Curr Microbiol (2011) 63:250–258
DOI 10.1007/s00284-011-9944-y

Preliminary In Vitro Insights into the Use of Natural Fungal Pathogens of Leaf-cutting Ants as Biocontrol Agents

Patricia Folgarait · Norma Gorosito · Michael Poulsen · Cameron R. Currie

Received: 11 April 2011 / Accepted: 21 April 2011 / Published online: 8 July 2011
© Springer Science+Business Media, LLC 2011

Abstract Leaf-cutting ants are one of the main herbivores of the Neotropics, where they represent an important agricultural pest. These ants are particularly difficult to control because of the complex network of microbial symbionts. Leaf-cutting ants have traditionally been controlled through pesticide application, but there is a need for alternative, more environmentally friendly, control methods such as biological control. Potential promising biocontrol candidates include the microfungi *Escovopsis* spp. (anamorphic Hypocreales), which are specialized pathogens of the fungi the ants cultivate for food. These pathogens are suppressed through ant behaviors and ant-associated antibiotic-producing Actinobacteria. In order to be an effective biocontrol agent, *Escovopsis* has to overcome these defenses. Here, we evaluate, using microbial in vitro assays, whether defenses in the ant-cultivated fungus strain (*Leucoagaricus* sp.) and Actinobacteria from the ant pest *Acromyrmex lundii* have the potential to limit the use of *Escovopsis* in biocontrol. We also explore, for the first time, possible synergistic biocontrol between *Escovopsis* and the entomopathogenic fungus *Lecanicillium lecanii*. All strains of *Escovopsis* proved to overgrow *A. lundii* cultivar in less than 7 days, with the *Escovopsis* strain isolated from a different leaf-cutting ant species being the most efficient. *Escovopsis* challenged with a *Streptomyces* strain isolated from *A. lundii* did not exhibit significant growth inhibition. Both results are encouraging for the use of *Escovopsis* as a biocontrol agent. Although we found that *L. lecanii* can suppress the growth of the cultivar, it also had a negative impact on *Escovopsis*, making the success of simultaneous use of these two fungi for biocontrol of *A. lundii* questionable.

P. Folgarait (✉) · N. Gorosito
Centro de Estudios de Investigaciones, Universidad Nacional de Quilmes, Bernal, Buenos Aires, Argentina
e-mail: patricia.folgarait@gmail.com

M. Poulsen · C. R. Currie
Department of Bacteriology, University of Wisconsin, Madison, WI, USA

Present Address:
M. Poulsen
Section for Ecology and Evolution, Department of Biology, University of Copenhagen, Copenhagen East, Denmark

Introduction

In the New World, leaf-cutting ants are dominant herbivores and major agricultural and agroforestry pests. In Argentina, *A. lundii* is one of the worst crop pests: it commonly occurs in gardens and horticultural fields, and is known to negatively affect plantations. Traditionally, attempts to control leaf-cutting ants have involved the use of chemical pesticides [10, 19], but despite continuous application, these ants are hard to eradicate [11]. This is likely because pesticides can affect natural enemies of leaf-cutting ants. For example, Fipronil kills parasitoids (www.beekeeping.com/intoxications/fipronil_en.htm), therefore, potentially killing specific phorid parasitoids of leaf-cutting ants. Although pesticides may kill thousand of workers, they are unlikely to reach the queen, which has to be killed in order for colonies to collapse. In addition to lack of specificity in the use of chemical pesticides, this approach is also not sustainable because of potential bioaccumulation problems and contamination of ground water and agricultural products [39]. Consequently, alternative control methods that can provide more sustainable and efficient control of leaf-cutting ant colonies are needed [23, 25].

An alternative, more environmentally friendly method is biocontrol. Natural enemies are expected to be particularly useful, as they have evolved to utilize the target pest, and to some extent, may be able to overcome host defenses. The leaf-cutting ant system offers a unique opportunity to exploit specific natural enemies that may represent promising biocontrol agents. One such pathogen is a group of microfungi (*Escovopsis*: Ascomycota), which target and utilize the ant-cultivated fungus (*Leucoagaricus*: Basidiomycota) the ants rely on for food for the queen and brood [15, 43]. *Escovopsis* species appear to be specific to fungus-growing ant cultivars, are potentially virulent, and have likely coevolved with the ant-cultivar association since the origin of ant fungiculture [13, 17, 40]. The ants use behavioral mechanisms [1, 14], glandular secretions [6, 21], production of infrabuccal pellets [34], and task partitioning [7] to reduce the spread and virulence of the parasites. Furthermore, the ants rely on mutualistic Actinobacteria (genus *Pseudonocardia*) that produce antibiotics that help suppress *Escovopsis* [9, 16, 18, 37, 41], and other Actinobacteria genera may play a similar role [5, 36]. Despite these defenses, *Escovopsis* species have the capacity to induce ant colony collapse [15, 41], and may consequently be an appropriate natural biocontrol agent.

To use *Escovopsis* as a biocontrol agent, the parasite needs to overcome ant defenses. Here, using microbial in vitro bioassays, we evaluate whether defenses in the mutualistic fungus and Actinobacteria isolated from *Acromyrmex lundii* have the potential to limit the use of naturally occurring *Escovopsis* sp. as biocontrol agents. Although no other specific pathogens of leaf-cutting ants are known, other fungi are present and could have an impact on colonies. For example, the most common fungus tested for biocontrol purposes against *Leucoagaricus* in vitro is *Trichoderma*, whereas *Metarhizium* and *Beauveria* are most commonly tested for ant mortality [e.g., 20, 38, 45]. Only one study explored the effect of *Metarhizium anisopliae* and *Trichoderma viride* in whole colonies, which was done using collection strains, not isolates obtained from leaf-cutting ants [35]. Therefore, we also explore potential antagonism between *Escovopsis* sp. and a generalist entomopathogenic fungus, *Lecanicillium lecanii,* which has been shown as an efficient agent against homopterans [12, 26, 28]. The co-inoculation with another microbe could make a biocontrol agent particularly promising because this potentially requires more extensive defenses in the ants than infection with just one fungus [cf. 31]. Furthermore, *L. lecanii*, tested for the first time on the leaf-cutting system, could also work as a mycoparasite or competitor of *Leucoagaricus*, and thereby potentially produce a synergistic negative effect in combination with *Escovopsis* on the cultivar.

Materials and methods

Escovopsis sp. was isolated from two colonies of *A. lundii* (hereafter referred to as ESA and ESB) and from one colony of *A. heyeri* (hereafter referred to as ESC). *Leucoagaricus* sp. and *L. lecanii* were isolated from the fungus garden of an *A. lundi* colony. Pieces from the ant garden were placed on PDA plates (potato dextrose agar, Britania) and allowed to grow for several weeks. Sub-culturing to new PDA plates was done until pure cultures of both strains were obtained, after which their identity was confirmed with molecular methods (U. G. Mueller, unpublished). In order to obtain mutualistic Actinobacteria from *A. lundii*, we performed isolation attempts from multiple ants from 10 different colonies using standard isolation methods [8]. *Pseudonocardia* has previously been the main actinobacterium isolated from other *Acromyrmex* leaf-cutting ant species [cf. 8, 41], but contrary to our expectations, *Pseudonocardia* was not obtained from *A. lundii* in our isolations. However, we consistently obtained morphologically similar *Streptomyces* and we confirmed their identity by sequencing 16S rDNA (match in BLAST search in GenBank: AY996829, *Streptomyces* sp. 80134). All isolates were obtained from colonies excavated at Santa Fé province in Argentina.

To explore microbial interactions in vitro that could inform on interactions within colonies, we performed four different in vitro assays: *Leucoagaricus-Escovopsis*, *Streptomyces–Escovopsis*, *L. lecanii–Escovopsis*, and *Leucoagaricus–L. lecanii*. For the *Leucoagaricus–Escovopsis* assay, an inoculum (0.20 cm^2) of *Leucoagaricus* was placed in the middle of 20 Petri dishes containing PDA (20 per *Escovopsis* strain). When the cultivar reached a diameter of 2–3 cm, it was challenged with an inoculum of 0.20 cm^2 of sporulating *Escovopsis* placed on the side of the Petri dish. We made controls of *Leucoagaricus* and each *Escovopsis* strain separately ($N = 6$). We monitored the challenges daily for 8 days. For *Streptomyces–Escovopsis* and *L. lecanii–Escovopsis* challenges, we inoculated *Streptomyces/L. lecanii* in the center of a Petri plate containing PDA and waited until it reached a diameter of 1.5–2 cm before inoculating *Escovopsis* at the edge. The progression of growth of *Escovopsis* was followed for 10–14 days ($N = 30$). We made the representative controls, being the growth of each of the microbes in the absence of the other microbe ($N = 6$). We used the same methodology for the *Leucoagaricus–L. lecanii* challenge ($N = 10$), except that interactions were evaluated 23 days after the initiation of challenges. Controls of *L. lecanii* were done by growing them on separate plates ($N = 6$). Experiments were finished when no changes in the interactions were observed for at least 4 consecutive days; thus,

the duration of challenges depended on the growth rates of the different microbe combinations.

For all challenges, the initial and the final area of each of the two microorganisms involved were measured using the software ImageJ 1.4 (Wayne Rasband, National Institute of Health, USA). We also measured the initial and final area of each of the controls. This information was used to calculate the following growth index:

$$\frac{\text{final area (challenge)} - \text{initial area (challenge)}}{\text{final area (control)} - \text{initial area (control)}}$$

If the growth index was not significantly different from a Student's T distribution or one sample T test (reported as t test or T) with a mean of 1, we concluded that there were no effects of one microorganism on the other (i.e., for *Leucoagaricus* vs. *Streptomyces, Escovopsis* vs. *L. lecanii*). If the index value was significantly <1, we interpreted this as one microorganism negatively affecting the other (i.e., expected for *Leucoagaricus* when challenged with *Escovopsis*, for *Escovopsis* when challenged with *Streptomyces*). If the index was significantly >1, we interpreted this as a benefit from the interaction (i.e., expected for *Escovopsis* when challenged with *Leucoagaricus*). Differences in the final mycelium growth areas were compared statistically among strains within challenges using non-parametric Kruskal–Wallis tests (reported as H and contrasts as z) if there were more than two factors or with Mann–Whitney tests if there were only two (reported as U). All tests were Bonferroni corrected to reduce the global error Type I when multiple comparisons are performed [44].

For *Escovopsis*, we registered the following categories of growth: I = growth area > 75% of the Petri dish area, II = growth area 50–75% of the Petri dish area, III = growth area 25–50% of the Petri dish area, and IV = growth area occupying < 25% of the Petri dish area. Four categories of conidiophore formation (i.e., sporulation) were also quantified if spores covered >50% of the Petri dish area, covered 20–50% of the Petri dish area, covered < 20% of the surface, or if no sporulation was observed. The number of days it took each *Escovopsis* to sporulate was counted, and appropriate post-hoc categories of elapsed time (number of days) were made in order to perform statistical comparisons. Finally, *Escovopsis–Streptomyces* interactions were scored as "clear inhibition zone", "contact between the two microorganisms", and "*Escovopsis* overgrew *Streptomyces*". Because all data are frequencies, they were analyzed using logit models with the Pearson goodness-of-fit statistics and reported as χ^2. Categories were joined when there were too many zeros and/or expected frequencies were lower than 5 in 25% of the cells [2].

Results

Leucoagaricus-Escovopsis Challenges

In all challenges involving *Leucoagaricus* and *Escovopsis*, we observed a negative effect of the pathogen on the cultivar, as all three indices calculated for *Leucoagaricus* were negative and significantly lower than 1 (DF = 15, 14, 12; $T = -26.6$, -17.4, -21.6; each $P < 0.00001$ for those challenged with ESA, ESB, ESC, respectively). The impact of *Escovopsis* on *Leucoagaricus* differed significantly depending on the different *Escovopsis* strains used in the challenges ($H = 14.46$; $P < 0.0007$), with the differences apparently being due to ant species origin, i.e., *Escovopsis* isolated from *A. lundii* (ESA and ESB) did not differ between each other, but were different from the *Escovopsis* isolated from *A. heyeri* (ESC) (contrasts $z = 2.79$, adjusted $P < 0.016$). Moreover, the difference in growth area (final minus initial) of each challenged *Leucoagaricus* also showed a negative effect of *Escovopsis*, as compared to that of the ant-cultivated fungi control (for ESA and ESB $P < 0.0001$, and for ESC $P < 0.00001$. Medians for challenges = -2.02, -2.00, -3.18. Medians for controls = 3.81, 3.81, 3.58, respectively).

Each of the *Escovopsis* isolates completely overgrew *Leucoagaricus* by the seventh day (Fig. 1), and the indices showed significant differences among the three isolates ($H = 34.9$, $P < 0.0001$).The index for ESC did not differ significantly from an index greater than 1 (DF = 12; $T = -1.95$; $P = 0.9628$), whereas indices for ESA or ESB were significantly greater than 1 (DF = 15, 14; $T = 18.1$, 11.9, respectively; both $P < 0.00001$), indicating a benefit from the interaction (Table 1). Similarly, the difference in area of the challenged *Escovopsis* was greater for ESA (Median = 72.72) and ESB (Median = 75.75) in comparison to their respective controls (MedianESA = 15.99, MedianESB = 41.6), whereas for ESC the control grew relatively more than the challenged (Median Control = 37.02, Median Challenged = 0.00). Only the greatest growth categories I and II were registered (none of the Petri plates exhibited growth corresponding to categories III or IV) for the three *Escovopsis* without any significant association between growth category and *Escovopsis* strains ($\chi^2 = 0.59$; DF = 2; $P > 0.74$). Similarly, there was no association between level of sporulation (1 and 2, only two levels that occurred across all three strains) and *Escovopsis* strains ($\chi^2 = 0.22$; DF = 2; $P > 0.89$). ESB was the only one that showed 6% of the challenges with levels of sporulation 3 and 4, which corresponded to less than 25% or no sporulation, respectively.

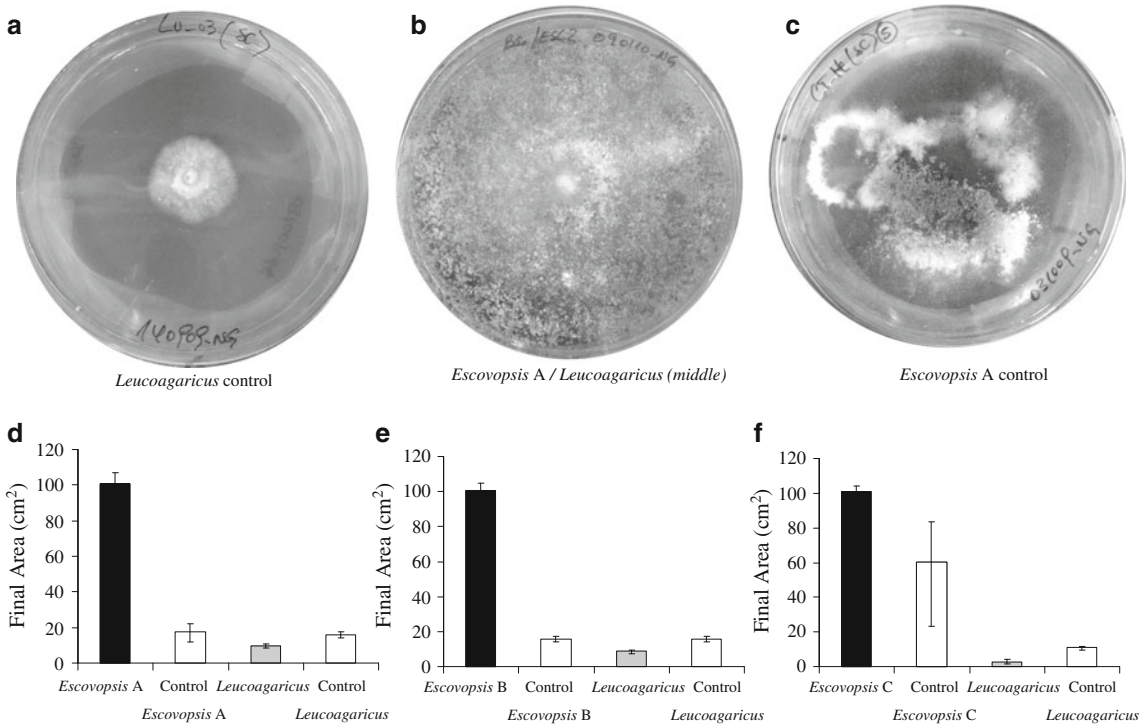

Fig. 1 *Leucoagaricus* versus *Escovopsis* strains ESA, ESB (from *A. lundii*) and ESC (from *A. heyeri*). **a** and **c** correspond to both controls whereas the middle image (**b**) exhibits a representative *Leucoagaricus-Escovopsis* challenge. The *bar graphs* show the median and quartiles of the final area for the pairings and controls (**d** *Escovopsis* A–*Leucoagaricus*, **e** *Escovopsis* B–*Leucoagaricus*, and **f** *Escovopsis* C–*Leucoagaricus*)

Table 1 Median and first and third quartile of the index calculated for each microorganism: *Escovopsis, Leucoagaricus, Streptomyces*, and *Lecanicillium* challenged with each of the three *Escovopsis* strains (ESA, ESB, and ESC)

	ESA	ESB	ESC
Escovopsis	4.12	1.9	0
	(3.66–4.54)	(1.81–2.20)	(0–1.20)
Leucoagaricus	−0.51	−0.50	−0.83
	(−0.31)–(−0.73)	(−0.22)–(0.75)	(−0.61)–(−1.11)
Escovopsis	0.91	0.66	0.89
	(0.52−0.95)	(0.58–0.92)	(0.79–0.95)
Streptomyces	0.58	0.53	0.55
	(0.48−0.72)	(0.40–0.71)	(0.48–0.66)
Escovopsis	0.49	0.63	0.94
	(0.44−0.94)	(0.47–0.69)	(0.77–1.01)
Lecanicillium	0.98	0.96	0.95
	(0.87−1.01)	(0.87–0.99)	(0.88–1.00)

Index values below one indicate growth suppression, above one growth enhancement, and close to one no effect of a microorganism on the other

Escovopsis–Streptomyces Challenges

We did not find differences among the growth indices calculated for the *Streptomyces* challenged with each *Escovopsis* ($H = 0.58$; $P = 0.74$). All indices had values around 0.5 and were significantly lower than 1 (DF = 12, 13, 15; $T = -10.0$, -3.82, -9.15; each $P < 0.001$ for those challenged with ESA, ESB, ESC, respectively), indicating that *Escovopsis* negatively affected *Streptomyces*. The difference in area was significantly smaller for the *Streptomyces* challenged with each of the three *Escovopsis* compared to its control (in all cases $U > 21$; $P < 0.0045$).

Escovopsis growth indices were significantly smaller than 1 (DF = 5, 7, 12; $T = -2.33$, -4.48, -4.87; each $P < 0.03$ for those challenged with ESA, ESB, ESC, respectively) but with much greater values than for *Streptomyces* (Table 1) indicating a much lower negative effect of *Streptomyces* on *Escovopsis* when compared to the reverse as outlined above. Indices did not show significant differences among strains ($H = 3.49$; $P = 0.175$). In fact, the difference in area showed the negative effect of the challenge as all *Escovopsis* had significantly smaller growth rates than their respective controls (all $U = 0.0$; $P < 0.0001$). There were no significant differences in growth ($\chi 2 = 0.38$; DF = 2; $P = 0.826$), sporulation categories ($\chi^2 = 0.44$; DF = 1; $P = 0.80$), dates ($\chi^2 = 0.46$, DF = 1; $P = 0.796$), or distance between *Escovopsis* and *Streptomyces* ($\chi^2 = 0.39$, DF = 2; $P = 0.83$) among the *Escovopsis* strains. Therefore, the three *Escovopsis* strains behaved similarly in the presence of *Streptomyces* sp.

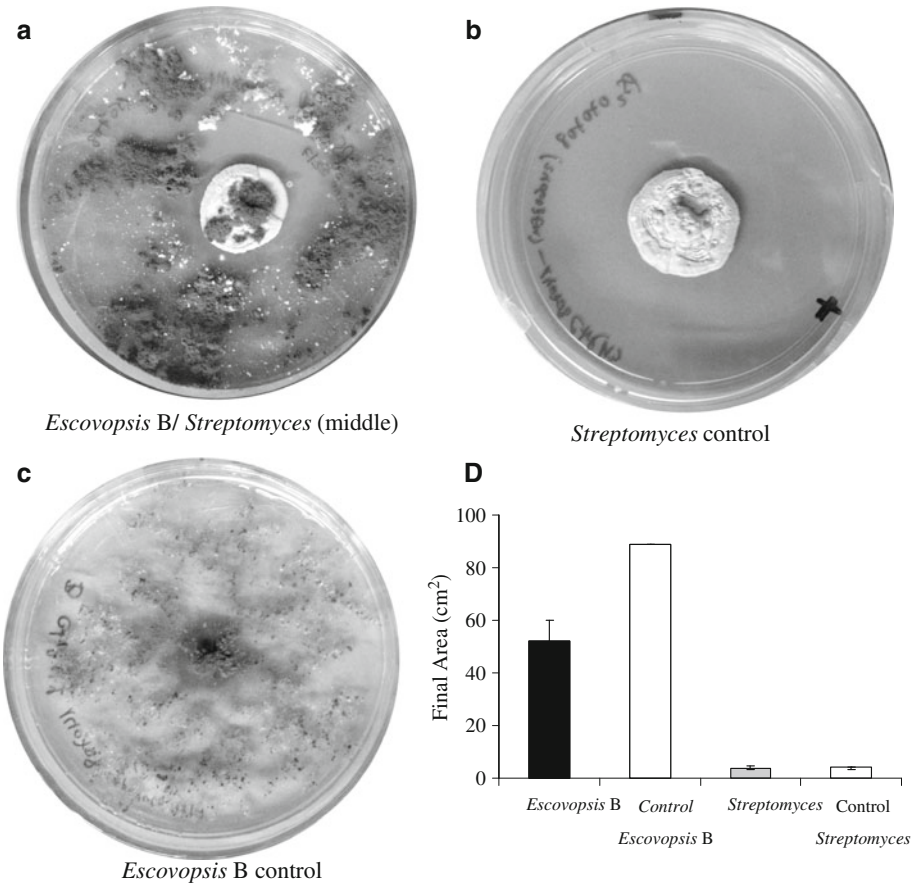

Fig. 2 *Escovopsis–Streptomyces* challenges. **a** Representative result from a pairing. Controls of *Streptomyces* and *Escovopsis* at the end of the experiment are shown in **b** and **c**, respectively. The *bar graph* shows the median and quartiles of the final area for the microorganisms challenged and their respective controls

However, taking into consideration all three *Escovopsis* strains together we did find differences among growth categories ($\chi^2 = 17.91$; DF = 2; $P = 0.0001$), and the chance of a level I response (>75% of Petri dish area covered) was 2.9 times greater than level III (25–50% of petri Dish area covered); level IV was zero in all cases. For all three strains, we only registered levels 1 (spores covering >50% of Petri dish area) and 2 (spores covering between 20–50% of Petri dish area), and sporulation dates differed significantly ($\chi^2 = 5.54$; DF = 1; $P = 0.0018$) such that the chance of sporulation occurring during the first 7 days was 1.6 times lower than later. The chance of *Escovopsis* spp. overgrowing *Streptomyces* sp. was 3.4 times greater than the chance of having an "inhibition zone" present, whereas the chance of "contact between microorganisms" was 2.3 less frequent than the formation of an "inhibition zone" ($\chi^2 = 22.05$; DF = 2; $P = 0.0001$) (Fig. 2).

Escovopsis controls at the end of the experiment were classified with growth I and we did not find significant differences in levels of sporulation ($\chi^2 = 1.02$, DF = 2; $P = 0.59$) between strains or in the date of onset of sporulation ($\chi^2 = 0.08$; DF = 1; $P = 0.78$).

L. lecanii–Escovopsis Challenges

Each of the indices calculated for *L. lecanii* were around 0.95 and not significantly different to 1 (DF = 6, 9, 7; $T = -1.27, -2.21, -2.13$; each $P > 0.05$ for those challenged with ESA, ESB, ESC, respectively) indicating no antagonistic effect of *Escovopsis* on *L. lecanii*. In addition, the three indices did not differ among themselves ($H = 0.376$, $P = 0.828$). In fact, the difference in area (all cases $U > 12$; $P > 0.02$; $P_{adj} = 0.016$) of the challenged *L. lecanii* (against each of the three *Escovopsis* strains) did not differ from the *L. lecanii* control (Fig. 3). *Escovopsis* growth indices were significantly different from 1 (DF = 12, 13, 14; $T = -4.67, 10.89, -2.60$; each $P < 0.02$ for those challenged with ESA, ESB, ESC, respectively) with all positive values but much lower than for *L. lecanii* (Table 1) showing an antagonistic effect of *L. lecanii* on *Escovopsis*. We did not find differences among the indices of the *Escovopsis* strains challenged with *L. lecanii* ($H = 6.78$; $P = 0.03 > P_{adj} = 0.016$). Final areas among *Escovopsis* strains were not significantly different ($H = 7.84$, $P = 0.019 > P_{adj} = 0.016$), although they showed the same tendency (Fig. 3) as the indices, i.e., greatest area

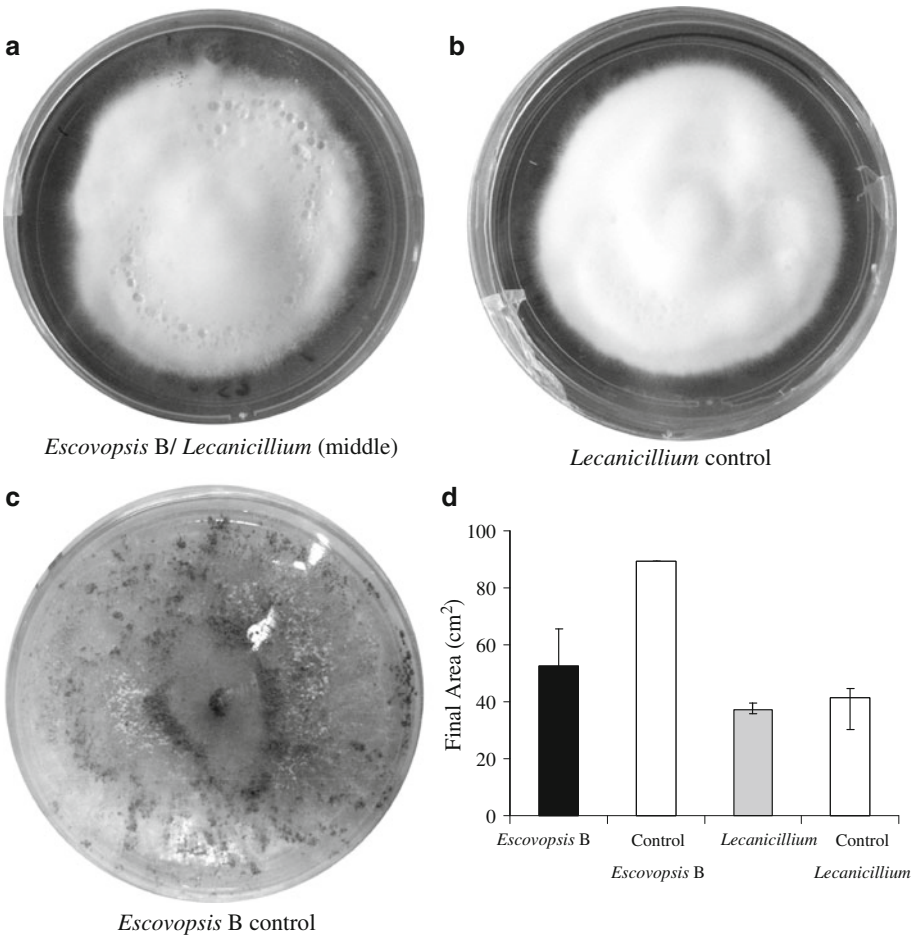

Fig. 3 *Escovopsis–L. lecanii* challenges. **a** Typical *Escovopsis–L. lecanii* result, and controls of *L. lecanii* and one strain of *Escovopsis*, at the end of the experiment, are given in **b** and **c**, respectively. The *bar graph* shows the median and quartiles of the final area for the microorganisms challenged and their respective controls

values for ESC (Median Challenged = 78.84) and smallest for ESA (Median Challenged = 40.15). For each *Escovopsis,* the difference in area were significantly lower than its respective control (all $U > 6$; $P < 0.0019$). There were no differences in growth, or amount or date of sporulation, among the different *Escovopsis* strains (all $\chi^2 < 3$; DF = 1 or 2; $P > 0.05$).

Leucoagaricus–L. lecanii Challenges

The indices calculated for *Leucoagaricus* in the presence of *L. lecanii* were significantly lower than 1 (DF = 5; $T = -11.56$; $P < 0.00001$), with a median of 0.45 (Q1 = 0.36, Q3 = 0.56). This indicates that the entomopathogen has a negative effect on the growth of *Leucoagaricus*. In fact, *L. lecanii* reduced the ant-cultivated fungi area by an average of 50% (Median Control *Leucoagaricus* = 15.25, Median Challenged *Leucoagaricus* = 6.63; $U = 30$, $P < 0.0022$). However, none of the *Leucoagaricus* challenges were completely overgrown by *L. lecanii*; in fact, in most cases, we observed antagonism between the two fungi (Fig. 4). The growth index calculated for *L. lecanii* was similar to that of *Leucoagaricus* (Median = 0.46, Q1 = 0.41, Q3 = 0.57), and also significantly lower than 1 (DF = 5; $T = -13.61$; $P < 0.00001$; *t* test). Again, there was a negative (and similar) effect of *Leucoagaricus* on *L. lecanii*, as the latter exhibited a 50% growth reduction in the presence of the ant-cultivated fungi (Median Control *L. lecanii* = 59.15, Median Challenged *L. lecanii* = 26.63; $U = 48$; $P < 0.0024$).

Discussion

Our results indicate that *Escovopsis* has the potential to be used as a promising biocontrol agent of the pest ant *A. lundii* by being attracted to and utilizing the cultivar maintained by these ants. In all pairings, the three *Escovopsis* strains used in this study rapidly overgrew the strain of *Leucoagaricus*, and exhibited similar growth parameters and virulence characteristics. This is the first time that interactions between *Escovopsis* and symbionts from *A. lundii* garden have been tested, and our findings indicate that the patterns of interactions are comparable to those observed in other *Acromyrmex* spp. For example, Taerum et al. [45] showed a lack of specificity of different *Escovopsis* strains towards cultivars from geographically diverse *Atta* and *Acromyrmex* leaf-cutting ants, and

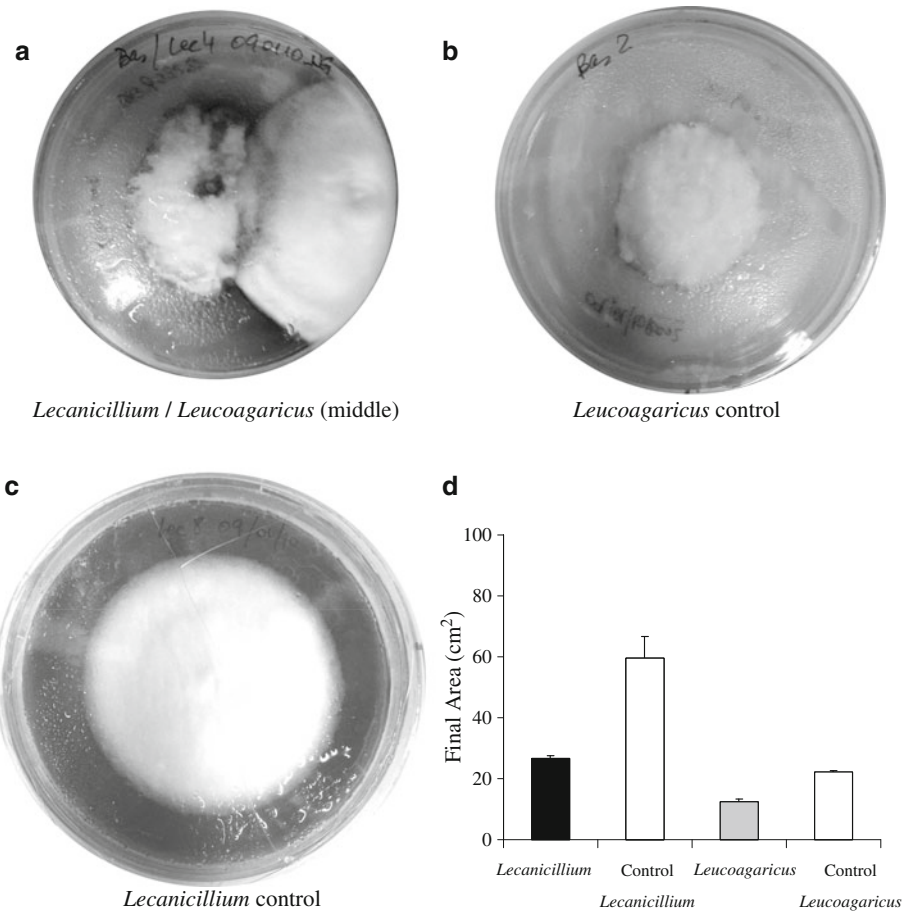

Fig. 4 *Leucoagaricus–L. lecanii* sp. Images show the result of the challenges after 23 days (**a**) and the final growth of *Leucoagaricus* (**b**) and *L. lecanii* (**c**) controls. The *bar graph* shows the median and quartiles of the final area for the fungi challenged and their respective controls

Poulsen et al. [41] found little to no specificity in *Escovopsis*-cultivar pairings of symbionts originating from 12 *Acromyrmex* colonies from Panama and Argentina. Thus, our in vitro findings support that *Escovopsis* has the potential to be a useful biocontrol agent of this leaf-cutting ant species, and probably also for other *Acromyrmex* leaf-cutting ant pests. However, we should stress that its potential will be pending on finding similar effects using entire colonies, which would encompass all possible interactions between *Escovopsis* and the ants and other microorganisms in the system.

Despite the consistent overgrowth that *Escovopsis* exhibited on cultivar strains, we did find some differences between the parasite strains. ESC (from the sympatric allospecific host *A. heyeri*) exhibited the fastest response in the presence of the cultivar; 2 days after initiating the challenge, the pathogen covered all of the Petri dish area and the ant-cultivated fungi. At the same time, the index for ESC did not differ from 1, while the other strains showed index values much greater than 1. These differences suggest the possibility that ESC obtains less benefit from the novel *Leucoagaricus* strain, or that it experiences a higher cost associated with overcoming the defense as compared to the other two strains. This variation in *Escovopsis* growth rates suggests that specific strains might be faster acting during colony infections, and this should be a consideration for strain choice for biocontrol. As mentioned before, colony and, ideally, field experiments need to be employed to verify the impact and outcome of *Escovopsis* infection on mature *A. lundii* nests.

Leucoagaricus and *L. lecanii* negatively affected each other, which was apparent through reduced growth rates and halted growth (i.e., one fungus never overgrew the other). This suggests that *L. lecanii* is competing for nutrients with the ants' mutualistic fungus [42], despite being entomopathogenic, at least under laboratory conditions. Consequently, it may be recommendable to use *L. lecanii* as an entomopathogen to both kill the ants and compete with the cultivar. As of yet, no data on the impact of *L. lecanii* on the ants themselves are available, but such studies would be a welcome addition to exploring the use of this fungus in biocontrol.

Lecanicillium lecanii also seemed to compete with *Escovopsis*, reducing the growth of the latter. This suggests that it might not be valuable to use *Escovopsis* and *L. lecanii* simultaneously as biocontrol agents. However, since our results are based on Petri dish challenges, simultaneous infections within intact colonies could

potentially increase pressure on ant defenses. For example, the presence of an entomopathogen may reduce the ability of the ants to remove *Escovopsis*. Hughes and Boomsma [31] showed that a mild pathogen of *Acromyrmex echinatior* ants (*Aspergillus flavus*) became significantly more virulent when co-infected with the virulent *M. anisopliae*. Therefore, offering two fronts of attacks (*Escovopsis* and *L. lecanii*) could diminish the efficacy with which leaf-cutting ants can maintain colony health.

An important consideration for biocontrol is the avoidance of impact on non-target organisms [24, 27, 30, and references therein]. Non-target impacts are unlikely for *Escovopsis* spp. because these fungi specifically attack fungus-growing ant cultivars and have not been to be associated with other types of ants [17]. High species richness of leaf-cutting ants coexisting is found in natural environment, where there is little or no disturbance [21]; however, in human-modified habitats where biocontrol is needed, species richness is significantly reduced [3]. Therefore even if *Escovopsis* could switch host, it is unlikely that other hosts than the target and pest leaf-cutter ant species would be present. Regarding *L. lecanii,* it is a mesophilic generalist entomopathogen found in many parts of the world [4] leading to higher expectations of potential spread and infection of non-target organisms, and future work is thus needed to establish such potential side-effects.

Pseudonocardia Actinobacteria have been shown to contribute to the suppression of *Escovopsis* in other *Acromyrmex* species [16, 17, 41]. The *Streptomyces* sp. isolated from *A. lundii* did not inhibit growth of *Escovopsis* significantly, and in fact *Escovopsis* strains showed maximum growth and sporulation in these challenges. In addition, *Streptomyces* grew significantly less (lower index, smaller area in comparison to control) when challenged with *Escovopsis* spp. This could be due to the potential extra cost of antibiotic production in the presence of the pathogen. There are other studies reporting no inhibition of *Escovopsis* spp. by some Actinobacteria [5, 32, 36], but the functional role of these additional bacteria has not been fully established. Since the putative role of *Streptomyces* sp. strains in the association with *A. lundii* is also not fully established, it is likely that this ant species either (i) has *Pseudonocardia* as other *Acromyrmex* spp. and that we missed these in our isolation attempts, or (ii) has lost or significantly reduced the association with Actinobacteria. If the latter applies, this would mirror the situation in *Atta* and *Sericomyrmex* fungus-growing ants, where the role of antibiotics from Actinobacteria has possibly been replaced with antibiotic secretions from the metapleural glands of the ants themselves [22]. Irrespective of the presence or absence of specific *A. lundii*–Actinobacteria associations, our findings indicate that the tested *Streptomyces* sp. is unlikely to reduce the impact of *Escovopsis*.

Damage produced by leaf-cutting ants in the Americas exceeds billon dollars per year [33]. Therefore, an urgent need for their control is warranted where pesticides have proven inefficient to solve this problem. Biocontrol strategies are also promising alternatives and the results obtained in this study are encouraging. Specifically, our findings favor the use of *Escovopsis* as a biological control agent, because it is specific to fungus-growing ant cultivars and potentially virulent, thereby fulfilling the main prerequisites of a biological control agent. It remains to be determined if it is effective at controlling colonies in nature and future studies should include in situ evaluations. Albeit potentially competing with *Escovopsis*, the competitive abilities of *L. lecanii* towards *Leucoagaricus* is promising and should be further evaluated using entire colonies, both alone as well as in combination with *Escovopsis*.

Acknowledgments We thank U. G. Mueller for molecular identification of *Leucoagaricus* and *Lecanicillium lecanii*, and Ariel Marfetán, Deborah Colman, and Gabriel Maceiras for their laboratory assistance. An anonymous reviewer, the associate editor Dr. Matías Cafaro, and Johanna Gelderman provided important editorial corrections. We are indebted to the Capovilla family who allowed us to sample on their property. This work was financed by the Agencia Nacional de Promoción Científica y Técnica, Argentina, PICT 20924 to PJF. P. Folgarait thanks CONICET and Universidad Nacional de Quilmes for continuous support.

References

1. Abramowski D, Currie CR, Poulsen M (2011) Caste specialization in behavioral defenses against fungus garden parasites in *Acromyrmex octospinosus* leaf-cutting ants. Insectes Soc 58:65–75
2. Agresti A (1991) Categorical data analysis. Wiley, New York
3. Anjos N, Moreira DDO, Della Lucia TMC (1993) Manejo integrado de formigas cortadeiras em reflorestamentos. In: Della Lucia TMC (ed) As formigas cortadeiras Mina Gerais. Universidade Federal de Viçosa, Brazil, pp 212–241
4. Ayala Zermeño MA, Mier T, Sánchez-Robles J, Toriello C (2005) Variabilidad intraespecífica del crecimiento de *Lecanicillium lecanii* por efecto de la temperatura. Revista Mexicana de Micología 20:93–97
5. Barke J, Seipkel RF, Grüschow S, Heavens D, Drou N, Bibb MJ, Goss RJM, Yu DW, Hutchings MI (2010) A mixed community of actinomycetes produce multiple antibiotics for the fungus farming ant *Acromyrmex octospinosus*. BMC Biol 8:109
6. Bettini S (1978) Arthropod venoms, Chap 25. Springer-Verlag, New York, pp 801–869
7. Bot ANM, Currie CR, Hart AG, Boomsma JJ (2001) Waste management in leafcutting ants. Ethol Ecol Evol 3:225–237
8. Cafaro MJ, Currie CR (2005) Phylogenetic analysis of mutualistic filamentous bacteria associated with fungus-growing ants. Can J Microbiol 51:441–446
9. Cafaro M, Poulsen M, Little AEF, Gerardo NM, Price S, Wong B, Stuart AE, Larget B, Abbot P, Currie CR (2011) Specificity in the symbiotic association between fungus-growing ants and protective *Pseudonocardia* bacteria. Proc R Soc B. doi:10.1098/rspb.2010.2118

10. Cherret JM (1986) History of the leaf-cutting ant problem. In: Lofgren CS, Vander Meer RK (eds) Fire ants and leaf-cutting ants: biology and management. Westview Press, Boulder, pp 10–17
11. Cherret JM (1986) The economic importance and control of leaf-cutting ants. In: Vinson SB (ed) Economic impact and control of social insects. Praeger, New York, pp 165–192
12. Cortez Madrigal H (2007) Producción de *Lecanicillium* (=*Verticillium*) *lecanii* en diferentes sustratos y patogenicidad. Agr Tec Mex 33:83–87
13. Currie CR (2001) Prevalence and impact of a virulent parasite on a tripartite mutualism. Oecologia 128:99–106
14. Currie CR, Stuart AE (2001) Weeding and grooming of pathogens in agriculture by ants. Proc R Soc Lond B 268:1033–1039
15. Currie CR, Mueller UG, Malloch D (1999) The agricultural pathology of ant fungus gardens. Proc Natl Acad Sci USA 96:7998–8002
16. Currie CR, Scott JA, Summerbell RA, Malloch D (1999) Fungus growing ants use antibiotic-producing bacteria to control garden parasites. Nature 398:701–704
17. Currie CR, Wong B, Stuart AE, Schultz TR, Rehner SA, Mueller UG, Sung GH, Spatafora JF, Straus NA (2003) Ancient tripartite coevolution in the attine ant–microbe symbiosis. Science 299: 386–388
18. Currie CR, Bot ANM, Boomsma JJ (2003) Experimental evidence of a tripartite mutualism: bacteria protect ant fungal gardens from specialized parasites. Oikos 101:91–102
19. Della Lucia TMC (1993) As formigas cortadeiras. Universidade Federal de Viçosa, Mina Gerais, Brazil
20. Della Lucia TMC, Vilela EF (1993) Métodos atuais de controle e perspectivas. In: Della Lucia TMC (ed) As formigas cortadeiras. Universidade Federal de Viçosa, Mina Gerais, Brazil, pp 163–190
21. Elizalde E, Folgarait PJ (2010) Host diversity and environmental variables as determinants of the species richness of the parasitoids of leaf-cutting ants. J Biogeogr 37:2305–2316
22. Fernández-Marín H, Zimmerman JK, Nash DR, Boomsma JJ, Wcislo WT (2009) Reduced biological control and enhanced chemical pest management in the evolution of fungus farming in ants. Proc R Soc B 276:2263–2269
23. Foguelman D (ed) (2003) Plagas y Enfermedades en Manejo Orgánico: Una Mirada Latinoamericana. IFOAM, Alemania
24. Follett PA, Duan JJ (1999) Non-target effects of biological control. Kluwer Academic Publishers, Dordrecht
25. Forest Stewardship Council (2007) FSC Guidance Document. FSC pesticides policy: guidance on implementation. Forest Stewardship Council A.C., Bonn, Germany
26. Ganassi S, Grazioso P, Moretti A, Sabatini MA (2010) Effects of the fungus *Lecanicillium lecanii* on survival and reproduction of the aphid *Schizaphis graminum*. Biocontrol 55:299–312
27. Hajek AE, Goettel MS (2007) Guidelines for evaluating effects of entomopathogens on non-target organisms. In: Lacey LA, Kaya HK (eds) Field manual of techniques in invertebrate pathology. Springer, Dordrecht, pp 815–834
28. Hall RA (1981) The fungus *Verticillium lecanii* as a microbial insecticide against aphids and scales. In: Burges HD (ed) Microbial control of pests and plants disease. Academic Press, New York, pp 483–498
29. Header SR, Wirth R, Hertz H, Spiteller D (2009) Candicin-producing *Streptomyces* support leaf-cutting ants to protect their fungus garden against the pathogenic fungus *Escovopsis*. Proc Natl Acad Sci USA 106:4742–4746
30. Hokkanen HMT, Zeng QQ, Menzler-Hokkanen I (2003) Environmental impacts of microbial insecticides. Kluwer Academic Publishers, Dordrecht
31. Hughes WOH, Boomsma JJ (2004) Let your enemy do the work: within-host interactions between two fungal parasites of leaf-cutting ants. Proc R Soc B 271:S104–S106
32. Kost C, Lakatos T, Bottcher I, Arendholz WR, Redenbach M, Wirth R (2007) Non-specific association between filamentous bacteria and fungus-growing ants. Naturwissenschaften 94: 821–828
33. Lima PP (1992) Palestra sobre formigas cortadeiras. Memoria de Reuniao de Especialistas en Controle Alternativo de Cupins e Formigas. Ibama, Brasil
34. Little AEF, Murakami T, Mueller UG, Currie CR (2006) Defending against parasites: fungus-growing ants combine specialized behaviours and microbial symbionts to protect their fungus gardens. Proc R Soc Biol Lett 2:12–16
35. Lopez E, Orduz S (2003) *Metarhizium anisopliae* and *Trichoderma viride* for control of nests of the fungus-growing ant, *Atta cephalotes*. Biol Control 27:194–200
36. Mueller UG, Dash D, Rabeling C, Rodrigues A (2008) Coevolution between attine ants and actinomycete bacteria: a reevaluation. Evolution 62:2894–2912
37. Oh DC, Poulsen M, Currie CR, Clardy J (2009) Dentigerumycin: a bacterial mediator of an ant-fungus symbiosis. Nat Chem Biol 5:391–393
38. Ortiz A, Orduz S (2000) In vitro evaluation of *Trichoderma* and *Gliocladium* antagonism against symbiotic fungus of the leaf-cutting ant *Atta cephalotes*. Mycopathologia 150:53–60
39. Pimentel D, McLaughlin L, Zepp A, Lakitan B, Kraus T, Kleinman P, Vancini F, John Roach W, Graap E, Keeton WS, Selig G (1991) Environmental and economic effects of reducing pesticide use. Bioscience 41:402–409
40. Poulsen M, Currie CR (2006) Complexity of insect-fungal associations: exploring the influence of microorganisms on attine ant-fungus symbiosis. In: Bourtzis K, Miller T (eds) Insect symbiosis, vol 2. CRC Press, Boca Raton
41. Poulsen M, Cafaro MJ, Erhardt D, Little AEF, Gerardo NM, Tebbets B, Klein B, Currie CR (2010) Variation in *Pseudonocardia* antibiotic defense helps govern parasite-induced morbidity in *Acromyrmex* leaf-cutting ants. Environ Microbiol Rep 2: 534–540
42. Read AF, Taylor LH (2001) The ecology of genetically diverse infections. Science 292:1099–1102
43. Reynolds HT, Currie CH (2004) Pathogenicity of *Escovopsis weberi*: the parasite of the attine ant-microbe symbiosis directly consumes the ant-cultivated fungus. Mycologia 96:955–959
44. Siegel S (1976) Estadística no paramétrica aplicada a las ciencias de la conducta. México, Trillas
45. Taerum SJ, Cafaro MJ, Little AEF, Schultz TR, Currie CR (2007) Low host-pathogen specificity in the leaf-cutting ant-microbe symbiosis. Proc R Soc B 274:1971–1978