isolates of *E. weberii* not only grew faster, but were also able to produce hooks. This may suggest that the production of these structures is not only involved in pathogenesis, but can also increase the damage caused by the pathogen to its target, probably because these hooks are used to penetrate the host hyphae (Fig 2B). Hook formation is widely distributed within the Hypocreales genera, and the hooks formed by *Stephanoma phaeospora* (Rakvidhyasastra and Butler, 1973) and by *Trichoderma* spp. (Elad et al., 1983a) are two of the most studied cases. Hooks present in the *Escovopsis-Leucoagaricus* interaction are morphologically very similar to the hooks formed by *Trichoderma* species when parasitizing *Rhizoctonia solani* and *Sclerotium rolfsii* (Elad et al., 1983a).

Traps are also widely distributed structures within the Basidiomycota and Ascomycota, and are mainly found in nematophagous fungi (Tzean and Estey, 1978; Nordbring-Hertz et al., 1989; Yang et al., 2007). Nevertheless, traps formed by *E. weberi* isolates were morphologically different from traps formed by nematophagous fungi. In the latter, traps are formed by three cells bound only by anastomosis (Nordbring-Hertz et al., 1989), whereas in *Escovopsis*, traps were formed by two hyphae bound by coiling and anastomosis (Fig 2C). Traps formed by *E. weberi* were also not able to generate pressure over their target nor degrade the *Leucoagaricus* sp. hyphae (Fig 2C). This could be the reason why *Escovopsis* traps did not cause an increase in virulence.

We expected that isolates of *E. weberi* obtained from *A. lundii* would be more virulent over the *Leucoagaricus* sp. cultivated by *A. lundii* due to their history of coevolution, but this was not the case. Although the most virulent *Escovopsis* (*E. weberi* E13 and E16) were isolated form *A. lundii*, one of the least virulent (*E. weberi* E16) was also isolated from the same ant species. More surprising, considering that *Escovopsis* isolates are purportedly transmitted horizontally (Currie et al., 1999a), one of the most virulent (E13) and one of the least virulent (E12) *E. weberi* isolates, were from the same field site (Mercedes, Corrientes); the virulence level did not seem to be related to the ant species or to the site from where the pathogen was isolated, but was a characteristic of *E. weberi* isolates.

To a lesser extent, the difference in virulence of the *E. weberi* isolates could depend on the *Leucoagaricus* isolate that was being parasitized. Although we reported the averaged virulence of *E. weberi* over three *Leucoagaricus* sp. isolates, when we evaluated separately the effect of *E. weberi* strain over each *Leucoagaricus*, a small difference was found depending on the *Leucoagaricus* isolates (data not shown). However, this effect needs to be further addressed.

The fact that *Escovopsis* isolates increased their mycelial density and conidiation in areas close to *Leucoagaricus* sp. suggests some kind of tropism, which is consistent with the ability of *Escovopsis* species to locate their target by chemotaxis (Gerardo et al., 2006). This tropism can in turn be related to the ability of *Escovopsis* spp. to change their physiological response by sensing compounds secreted by species of *Leucoagaricus*. For example, previous work has shown that *E. weberi* was able to maximize its growth rate and induce an increase in conidiation by sensing soluble compounds produced by *Leucoagaricus* sp. (Folgarait et al., 2011b). We thus propose that this tropism is actually a chemotropism in response to secretions from *Leucoagaricus*.

Furthermore, *Leucoagaricus* hyphae showed degradation when near to but not in contact with *E. weberi* hyphae. This could suggest the use of physiological mechanisms to parasitize *Leucoagaricus* sp. isolates. Several species of Hypocreales employ physiological mechanisms, including the production

of exoenzymes like β-glucanases, cellulases, chitinases and proteinases to parasitize their hosts (Gupta et al., 1993; Rocero et al., 2000; Berto et al., 2001; Steyaert et al., 2003). These enzymes are extensively used in the pathogenic process by mycopathogenic fungi such as *Trichoderma* spp., *Fusarium oxysporum* and *Purpureocillium lilacinum* (=*Paecilomyces lilacinus*), and confer the ability to degrade the host cell wall and to absorb the released nutrients (Gupta et al., 1993; García-Maceira et al., 1997; Savoie et al., 1998). The utilization of enzymes by *Escovopsis* species is consistent with the information reported for *E. weberi* consuming *Leucoagaricus* spp. from *At. colombica* and *A. octospinosus* (Reynolds and Currie, 2004). Our results showed that both structural and physiological mechanisms are commonly used by *E. weberi* during parasitism.

Mycoparasites may be classified into two large groups based on the mode of parasitism and on its effects on the host. The biotrophic parasites are those which secure nutrients from the living cells of the host and get nutrients via structural mechanisms or by close hyphal contact (Boosalis, 1964). The second group of mycoparasites are necrotrophic, encompassing those fungi which derive nutrients from dead hosts, usually killed by the parasite before it invades the host. Reynolds and Currie (2004) proposed that *E. weberi* was a necrotrophic parasite because they did not observe contact between the hyphae, and they assumed that *E. weberi* derived nutrients from the dead host. Our results clearly show that contact between hyphae and the presence of structural mechanisms such as hooks, as well as the penetration of the host hyphae, to be fundamental in the parasitic process. Therefore, we conclude that *E. weberi* is a destructive biotrophic parasite that kills its host through the parasitic process, deriving nutrients from living cells.

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