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Antagonistic interactions between garden yeasts and microfungal garden pathogens of leaf-cutting ants

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Abstract We investigate the diversity of yeasts isolated in gardens of the leafcutter ant *Atta texana*. Repeated sampling of gardens from four nests over a 1-year time period showed that gardens contain a diverse assemblage of yeasts. The yeast community in gardens consisted mostly of yeasts associated with plants or soil, but community composition changed between sampling periods. In order to understand the potential disease-suppressing roles of the garden yeasts, we screened isolates for antagonistic effects against known microfungal garden contaminants. In vitro assays revealed that yeasts inhibited the mycelial growth of two strains of *Escovopsis* (a specialized attine garden parasite), *Syncephalastrum racemosum* (a fungus often growing in gardens of leafcutter lab nests), and the insect pathogen *Beauveria bassiana*. These garden yeasts add to the growing list of disease-suppressing microbes in attine nests that may contribute synergistically, together with actinomycetes and *Burkholderia* bacteria, to protect the gardens and the ants against diseases. Additionally, we suggest that garden immunity against problem fungi may therefore derive not only from the presence of disease-suppressing *Pseudonocardia* actinomycetes, but from an enrichment of multiple disease-suppressing microorganisms in the garden matrix.

Keywords *Atta* · Symbiosis · Fungus garden · Yeast antagonism

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Introduction

Yeasts play an important role in the stability of many ecosystems (Latham 1998; Inácio et al. 2002; Gadanho et al. 2006). Because of their ubiquity and abundance in nature, yeasts are likely to interact with diverse microbes and eukaryotic organisms, and yeast-invertebrate associations are therefore thought to be common in nature; such as the associations between insects and gut-inhabiting yeasts (Nguyen et al. 2006; Suh et al. 2008). In addition, insects may harbor yeasts on other parts of their bodies, including their exoskeleton and legs (Ganter 2006).

Several types of yeast-insect interactions have been described to date (Vega and Dowd 2005). Some

may involve the passive dispersal of yeasts by fly vectors in tropical forests (Morais et al. 1992). Other yeasts are clearly mutualistic associates that are adaptively dispersed by their insect host, such as the cultivated yeast fungi of a specialized group of fungus-growing ants in the genus *Cyphomyrmex* (Mueller et al. 1998), and specialized yeasts dispersed by insects on ephemeral flowers (Lachance et al. 2001). In the present study we investigate whether yeasts could act as mutualists in the leaf-cutting ant-microbe association.

Leaf-cutting ants (Hymenoptera, Formicidae, tribe Attini) are involved in an ancient and obligate mutualism with basidiomycetous fungi which are cultivated as food (Mueller and Rabeling 2008; Schultz and Brady 2008). Leaf-cutting ants forage for fresh leaves and flowers as substrate for fungal growth, transport the plant material to underground garden chambers, then process the material for incorporation in their gardens. Over the past decade, filamentous bacteria of the genus *Pseudonocardia* were discovered to be associated with attine ants (Currie et al. 1999a; Cafaro and Currie 2005). These bacteria produce antibiotics to suppress the growth of a specialized fungal parasite in the genus *Escovopsis* (*Ascomycota*: anamorphic *Hypocreales*) that naturally infects gardens of several attine ant species (Currie et al. 1999b). Multiple lines of evidence indicate that *Escovopsis* likely coevolved in the attine ant-microbe symbiosis (Currie et al. 2003). *Pseudonocardia* symbionts apparently coevolved within this system (Cafaro and Currie 2005), however, recent studies have shown that these bacteria may exhibit a more diffuse association with their ant hosts (Kost et al. 2007; Mikheyev et al. 2008; Mueller et al. 2008).

The first systematic study on attine garden yeasts was carried out by Carreiro et al. (1997) who investigated yeasts in laboratory nests of *Atta sexdens rubropilosa.* Carreiro et al. (1997) found seven yeast genera in leafcutter gardens and in the waste material produced by the ant colony, including the widely distributed genus *Candida*. Later studies described two new yeast species from ant gardens, *Cryptococcus haglerorum* (Middelhoven et al. 2003) and *Sympodiomyces attinorum* (Carreiro et al. 2004); both isolated from *A. sexdens rubropilosa* nests. Yeasts were also found in infrabuccal pellets carried by gynes of the leaf-cutting ants *Atta texana* (Cable et al. in preparation) and *A. laevigata* (Pagnocca et al. 2008) as well as on the exoskeleton of dispersing females of *A. laevigata* and *A. capiguara* (Pagnocca et al. 2008). Moreover, black yeasts (closely related to *Phialophora* sp.) found in the exoskeleton of *Apterostigma* ants and also on other attine species were shown to have negative indirect effects on *Pseudonocardia* (Little and Currie 2008).

Several roles have been hypothesized for yeasts in gardens of leaf-cutting ants. First, a number of yeasts produce hydrolytic enzymes that help digest the major plant polysaccharides present in the fungus gardens and thus aid the cultivated fungus to access more nutrients from the plant substrate (Carreiro 2000). Second, yeast found in attine gardens secretes low-weight proteins (i.e. mycocins) that inhibit the growth of yeast sensitive strains (Carreiro et al. 2002). Toxin-secreting yeasts are thought to maintain the stability of some microbial communities (Starmer et al. 1987), mediated through interference competition against other yeasts and also plant pathogenic fungi (Ganter and Starmer 1992; Walker et al. 1995). Carreiro et al. (2002) suggested that interference competition as a possible mechanism controlling yeast population and other antagonistic microbes in attine gardens.

Fungus gardens of attine ants are continuously threatened by two different types of microbes: (1) unspecialized antagonist fungi such as *Cunninghamella elegans*, *Syncephalastrum racemosum* and *Trichoderma harzianum* (saprophytic fungi commonly found in both laboratory or field nests of attine ants) that are imported by the ants with the plant material they collect or are acquired from the soil (Rodrigues et al. 2005, 2008a), and (2) specialized garden pathogens, such as *Escovopsis* spp., which are horizontally transmitted among nests (Currie et al. 1999b). Attine ants remove or suppress the growth of such invading microbes by weeding and grooming nest parts (Currie and Stuart 2001), antimicrobial secretions of the ants (Bot et al. 2002; Rodrigues et al. 2008b) or by application of antibiotics derived from disease-suppressing bacteria on the ants or in the garden matrix (Currie et al. 1999a; Santos et al. 2004; Mueller et al. 2005). In the present study, we profiled the yeast diversity in gardens of the leafcutter ant *A. texana* and tested whether yeasts could serve disease-suppressing roles against microfungal garden pathogens.

Methods

Fungus garden collection

To profile the yeasts associated with leaf-cutting ants and to investigate the possible effects of sampling regime on the yeast garden community, we studied four mature field nests of *A. texana*. One nest (colony UGM051218-02) was sampled near Buescher State Park, Bastrop County, Texas (GPS locality: N30°5.808′, W97°13.462′), whereas the other three nests (colonies UGM060121-01, UGM060121-02 and AR060123-01) were sampled at Hornsby Bend Environmental Research Center, Austin, Texas (GPS localities: N30°13.973′, W97°39.101′; N30°13.94′, W97°39.18′; N30°14.008′, W97°39.039′). Each nest was sampled every 3 months during a 1 year study (winter, spring, summer, and fall of 2006).

Nests were excavated by opening a 1–2 m deep trench (depending on the expected depth of gardens) close to the colony mound area, where most ant digging-activity was concentrated. Once the trench was dug, excavation continued in direction of the expected location of gardens until a fungus chamber was exposed. This lateral excavation ensured that gardens were accessed with minimal contamination. Mature fungus gardens (cream-colored parts) together with tending ants were aseptically collected and stored in sterile containers for transport to the laboratory. Garden texture remained intact during the transport, and samples were processed for yeast isolation within 8 h after collection. For some nests in spring and fall, collected fungus gardens were composed of dark-green-colored parts, indicating recent addition of plant substrate by the ants; in contrast, in winter and summer, ants decrease their foraging activities, and we did not find gardens with recent plant addition. Excavation trenches were closed after each collection to minimize disturbance and prevent colony migration to an undisturbed nest site.

Three nests could be sampled four times in the above manner, but after three excavations in winter, spring, and summer, no live garden could be found in one nest (AR060123-01) at this nest's original site; this nest either had deteriorated because of the repeat disturbances, or moved to a new location. Because no fresh garden could be collected in fall 2006 for this nest, we instead use dried fungus gardens found in chambers underneath the original mound (here after named "collapsed garden") that we collected and processed as described for the samples from healthy garden.

Yeast isolation and enumeration

Ten-fold dilution series were carried out in 0.05% Tween 80 and 0.2% peptone water using one gram of each sample, which were first disrupted using a sterile glass rod and vortexed for 1 min. Aliquots of 150 µl from a dilution were then surface spread on each of four plates with yeast-malt agar medium (YMA: yeast extract 3.0 g l^{-1}, malt extract 3.0 g l^{-1}, peptone 5.0 g l^{-1}, dextrose 10 g l^{-1} and agar 15 g l^{-1}) supplemented with 150 µg ml^{-1} of chloramphenicol (US Biological Inc.) and 30 µg ml^{-1} of rose bengal (Fisher Scientific Inc.). After an incubation time of 5 days at 25°C, plates were screened for yeasts under a stereomicroscope and the number of yeast-colony-forming units (CFU) per gram of fungus gardens was determined. Yeast colonies were selected from YMA plates and each colony was considered an isolate in this study (see Tables 2, 3). Pure strains were suspended in 30% glycerol and stored at −80°C for identification. Representative strains were deposited at Centraalbureau voor Schimmelcultures—CBS (see Table 2 for accessions #).

Yeast identification and sequencing

Yeast strains were identified using sequencing information from the variable D1/D2 regions of the nuclear large subunit ribosomal DNA (Kurtzman and Robnett 1998). DNA extractions followed the protocol of Mikheyev et al. (2006) and 1.0 µl DNA extracts were used as template in 10 µl PCR reactions. Reactions included 10× PCR-buffer, 0.8 µM of each primer (NL1 and NL4), 1 mM of each dNTP, 2.5 mM of $MgCl_2$, and 1 U of pure *Taq* polymerase (Bioline). PCR conditions were as follows: 96°C for 3 min, 35 cycles at 96°C for 30 s, 61°C for 45 s, and a final extension step at 72°C for 1 min. Purification and cycle sequencing procedures of amplicons followed Mikheyev et al. (2006). Both forward and reverse sequences were generated on a 3100 ABI automated sequencer (Applied Biosystems).

Sequences were edited and contigs assembled in Bioedit v.7.0.5.3 (Hall 1999), then used in BLASTn analysis at NCBI-GenBank (Altschul et al. 1997).

Sequences with 99% similarity to sequences deposited at GenBank were considered conspecific (Kurtzman and Robnett 1998), whereas sequence similarity scores of 98% or lower were labeled with the closest relative found in the database (Table 2). Sequences of representative taxa were deposited at GenBank under accession #: FJ743599–FJ743631.

Yeast antagonism bioassays

To test the potential defensive role of yeasts in fungus gardens, 14 yeast strains commonly found in *A. texana* gardens as well as strains that occurred in minor proportions (Table 2) were challenged in in vitro bioassays against microfungal antagonists (Currie et al. 1999a; Rodrigues et al. 2008a). Bioassays challenges followed an adapted version of the experimental set up used by Gerardo et al. (2006). Yeasts were grown on YMA medium for 3 days at 25°C before the experiments. YMA plates were divided into four tracks (Fig. 2), three were point-inoculated at the edges with the test yeasts and one track was left blank as a control (no yeast inoculated). Yeasts were allowed to grow for 3 days at 25°C, and then an agar plug (5 mm of diameter) of the test microfungus was inoculated in the center of the Petri dish. Microfungi isolated from different attine sources (Table 1) were grown in 2% malt agar medium for 7 days at 25°C before cutting plugs for the experiments.

The insect pathogen *Beauveria bassiana* and the microfungal garden antagonists *C. elegans*, two *Escovopsis* strains, *S. racemosum* and *T. harzianum* (Table 1) were each confronted four times with yeast strains. The experiments were monitored daily and measurements (from the edge of the central agar plug to the mycelial growth edge) where taken when mycelial growth of the tested microfungus reached the end of the control track; or when the mycelium stopped growing in the control track (Fig. 2).

Effects on microfungi growth were assessed by dividing the distance of the mycelium growing in the presence of the test yeast by the distance of the mycelium growing in absence of the test yeast (control). Ratios were average for each microfungi and overall differences in suppression or increase of mycelial growth were tested using Kruskal–Wallis non-parametric test in BioEstat 5.0 (Ayres et al. 2007).

Mycocin activity

To check the mechanism by which yeasts inhibited the mycelial growth of the tested microfungi, the extracellular production of mycocins by yeasts was evaluated using the method of Walker et al. (1995). Briefly, modified Sabouraud medium (20 g l^{-1} of dextrose, 10 g l^{-1} of peptone, 20 g l^{-1} agar and 0.03 g l^{-1} of methylene blue in citrate-phosphate buffer, pH 4.65) was mixed with 1 ml of sensitive yeast suspension and poured in Petri dishes. Five sensitive strains were used: *Candida albicans* 3153 (retrieved from The London School of Hygiene & Tropical Medicine), *Candida glabrata* NCYC 388 (Starmer et al. 1987), *Pichia kluyveri* UFMG-A15, *Saccharomyces cerevisiae* NCYC1006 and *Cryptococcus neoformans* HSL3 (Fuentefria et al. 2008).

Yeast strains to be tested for killer activity were point-inoculated onto the lawn of the sensitive yeast strains, and then incubated at 25°C for 48 h. Production of mycocins was identified by the formation of an inhibition halo surrounding the yeast colony. Test

Table 1 Microfungal species used in yeast antagonism bioassays

IDs	Microfungal isolates	Ant species[a]	Origin
RC003	*Beauveria bassiana*	*Atta texana*	Worker corpse in an "external dump", USA
TR001	*Cunninghamella elegans*	*Trachymyrmex septentrionalis*	Field nest, USA
RC005	*Escovopsis* sp.	*Trachymyrmex turrifex*	Laboratory nest, USA
RC007	*Escovopsis* sp.	*Atta sexdens*	Laboratory nest, originally from Peru
RC001	*Syncephalastrum racemosum*	*Acromyrmex coronatus*	Laboratory nest, originally from Panama
TR003	*Trichoderma harzianum*	*Trachymyrmex septentrionalis*	Field nest, USA

[a] Ants species which the microfungi were isolated from

and sensitive yeasts strains were 24 h old at the time of testing. Ten mycocin-positive strains were used as control.

Results

Diversity and abundance of yeasts on *A. texana* gardens

A total of 64 yeasts strains were recovered from gardens of four *A. texana* nests sampled during the 1-year collection period (Table 2). Based on morphological characteristics, 59 strains were recognized as yeasts and 5 strains were classified as yeast-like fungi. On culture plates, the latter isolates resemble yeasts morphologically; however, they are not closely related with this group accordingly to the BLASTn results (Table 2).

The number of basidiomycetous yeast strains ($n = 46$) were almost four times higher than the number of ascomycetous yeasts strains ($n = 13$). Overall, 32 yeast species from 18 genera were identified with morphological and molecular criteria. *Cryptococcus* (47%) was the most abundant genus, followed by *Rhodotorula* (9%) and *Kodamaea* (8%) (Table 2). The most-abundant species was *Cryptococcus magnus* (12.5%), followed by *Kodamaea ohmeri* (7.6%).

Five yeast strains (*Candida* cf. *melibiosica*, $n = 1$ isolate; *Cryptococcus* cf. *luteolus*, $n = 3$; *Cryptococcus* cf. *taibaiensis*, $n = 1$; *Rhodotorula* cf. *javanica*, $n = 1$; *Rhodotorula* cf. *taiwaniana*, $n = 1$) had sequence similarity scores of 97% when compared with sequences deposited at GenBank (Table 2). In yeast taxonomy, more than 1% of substitutions in the D1/D2 region is usually assumed as a criterion for identification of a putative new yeast species (Kurtzman and Robnett 1998). Thus, in the present study these yeasts isolates may represent undescribed yeast species. Further studies to investigate their taxonomic relationships will be presented elsewhere.

Fungus gardens of *A. texana* appear to have a transient yeast microbiota, with exception of *Cr. magnus* that was recovered consistently during the winter, spring, and summer collections (Table 2). Yeasts were isolated from nests UGM051218-02 and UGM060121-02 from all four seasons (Table 3). On the other hand, no yeast was isolated from nest UGM 060121-01 during summer and from nest AR060123-01 during spring and fall collections. No correlation between sampling periods and the number of yeasts CFU per gram was observed (Table 3). In fact, we observed a wide fluctuation in the number of CFUs from different nests sampled at different seasons. For instance, yeast populations ranged from 0.28×10^2 to 80×10^2 CFU of yeasts per gram of fungus garden in nest UGM 051218-02 (Table 3). Interestingly, no yeast was isolated from collapsed, abandoned garden collected in fall 2006 from nest AR060123-01, in contrast to gardens in other nests that were tended by workers; instead filamentous fungi such as *Absidia* cf. *glauca*, *Aspergillus flavus*, *Cunninghamella* sp. and *Mucor* sp. were isolated from this type of substrate.

Antagonism against garden pathogens

In the 82 bioassay challenges, the mycelial growth of all microfungi was affected by at least one yeast strain (Figs. 1, 2). Comparisons among the tested yeasts revealed that some strains either increased or inhibited the mycelial growth of *C. elegans* (Kruskal–Wallis test, $H = 31.67$, $df = 14$, $P < 0.05$, Fig. 1b); however, when comparing each yeast with the control treatment the differences were not significantly different ($P > 0.05$). Apparently, the mycelial growth of *C. elegans* was significantly reduced by *K. ohmeri* in relation to *Cryptococcus* cf. *cellulolyticus* A (Dunn test, $z = 3.59$, $P < 0.05$, Fig. 1b); but this reduction was not significant when compared to the control experiments ($z = 3.05$, $P > 0.05$). In addition, the filamentous growth of *T. harzianum* was significantly affected among yeast treatments ($H = 30.77$, $df = 11$, $P < 0.05$), however, the treatments were not significantly different when compared to the control (Fig. 1d).

Differences in the mycelial growth of *S. racemosum* were observed among treatments ($H = 41.52$, $df = 14$, $P < 0.05$, Figs. 1a and 2). Particularly, the observed differences relied in two yeasts species, *Bulleromyces albus* and *Cr. magnus*, that significantly inhibited *S. racemosum* when compared with the control ($z = 3.41$ and $z = 3.34$, $P < 0.05$, respectively). All other yeasts isolates did not significantly inhibited the mycelial growth of *S. racemosum*.

Moreover, *Beauveria bassiana* filamentous growth was significantly inhibited by some yeasts strains

Table 2 Yeasts species isolated from gardens of field nests of *Atta texana*

Yeast species	Closest relative[a]		# of isolates				Frequency[b] (%)
	Similarity %	Accession #	Winter	Spring	Summer	Fall	
Ascomycota							
Aureobasidium pullulans	100	DQ377657				3C[d]	4.7
Candida membranifaciens CBS11365[c]	100	EF362752	3A				4.7
Candida cf. *melibiosica*	96	U44813	1A				1.6
Kodamaea ohmeri[c]	100	AF335976	1A		1A,2C,1D		7.8
Saccharomyces exiguus	100	AY007906			1A		1.6
unidentified yeast-like fungus[c]	90	DQ384104		1A	2A		4.7
Basidiomycota							
Bullera sinensis[c]	100	AF189884	1B				1.6
Bulleromyces albus[c]	100	AF416643	2B				3.1
Cryptococcus cf. *cellulolyticus*[c]	98	AF075525	2C				3.1
Cryptococcus flavescens CBS11364[c]	100	AF487885		1C			
	99	AM160631	1B				3.1
Cryptococcus flavus CBS11397	100	AF075497				3ABC	4.7
Cryptococcus laurentii[c]	99	AJ876597	2B			1A	4.7
Cryptococcus luteolus	100	AM160632		1C			6.3
Cryptococcus cf. *luteolus*[c]	97	AM160633	2BC			1B	
Cryptococcus magnus CBS11366[c]	100	AF189872	1C	2BC			
	100	AY242120		2BC	1D		12.5
	99	DQ377663	2BC				
Cryptococcus cf. *podzolicus*	98	AF075481	1B		1A		3.1
Cryptococcus cf. *taibaiensis*[c]	97	AY557601	1C				1.6
Cryptococcus terreus	99	AF444694		1A			1.6
Cryptococcus sp. 1	100	AF416643	1C				1.6
Cryptococcus sp. 2	99	AY646103	1B				1.6
Cryptococcus sp. 3	98	DQ377673		1C			1.6
Cryptococcus sp. 4	98	AF444699		1A			1.6
Pseudozima sp.[c]	99	AM160637	1C				1.6
Rhodosporidium cf. paludigenum	98	AF514863				1C	1.6
Rhodotorula cf. *javanica*	97	AF189935		1A			1.6
Rhodotorula lactosa	99	AF189936				1A	1.6
Rhodotorula mucilaginosa	99	AF335987				1A	1.6
Rhodotorula nothofagi	100	AF444736		1A			1.6
Rhodotorula cf. *taiwaniana*	97	AY551270	1C				1.6
Rhodotorula sp. 1	99	AM160641		1C			1.6
Sporidiobolus ruineniae	99	AF070438				2A	3.1
Sporisorium penniseti (yeast-like fungi)	99	AY740130				2A	3.1
Sympodiomycopsis paphiopedili CBS11396	99	DQ832238				2B	3.1
Trichosporon porosum	100	AF308656			1A		1.6
Subtotal			24	13	10	17	
Total # of isolates			64				

[a] Accordingly to BLAST (Altschul et al. 1997). Isolates with same similarity are identical

[b] Frequency (in percentage) of yeast species within total sample of 64 yeast isolates

[c] Yeast species used in the in vitro bioassay challenges ($n = 14$ includes, 2 strains of *Cryptococcus* cf. *cellulolyticus* and 2 strains of *Cryptococcus* cf. *luteolus*)

[d] Letters indicate nest origin for yeast isolates—A: UGM051218-02, B: UGM060121-01, C: UGM060121-02, D: AR060123-01

Table 3 Mean number of yeast colony forming units (CFU g^{-1}) per gram of fungus garden and number of yeasts strains isolated from four *Atta texana* nests over 1 year

Ant nest	CFU g^{-1} (mean × 10^2 ± SE)[a]			
	Winter	Spring	Summer	Fall
UGM 051218-02	12.0 ± 6.05 (*n* = *4*)	0.41 ± 6.05 (*n* = *4*)	80.0 ± 6.05 (*n* = *4*)	0.28 ± 6.99 (*n* = *3*)
	5[b]	5	6	8
UGM 060121-01	0.63 ± 0.07 (*n* = *4*)	ND[c]	0	0.61 ± 0.07 (n = *4*)
	10	2	0	4
UGM 060121-02	0.74 ± 1.06 (*n* = *3*)	30.0 ± 0.73 (*n* = *3*)	67.0 ± 0.58 (*n* = *4*)	9.75 ± 0.17 (*n* = *4*)
	9	6	2	5
AR 060123-01	1.80 (*n* = *2*)	0	0.32 ± 0.66 (*n* = *3*)	0[d]
	0	0	2	0
Total # of isolates	24	13	10	17

[a] Figures in parentheses indicate the number of replicates used in each assay

[b] Number of yeasts isolates recovered in each sampling period

[c] *ND* no data available due to contamination by foreign fungi

[d] Isolation attempts were carried out on collapsed garden as no healthy fungus garden was available in this nest at the time of collection (see "Methods")

($H = 40.40$, $df = 12$, $P < 0.05$, Fig. 1c). *Candida membranifaciens* and one unidentified yeast-like isolate significantly inhibited *B. bassiana* mycelium growth when compared to the control ($z = 3.13$ and $z = 3.93$, $P < 0.05$, respectively). *Bullera sinensis* and *Pseudozyma* sp. apparently inhibited *B. bassiana*, but their effects in the mycelial growth were not significantly different from the control ($z = 2.84$ and $z = 3.04$, $P > 0.05$, respectively).

The mycelial growth of *Escovopsis* sp. RC005 and *Escovopsis* sp. RC007 were significantly different among yeast treatments ($H = 80.92$ and $H = 73.01$, $df = 14, P < 0.05$, Fig. 1e, f respectively). *Escovopsis* sp. RC005 was inhibited by seven yeasts strains, such as *B. sinensis* ($z = 3.38, P < 0.05$), *B. albus* ($z = 3.83$, $P < 0.05$), *C. membranifaciens* ($z = 3.29$, $P < 0.05$), *Cr. flavescens* ($z = 3.57$, $P < 0.05$), *Cr. magnus* ($z = 3.87$, $P < 0.05$), *Cr. cf. taibaiensis* ($z = 3.30$, $P < 0.05$) and *Pseudozyma* sp. ($z = 4.14$, $P < 0.05$). Moreover, the mycelial growth of *Escovopsis* sp. RC007 was significantly reduced when confronted with six yeasts strains: *B. sinensis* ($z = 4.14$, $P < 0.05$), *C. membranifaciens* ($z = 3.44$, $P < 0.05$), *Cr. flavescens* ($z = 3.16$, $P < 0.05$), *K. ohmeri* ($z = 3.52$, $P < 0.05$), *Pseudozyma* sp. ($z = 4.48$, $P < 0.05$) and the unidentified yeast-like isolate ($z = 3.87, P < 0.05$).

None of the 14 selected garden yeasts species used in the bioassays showed the killer phenomenon when confronted with the five standard killer-sensitive strains.

Discussion

Insects generally harbor several yeast species that interact with their host in commensal or beneficial ways (Mueller et al. 1998; Vega and Dowd 2005). Like virtually all other insects, leaf-cutting ant's workers accumulate yeasts on their cuticle (Little and Currie 2008; Pagnocca et al. 2008) but leafcutter ants, also harbor a diverse assemblage of yeasts in the fungus gardens, particularly basidiomycetous yeasts in the genus *Cryptococcus* in *A. texana* gardens. This finding contrasts with Carreiro et al. (1997), who found more ascomycetous yeasts (26 of 39 isolates) than basidiomycetous yeasts (13 of 39 isolates) in both old and new fungus gardens of *A. sexdens rubropilosa* colonies reared in the laboratory.

Yeasts in the garden microbial community of *A. texana* probably have diverse origins. For example, *Cryptococcus*, the most abundant yeast genus found in this study, is commonly associated with epiphytic microbial communities on plants (Inácio et al. 2002). Other genera such as *Candida*, *Rhodotorula* and *Trichosporon* are typically isolated from soil, including soils next to mounds of the fire ant

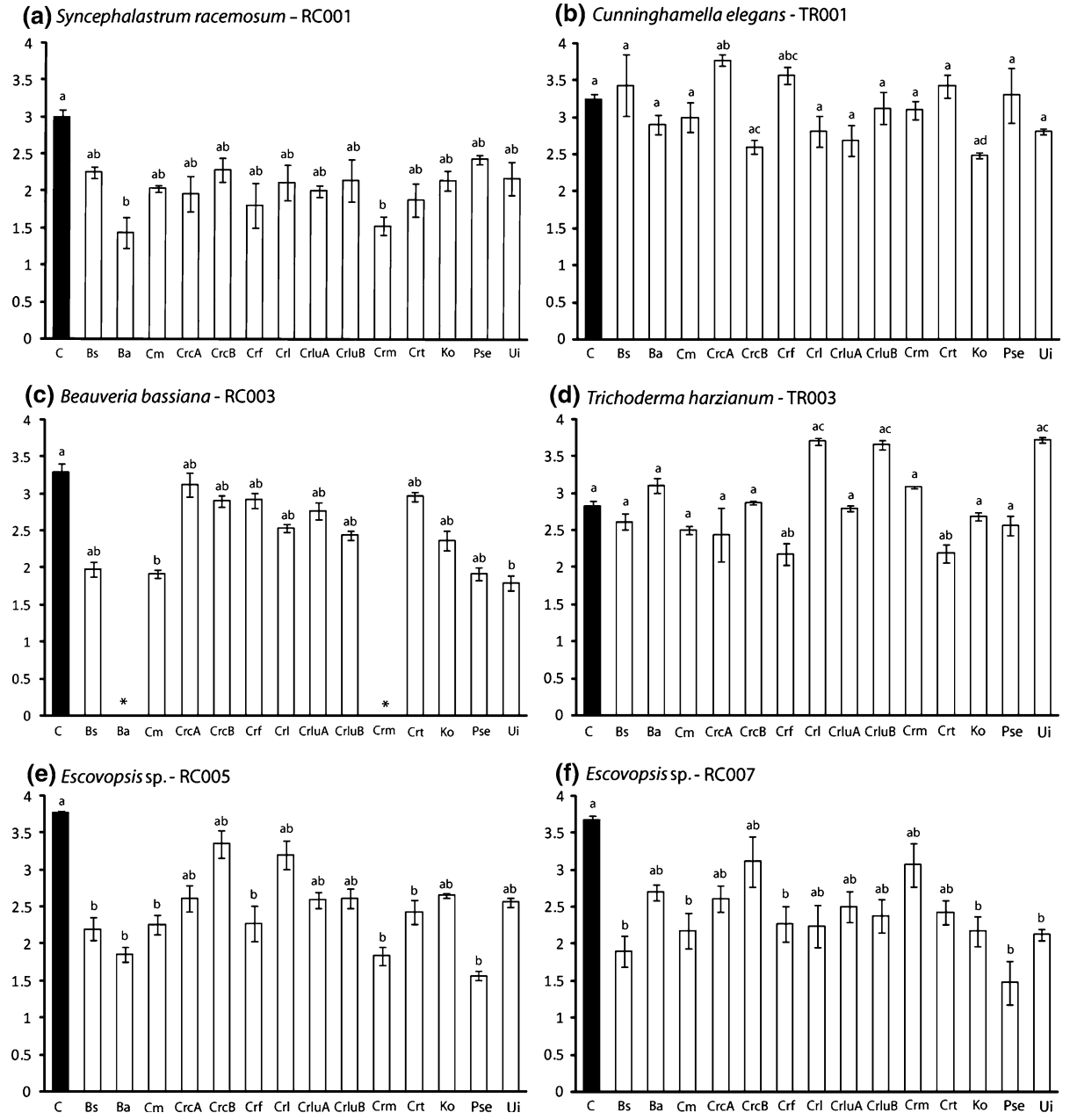

Fig. 1 Inhibitory and growth-enhancing effects of test yeasts on mycelial growth of six problem fungi of attine gardens (*Beauveria bassiana*, *Cunninghamella elegans*, *Escovopsis* sp., *Syncephalastrum racemosum* and *Trichoderma harzianum*). *Columns shaded in black* indicate control experiments. *: Absence of *bars* means that no bioassay challenge was performed. Yeast species, Bs: *Bullera sinensis*; Ba: *Bulleromyces albus*; Cm: *Cryptococcus magnus*; CrcA: *Cr.* cf. *cellulolyticus* A; CrcB: *Cr.* cf. *cellulolyticus* B; Crf: *Cr. flavescens*; Crl: *Cr. laurentii*; CrluA: *Cr.* cf. *luteolus* A; CrluB: *Cr.* cf. *luteolus* B; Crm: *Cr. magnus*; Crt: *Cr.* cf. *taibaiensis*; Ko: *Kodamaea ohmeri*; Pse: *Pseudozyma* sp.; Ui: unidentified yeast-like fungi. *Bars* labeled with different *letters* are significantly different ($P < 0.05$)

Solenopsis invicta (Ba et al. 2000). Thus, the occurrence of plant- and soil-associated yeasts in gardens of *A. texana* is perhaps not surprising, as the ants import yeasts with the plant material used to sustain garden growth (Carreiro et al. 1997). In contrast, isolation of the yeast *Sympodiomycopsis*

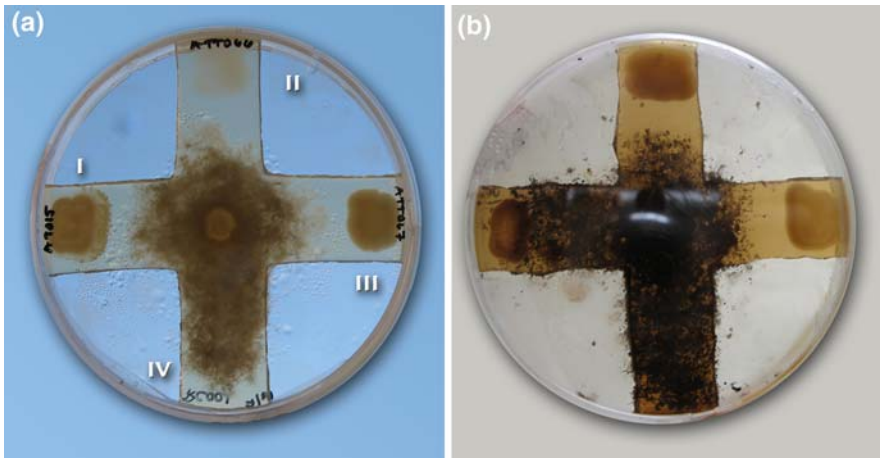

Fig. 2 Yeast antagonism bioassays against garden pathogens of leaf-cutting ants. Growth lanes were cut from the agar and then inoculating the lanes peripherally with a garden-yeast. **a** yeasts inoculated in (I) *Kodamaea ohmeri* (II) *Cryptococcus luteolus* A and (III) *Cryptococcus* cf. *cellulolyticus* A. One track (IV) was left as a control which no yeast was inoculated. **b** yeasts inoculated in the same order (I) *Cryptococcus* cf. *cellulolyticus* B(II) *Bulleromyces albus* and (III) *Pseudozyma* sp. After 3 days of growth, garden-yeasts were confronted with microfungi inoculated centrally onto the plate: **a** S*yncephalastrum racemosum* and **b** *Escovopsis* sp. RC005

paphiopedili in our study was unusual, because this yeast had previously been associated only from orchid's flowers in Japan (Sugiyama et al. 1991). Leafcutter ants are not known to forage on orchids, but a number of orchids are known to occur in Central Texas (Liggio and Liggio 1999), including the forested areas near the studied *A. texana* nests.

The abundance of yeasts (e.g., CFU counts) in leafcutter gardens is extremely variable regardless of garden state (garden with old/exhausted vs. garden with fresh leaf substrate added; Fernando C. Pagnocca, unpublished). The yeast abundance in mature colonies of *A. texana* evaluated in this study was also variable (Table 3), ranging from no yeast to 80×10^2 CFUs per gram of fungus gardens. These figures are much lower compared to adult nests of *A. sexdens rubropilosa* from Brazil (average of 1.4×10^5 yeast CFUs per gram of old fungus garden; Pagnocca et al. 1996) and lower than old fungus gardens from adults nests of *Atta cephalotes* and *Acromyrmex octospinosus* reared in the laboratory (average of 1.4×10^5 and 7.2×10^4 CFUs per gram, respectively, Craven et al. 1970). These results suggest the following possible explanations for the observed differences in yeast abundances between *A. texana* gardens: (1) garden-yeast populations are dependent on the yeast influx with specific kinds of plant substrate during different periods of the year or in different habitats; (2) garden age (state) or health, two factors that were not completely controlled in our study, could affect yeast growth in *A. texana* gardens; (3) ants could use resident garden-yeasts in some periods of the annual cultivation cycle, thus assembling a yeast community for diverse purposes in the fungus gardens, such as production of enzymes (Carreiro 2000) or defensive purposes; or (4) population size of yeast may vary as a function of the concentration of simple sugars generated by extracellular hydrolytic enzymes produced by the symbiotic fungus (Silva et al. 2006). Future studies should test more gardens to better understand yeast community structure in attine nests.

Antagonistic effects of yeasts on filamentous fungi have been reported in several studies (Petersson and Schnüner 1995; Walker et al. 1995; Adams et al. 2008). For example, the yeasts *Pichia anomala, P. guilliermondii and P.* (= *Kodamaea*) *ohmeri* are used effectively as biological control agents against fungal rot of fruits (Coelho et al. 2007). Interestingly, *P. guilliermondii* was isolated at high abundance from several fungus-growing ants reared in the laboratory, and preliminary bioassays revealed antagonistic interactions against common microfungal pathogens in attine gardens (Fernando C. Pagnocca and Ulrich G. Mueller, unpublished data). However, in contrast to the lab gardens, no *P. guilliermondii* was found in our survey of field gardens of *A. texana*.

In the present study, some yeasts strains apparently promoted the mycelial growth of the tested microfungi, but those effects were not statistically significant. On the other hand, garden yeasts suppressed the mycelial growth of some problem fungi of attine nests (Fig. 1). In this context, mycocin secretion by yeasts is generally thought to mediate antagonism against other yeasts and filamentous fungi (Walker et al. 1995). Although, Carreiro et al. (2002) observed a high proportion of killer yeasts in *A. sexdens rubropilosa* nests reared in the laboratory, mycocin-secreting yeasts were not identified in our survey. Thus, the antagonism exhibited by the garden-yeasts toward the microfungi would be due to others factors, such as (1) exploitation competition for nutrients (2) changes in pH caused by other metabolic products, or (3) production of antimicrobial compounds other than the killer toxins.

The microfungi used in this study are usually found in lab or field leafcutter ant nests and are potential "weeds" of attine gardens (Rodrigues et al. 2005, 2008a). The fact that *B. bassiana* and *S. racemosum* were inhibited, whereas *C. elegans* and *T. harzianum* were not affected by garden yeasts, suggests that the control of alien microorganisms in attine gardens is complex and may require additional defenses such as the antimicrobial secretions of the ants' metapleural and mandibular glands (Bot et al. 2002; Rodrigues et al. 2008b).

Most interestingly, garden yeasts inhibited *Escovopsis* strains. Specifically, the yeasts *Bullera sinensis*, *Cryptococcus magnus* and a species of *Pseudozyma* significantly inhibited the two *Escovopsis* strains tested (Fig. 1e, f). Currie et al. (1999a) studying several attine ant genera demonstrated that actinomycetes isolated from the workers' cuticle selectively inhibited *Escovopsis* strains but not other microfungal garden contaminants. Recently, several studies reported that *Escovopsis* is inhibited by bacteria of the genus *Burkholderia* found in *A. sexdens rubropilosa* gardens (Santos et al. 2004) and by secretions of the ant metapleural glands (Bot et al. 2002). However, the exact abundance of such protective microorganisms in the attine ant-microbe symbiosis is not known. Thus, the fact that *Escovopsis* can be inhibited by a variety of microorganisms, including actinomycete bacteria, *Burkholderia* bacteria, ant glandular secretions and now also yeasts, expands our view on the co-evolution of this fungus in the attine ant-microbe mutualism, since these diverse disease-suppressing microbes may contribute synergistically to defense against *Escovopsis*. This may be particularly beneficial for leaf-cutting ants, such as *Atta* spp., in which *Pseudonocardia* bacteria are absent or found in very low frequency (Currie et al. 1999b; Zhang et al. 2007; Mueller et al. 2008). Garden immunity against problem fungi like *Escovopsis* therefore may not derive exclusively from a co-evolutionary arms race between *Escovopsis* and specialized antibiotic-producing actinomycetes (Currie et al. 1999a), but may derive in part from an enrichment of multiple disease-suppressing microbes in the garden matrix.

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