Variation in the ability of fungi in the extrafloral nectar of *Mallotus paniculatus* to attract ants as plant defenders

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Abstract

Many plant–ant interactions are considered mutualisms. In *Mallotus paniculatus* (Euphorbiaceae) (also known as Turn-in-the-wind), the extrafloral nectaries (EFNs) on the base of the leaf laminas can produce sugar-rich secretions to attract ants as effective agents against herbivores or plant competitors. Growing evidence reveals that microorganisms are important "hidden players" in insect–plant interactions. Understanding which microorganisms act as such third-party species and how they operate is a major challenge in the study of mutualistic interactions. In this study, we showed that two dominant fungal species, the yeast *Jaminaea angkorensis* and the hyphal fungus *Gibellulopsis nigrescens* both from EFNs and the interiors of bodies of the ant *Pheidole megacephala* (Formicidae) on their own were sufficient for ant attraction. Our results also revealed that different fungal species on the host plant influenced ant behavior differently. These results can be applied in agriculture to increase ants or herbivore predators to protect plant hosts using fungal baits. Moreover, they indicate that fungal odors represent the critical signal to establish the plant–microbe–insect interactions. The traditional plant–defender concept must be updated to include the role of microorganisms.

Keywords – plant–ant interaction – extrafloral nectar – mutualism – olfaction – yeast

Introduction

Olfaction plays an important role in many behavioral contexts in insects. Insects receive odors emitted from individuals of the same species, such as sex pheromones for mate assessment (Johnson et al. 2017, Li et al. 2017), alarm pheromones for warning or alerting another of the same species of impending danger (Mizunami et al. 2010), and aggregation pheromones for attracting insects to favorable locations (Leonhardt et al. 2016). The insects also receive odor signals from other organisms such as enemies, potential hosts, preys, and food source (Gadenne et al. 2016). To successfully perform these behaviors, insects modulate their olfactory system according to their physiological state upon interaction with the environment. The changes in olfactory sensory pathways may even contribute to sympatric speciation (Scheidler et al. 2015). For example, *Rhagoletis pomonella*, apple maggot, is the pest of the apple tree. Studies have indicated that the shifts in neuronal pathways carrying information of behavioral relevance may represent a general phenomenon contributing to rapid shifts in habitat and mate choice, potentially catalyzing speciation (Linn et al. 2003).

Plants also release pheromone cues that dictate insect behaviors. For example, the carnivorous plant *Dionaea muscipula* attracts insects to visit the deadly capture organ of flyflap by mimicking fruit and flower smells (Kreuzwieser et al. 2014). Maize (*Zea mays*) defends itself from pest feeding and egg deposition by emitting herbivore-induced plant volatiles. These volatiles attract parasitic wasps to attack moth eggs (Tamiru et al. 2017). Although other organisms can influence insect behavior by producing volatile organic compounds, only few studies have investigated the role of such compounds in interactions between symbiotic microorganism on the plants and insects (Aleklett et al. 2014). Symbiotic microbes on the plant host can help defend against herbivores by interfering with the chemical compounds produced by the host. The plant *Arabidopsis thaliana* produces volatiles in response to herbivory to defend itself against herbivorous insects. Pineda et al. showed that the volatile blend from rhizobacteria-treated aphid-infested *Arabidopsis* plants is less attractive to an aphid parasitoid than the volatile blend from aphid-infested plants without rhizobacteria (Pineda et al. 2013).

Ants are arguably the greatest success story in the history of terrestrial metazoa. They monopolize 15%–20% of the terrestrial animal biomass, particularly in tropical regions (Schultz 2000). Because of their widespread presence, they have different ways of interacting with other organisms. Many plants in the families Euphorbiaceae and Fabaceae have glands that secrete nectar and attract nearby ants to be their day-and-night patrol for mutualism. This is a plant–ant facultative mutualistic association (Pemberton 1998). In general, extrafloral nectar (EFN) is rich in sugars with small quantities of amino acids, especially essential amino acids. Because yeasts are common inhabitants of angiosperm floral nectar (FN) and EFN, they may act as causal agents of fermentation odors in plants and, therefore, as possible intermediate agents in plant signaling to pollinators (e.g., bees) or antiherbivory defense (e.g., ants) (Goodrich et al. 2006, Schaeffer et al. 2017). Nevertheless, studies on the potential role of yeasts in the origin of FN or EFN scents generally have not directly explored their presence or abundance in these two niches.

Mallotus paniculatus (Euphorbiaceae) (also known as Turn-in-the-wind) is a common tree at secondary forest edges and can be easily identified by its leaf morphology. It is distributed throughout India, Burma (Myanmar), Indo-China, southern China, Taiwan, and Thailand (Plants of the world online, http://powo.science.kew.org/; searched on 27. February 2018). The leaves have ovate laminas with pointed tips, white undersides, and long petioles (Fig. 1A). The lamina margins are simple to 3-lobed. The base of the leaf lamina has a pair of extrafloral glands and secretes a sugary fluid that attracts ants (Fig. 1B); this may be a mutualistic relationship, with the ant protecting the plant against herbivorous insects in return (So 2004). Thus, our aim was to provide critical empirical evidence in support of the hypothesis of fungus-mediated signaling in plant–ant interactions on the plant *M. paniculatus*. Herein, we show that fungi isolated from EFN on their own are sufficient for ant attraction and that ants behave differently to different fungus baits.

Materials and methods

Study sites

We conducted this study in Changhua County (24°02'30.3"N, 120°35'43.8"E) in central Taiwan. The annual average temperature of Changhua is 22.4°C, with July being the hottest month and January being the coolest. The annual average rainfall is 1723.4 mm. The study site was located on a gentle slope in the Tri-Mountain National Scenic Area, an open shrubland dominated by *M. paniculatus*.

Yeast collection

We observed a greater nectar secretion rate in EFNs of young *M. paniculatus* individuals than in older plants, indicating that they are possibly more protected by ants. In addition, nectar secretion was higher at early morning (approximately 5 a.m. to 7 a.m.), a pattern that could be associated with an increase in herbivore pressure and water stress during the daylight hours. Thus, we collected the liquid secretion in EFN of three young *M. paniculatus* individuals by using a 0.2–

2-μL pipette, which was immediately put in a sterilized 2-mL microcentrifuge tube. The samples were maintained at 4°C until yeast isolation procedures. After the samples were transported to the laboratory, 100 μL of sterilized water was added to the microcentrifuge tubes and vortexed for 5 min at 3,000 revolutions/min (rpm) using a vortex mixer (AL-VTX3000L, CAE technology Co., Ltd.), and the samples were spread uniformly by using sterile 4 mm glass beads on the YPD agar plates (1% yeast extract, 2% peptone, 2% dextrose, and 2% agar) supplemented with approximately 2–3 mL of 100% lactic acid. The YPD plates were then incubated at 28°C. Purified yeast strains were suspended in YPD medium supplemented with 15% v/v glycerol and maintained at −80°C.
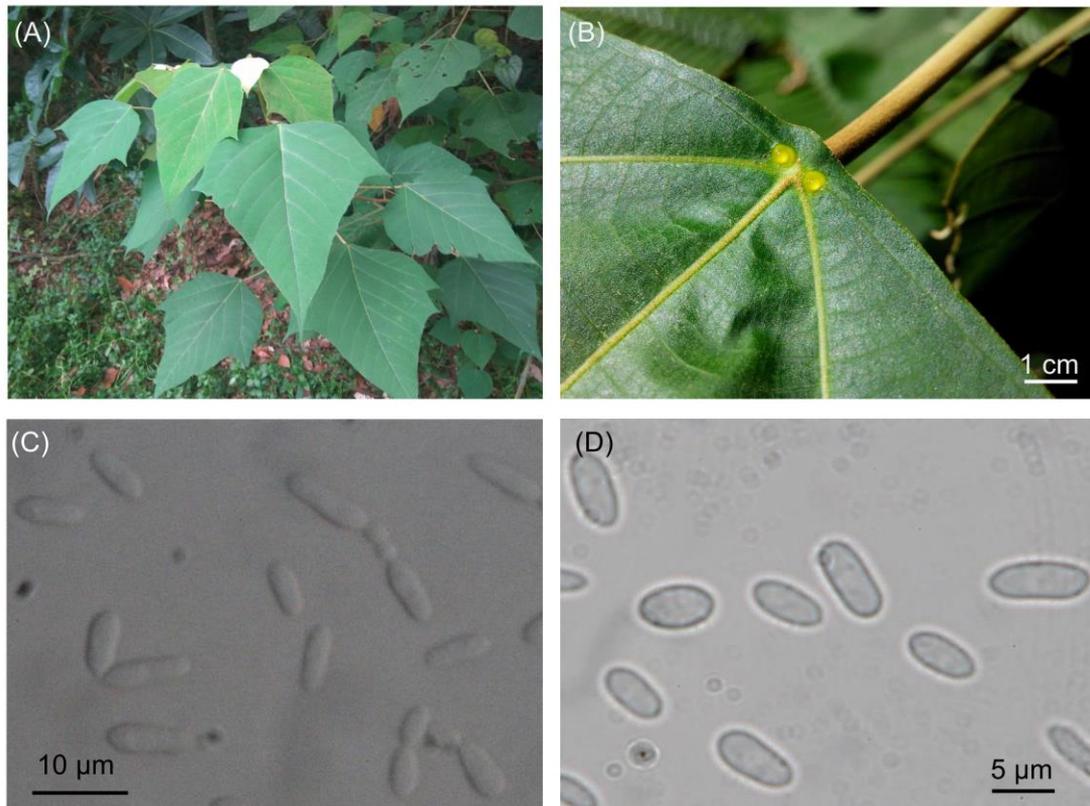
Figure 1 – (A) Characteristic leaves of the plant *Mallotus paniculatus* (also known as Turn-in-the-wind). (B) Extrafloral nectaries of *M. paniculatus* at the base of the upper leaf lamina. (C) Microscopic morphology of the yeast *Jaminaea angkorensis* used as bait here. (D) Microscopic morphology of conidia of the hyphal fungus *Gibellulopsis nigrescens* used as bait here.

Three defender ants were also transported to the laboratory and then frozen at −20°C to kill them. The corpses were transferred into sterilized microcentrifuge tubes (2 mL) with 70% ethanol to sterilize their body surface. They were then washed twice with 500 μL of sterilized water, and the liquid was removed. Subsequently, 200 μL of sterilized water was added, and the supernatant was spread on the YPD plate by gentle shaking to confirm the absence of any surviving yeast on the surface of the ants after sterilization. Finally, the ants were homogenized in 200 μL of water, and the mixture was spread on the YPD plate to obtain the yeasts from the interiors of the ant bodies. The YPD plates were then incubated at 28°C. Purified yeast strains were suspended in YPD medium supplemented with 15% v/v glycerol and maintained at −80°C.

Yeast identification

Yeast cultures (1 mL) were transferred to a 1.5-mL microcentrifuge tube and centrifuged at 13,000–16,000 g for 1 min. The supernatant was discarded, and the cell pellet was suspended in 200 μL of lysis buffer (2% Triton X-100, 1% sodium dodecyl sulfate, 100 mM sodium chloride [NaCl], 10 mM Tris [pH 8.0], and 1 mM ethylenediaminetetraacetic acid [EDTA]). Subsequently,

200 µL of phenol:chloroform:isoamyl alcohol (25:24:1 [the isoamyl alcohol being optional]) and 0.3 g of acid-washed glass beads (0.45–0.52 mm) were added and gently mixed. The samples were vortexed for 5 min to disrupt cells and then centrifuged at 13,000–16,000 g for 5 min. The aqueous layer of each sample was transferred to a clean tube, and 400 µL of 95% ethanol and 16 µL of 3 M sodium acetate (pH 5.2) were added. The samples were mixed by inversion and centrifuged at 13,000–16,000 g for 5 min. The pellets were washed with 300 µL of 70% ethanol, and the samples were centrifuged at 13,000–16,000 g for 2 min before the supernatant was discarded. Ethanol was aspirated for 30 min to dry the pellets. Finally, the genomic DNA from each sample was suspended in 100 µL of Tris-EDTA buffer (pH 8.0).

Yeast identification was performed by sequencing the polymerase chain reaction (PCR) products of large subunit (LSU) rDNA, including the D1/D2 domain, 5.8S rDNA, and internal transcribed spacer (ITS) regions amplified by the universal primers ITS-1 (5′-TCCGTAGGTGAACCTGCG-3′) and NL-4 (5′-GGTCCGTGTTTCAAGACGG-3′) (Kurtzman & Robnett 1997). PCR proceeded as follows: initial denaturation at 95°C for 5 min followed by repeated denaturation at 95°C for 1 min, annealing at 53°C for 30 s, and elongation at 72°C for 1 min 40 s for 35 cycles; the final elongation lasted 5 min. DNA sequencing of these samples was performed at Tri-I Biotech, Inc. A BLAST search of the nucleotide sequences was conducted through the National Center for Biotechnology Information homepage (http://www.ncbi.nlm.nih.gov). The voucher specimens of fungi used in this study are deposited in the Bioresource Collection and Research Center, Hsinchu City, Taiwan (http://www.bcrc.firdi.org.tw/). Any requests should be addressed to the corresponding author.

Behavioral assays by yeast bait attraction

To evaluate the variation in the ability of yeasts in EFN of *M. paniculatus* to attract ant defenders, we randomly selected *M. paniculatus* individuals. All selected plants were at least 10 m apart. Three randomly selected plants served as replicates in each experiment.

To prepare yeast cells for the attraction assay, actively growing yeast cells cultured in YPD medium at 28°C for approximately 12 hours were collected. The inocula were adjusted by YPD medium to an optical density of 0.8 at 660 nm. We spotted 20 µL of yeast culture or YPD medium only on the center between the midrib and leaf margin for the binary choice assay (Fig. 2). Furthermore, to investigate the difference between volatiles produced by young and old yeast cultures in terms of their attractiveness, actively growing yeast cells and 3-day-cultured (old, stationary stage) yeasts were used for comparison. The experiments were recorded using digital video recorder (SONY HDR-CX430V) to document the ant behavior until one of the baits was consumed completely. Once the baits were consumed completely, the ants dismissed and then returned to the nest. Thus, we can conclude that the baits were "consumed completely" by the ant behavior. The movie was divided into three equal parts (first, middle, and last) to analyze the differences between the responses of ants in different periods during the whole attraction assay. Two types of ant behaviors were analyzed. Attraction rate: We calculated the number of ants going into the counting area (the length between the midrib and leaf margin as the diameter) during the different periods (Fig. 2). We then divided the number of ants attracted in each period by the total number of ants attracted in the whole assay procedure to compare the attraction index between two baits. Stay rate: We calculated the difference between the number of ants attracted in each period and the number of ants left in each period and divided this difference by the number of ants attracted in each period.

Data analysis

Data are expressed as mean ± standard deviation. The significance of differences between groups was determined using Student t tests and analysis of variance. $P < 0.05$ was considered statistically significant.

Results

Yeast and ant species used

The total counts of yeast associated with the glands and ants can be determined by colony forming units (CFUs) on agar plates. In this study, we found two yeast species to be dominant in both EFN of *M. paniculatus* and the interiors of the ant bodies—*Jaminaea angkorensis* (accession number: MF765802) (Fig. 1C) and *Gibellulopsis nigrescens* (MF765803) (Fig. 1D). *J. angkorensis* is an anamorphic basidiomycetous yeast species originally isolated from decaying leaves in the world heritage site Angkor, Cambodia. Taxonomically, *J. angkorensis* is affiliated with Microstromatales (Exobasidiomycetes, Ustilaginomycotina, Basidiomycota) and represents a basal phylogenetic lineage of this fungal order (Sipiczki & Kajdacsi 2009). *G. nigrescens* was originally described on potato tubers in England (Zare et al. 2007). The ant species in this study was *Pheidole megacephala*, judging by morphological characteristics.
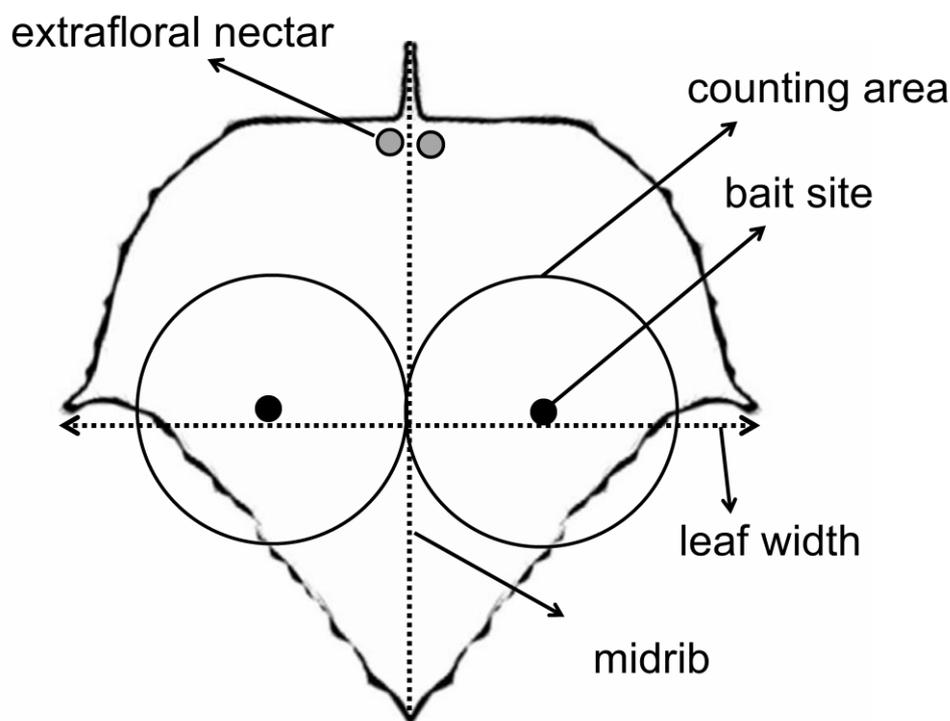
Figure 2 – Schematic of binary choice assay. The baits (yeast culture + YPD medium or pure YPD medium) were spotted on the center between the midrib and leaf margin for binary choice assay. Only ants going in the counting area (the length between the midrib and leaf margin as the diameter) were counted in this assay.

Ant behavior

Attraction rate:

In the binary choice assay between yeast + YPD medium and YPD medium alone, we found that both yeast species could significantly attract more ants than YPD medium. The average ant attraction rate for *J. angkorensis* was 56% ± 2%, which was higher than that for the control medium (44% ± 2%) (Fig. 3A); however, a statistically significant difference between the two was found only during the last period (Table 1). The ant attraction rate of *G. nigrescens* was 57% ± 2% and that of YPD medium was 43% ± 2% (Fig. 3B); however, a statistically significant difference was found only during the middle period (Table 1). Regarding the ability of attraction, *G. nigrescens* (56% ± 5%) attracted significantly more ants than *J. angkorensis* (44% ± 5%) (Fig. 3C), indicating

that ants preferred *G. nigrescens* when both *J. angkorensis* and *G. nigrescens* yeasts were present. However, a statistically significant difference between *G. nigrescens* and *J. angkorensis* was found only during the middle period (Table 1).

We found that older yeast attracted significantly fewer ants than younger yeast. Comparing the 1-day- and 3-day-cultured yeast *J. angkorensis*, we found that the ant attraction rate of the younger yeast (65% ± 6%) was higher than that of the older yeast (35% ± 6%) (Fig. 3D). A statistically significant difference was found during the middle and last periods (Table 1). For *G. nigrescens*, the ant attraction rate of the 1-day-cultured yeast (59% ± 2%) was higher than that of the 3-day-cultured yeast (41% ± 2%) (Fig. 3E). A statistically significant difference was found only during the last period (Table 1). On comparing the attraction rate between the 3-day-cultured yeasts of *J. angkorensis* (49% ± 7%) and *G. nigrescens* (51% ± 7%), we observed no significant difference (Fig. 3F); this indicates that ants did not exhibit bait preference for 3-day-cultured yeasts. Moreover, ants were less attracted to older yeasts.

Stay rate:

The average stay rate of *J. angkorensis* (9% ± 1%) was higher than that of the control medium (5 ± 1%) (Table 2). By contrast, the control medium had a higher average stay rate (10 ± 2%) than *G. nigrescens* (6 ± 2%) (Table 2). In different periods of stay rate analysis, we noted a significant difference only in the late period of the assay between the control medium and yeast. No significant difference was found between *J. angkorensis* and *G. nigrescens*. In the experiment of different growth stages of yeasts, no difference in the stay rate was found between older and young yeasts.

Direction preference assay

In the previous experiment, ants exhibited different behaviors when they fed on different yeast baits. To determine whether ants had direction preference, we spotted YPD medium onto both spot sites on the leaf. Our results showed that ants did not have direction preference during the bait feeding experiment. Both left- and right-side baits attracted 50% ± 2% of ants (Table 1), indicating that ants do not have direction preference when searching for food. In addition, we observed no difference in the stay rate between left- and right-side baits (Table 2).

Discussion

The study of plant–microbe–insect interactions has implications in fields of economic importance such as agriculture, biological invasion, and conservation (Biere & Bennett 2013). Several important metabolic pathways are utilized by microorganisms to produce certain classes of end-product volatiles that have ecological relevance to insects (Davis et al. 2013). Among these microorganisms, yeasts have widespread associations with insect herbivores, and these interspecific interactions play a critical role in both yeast and insect evolution. Yeast cells produce various volatile metabolites that are key contributors to pleasing fruity and flowery aromas such as ethyl acetate (pear aroma), isoamyl acetate (banana aroma), and phenylethyl acetate (flowery aroma) (Pretorius et al. 1999, Nordström 1966, Saerens et al. 2008). Becher et al. (2012) revealed that yeast volatiles, rather than fruit volatiles, were principally responsible for the attraction of *Drosophila melanogaster* to food resources. Studies have also reported *D. melanogaster* to play a vital role in the ecosystem as an efficient vector for transfer of yeast species (Stamps et al. 2012, Reuter et al. 2007). Hunt & Borden (1990) showed that two yeasts, *Hansenula capsulata* and *Pichia pini*, are responsible for the conversion of *cis*- and *trans*-verbenol to verbenone produced by female beetles (*Dendroctonus ponderosae*) and serves as an antiaggregation pheromone. Witzgall et al. (2012) found that the key pests of apple, codling moths (*Cydia pomonella*), are attracted to a fermenting yeast and lay more eggs on yeast-inoculated apples than on yeast-free apples. They also revealed that the yeast is an essential part of the larval diet and promotes larval survival by reducing the incidence of fungal infestations in the apple (Witzgall et al. 2012). Numerous studies have shown that yeasts can serve as a protein-rich food source for these insects and are beneficial to their

development and immunocompetence (Anagnostou et al. 2010, Stamps et al. 2012). Insects can disperse yeasts through ingestion or carrying them externally (Reuter et al. 2007). Reuter et al. (2007) demonstrated that the outbreeding rate increased in *Saccharomyces* yeast in response to dispersal by fruit flies. Thus, a mutualistic interaction between these insect vectors and yeasts has been suggested (Witzgall et al. 2012).
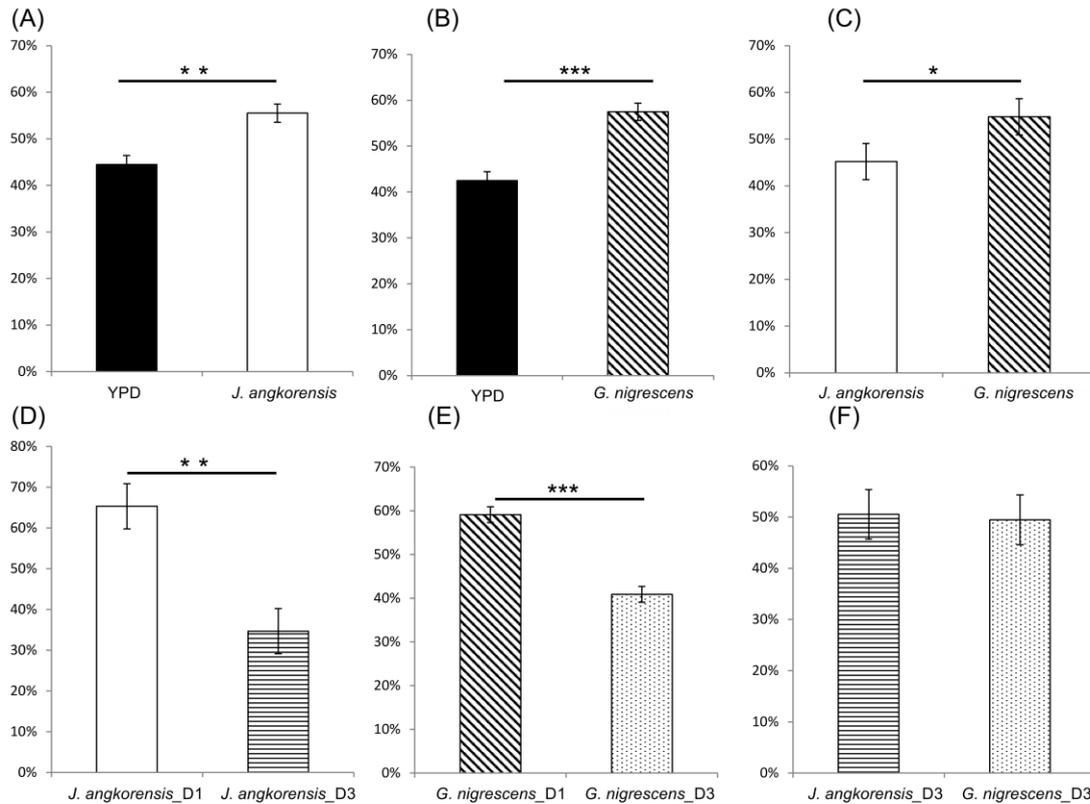
Figure 3 – Attraction rate of binary choice assay. (A) *Jaminaea angkorensis* compared with control medium. (B) *Gibellulopsis nigrescens* compared with control medium. (C) *J. angkorensis* compared with *G. nigrescens*. (D) 1-day-cultured *J. angkorensis* compared with 3-day-cultured *J. angkorensis*. (E) 1-day-cultured *G. nigrescens* compared with 3-day-cultured *G. nigrescens*. (F) 3-day-cultured *J. angkorensis* compared with 3-day-cultured *G. nigrescens*. Data are expressed as mean ± SD. $P < 0.05$ was considered significant. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

In the present study, we found that two fungal species were dominant both in EFN of *M. paniculatus* and the interiors of defender ant bodies; this indicates that the ants have close associations with these fungi. The ant feeding behavior presents the opportunity for acquiring fungi from EFN/or phyllosphere to the transmission of fungal population. For *M. paniculatus*, EFN defend plants indirectly by attracting patrolling ants that may reduce herbivory. The presence of ants on many EFN-bearing plants can reduce herbivory have been reported in many studies (reviewed in Heil 2015). Prof. Yamawo and his colleges have also indicated that the aggressiveness of ant species against herbivores on the EFN-bearing plant, *M. japonicus* and *M. philippensis* which are species close to *M. paniculatus* (Yamawo et al. 2012, 2014, 2017). The indirect anti-herbivore defense in symbiotic and non-symbiotic ant-plant associations in three *Macaranga* species have also been observed by Heil et al. (2001). *Macaranga* is a large genus of Old World tropical trees of the family Euphorbiaceae, the same as *M. paniculatus*. Although there is no direct evidence so far to show the defense specifically against herbivores by the ants on *M. paniculatus*, it is well accepted that the role of extrafloral nectaries of these plants is in attraction of ants and herbivore exclusion by ants (Rosumek et al. 2009, González-Teuber et al. 2012). Furthermore, in this study, we were unable to determine the importance of fungi to ant defenders. However, evidence suggests

that yeasts transported by ants can change the sugar content of nectar (de Vega & Herrera 2012, 2013). Thus, it may further change the pollination dynamics of ants or other pollinators. Mendes et al. demonstrated that yeasts growing in leaf-cutting ant nests may play the role of nutrient assimilation and removal of potentially toxic compounds that may be deleterious to the ants and their fungal cultivar (Mendes et al. 2012). This gives rise to intricate plant–microbe–insect interactions.

Table 1 Attraction rate of binary choice assay. D1 and D3 indicate 1-day-cultured (actively growing yeast cells) and 3-day-cultured (old, stationary stage) yeasts. Data are expressed as mean ± SD. $P < 0.05$ was considered significant. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Baits type	First (±SD)	Middle (±SD)	Last (±SD)	Total
YPD	3% (±2%)	20% (±4%)	22% (±4%)	44% (±2%)
J. angkorensis	4% (±3%)	23% (±1%)	30% (±5%)*	56% (±2%)**
YPD	4% (±4%)	15% (±1%)	23% (±4%)	43% (±2%)
G. nigrescens	5% (±5%)	24% (±3%)*	29% (±5%)	57% (±2%)***
J. angkorensis	7% (±1%)	16% (±4%)	20% (±4%)	44% (±5%)
G. nigrescens	9% (±5%)	24% (±1%)*	25% (±4%)	56% (±5%)*
J. angkorensis D1	10% (±4%)	28% (±7%)*	28% (±3%)**	65% (±6%)**
J. angkorensis D3	4% (±3%)	14% (±0%)	16% (±3%)	35% (±6%)
G. nigrescens D1	14% (±10%)	21% (±8%)	24% (±5%)*	59% (±2%)***
G. nigrescens D3	11% (±10%)	16% (±4%)	15% (±4%)	41% (±2%)
J. angkorensis D3	8% (±7%)	19% (±4%)	26% (±6%)	49% (±7%)
G. nigrescens D3	4% (±4%)	16% (±4%)	26% (±5%)	51% (±7%)
YPD Left	9% (±3%)	18% (±2%)	24% (±3%)	50% (±2%)
YPD Right	8% (±2%)	16% (±1%)	25% (±3%)	50% (±2%)

Table 2 Stay rate of binary choice assay. Data are expressed as mean ± SD. $P < 0.05$ was considered significant. * $P < 0.05$; ** $P < 0.01$.

Baits type	First (±SD)	Middle (±SD)	Last (±SD)	Total
YPD	46% (±31%)	16% (±3%)	10% (±1%)	5% (±1%)
J. angkorensis	53% (±18%)	17% (±6%)	17% (±2%)*	9% (±1%)**
YPD	46% (±22%)	23% (±7%)	18% (±0%)*	10% (±2%)*
G. nigrescens	47% (±30%)	21% (±5%)	13% (±2%)	6% (±2%)
J. angkorensis	18% (±6%)	15% (±7%)	9% (±2%)	4% (±1%)
G. nigrescens	15% (±7%)	11% (±7%)	6% (±3%)	3% (±1%)
J. angkorensis D1	39% (±8%)	17% (±10%)	16% (±9%)	5% (±3%)
J. angkorensis D3	30% (±11%)	14% (±10%)	17% (±12%)	5% (±3%)
G. nigrescens D1	22% (±5%)	21% (±21%)	18% (±14%)	7% (±4%)
G. nigrescens D3	15% (±9%)	16% (±11%)	13% (±13%)	8% (±6%)
J. angkorensis D3	32% (±30%)	18% (±6%)	9% (±1%)	6% (±4%)
G. nigrescens D3	16% (±4%)	12% (±3%)	8% (±3%)	4% (±3%)
YPD Left	14% (±3%)	10% (±3%)	9% (±4%)	5% (±2%)
YPD Right	25% (±20%)	12% (±9%)	11% (±5%)	4% (±1%)

Niche construction refers to the process by which an organism alters its own (or another species') environment and this modification affects it evolutionary trajectory. This intricate back-and-forth process creates a feedback relationship between natural selection and niche construction. Thus, some biologists have argued that niche construction may be a general mechanism driving the evolution of mutualisms. Buser et al. (2014) revealed that the fruit fly *Drosophila simulans* was more attracted to *S. cerevisiae* yeast strains that were naturally associated with *D. simulans* when compared with other strains of *S. cerevisiae*. More attractive yeasts are dispersed more frequently, and the flies associated with more attractive yeasts have higher fecundity (Buser et al. 2014).

Similarly, yeasts isolated from fruit-associated niches were more attractive than those from non-fruit-associated niches (Palanca et al. 2013). Because the fungal species used in the present study were isolated from EFN and interiors of ants, our findings suggest a general positive preference/attractiveness toward naturally associated fungi by ant defenders. However, only two fungal species isolated were used, and how differences in host ecology affect the ant–yeast relationship remains unknown. Determining whether fungi isolated from ant-associated environments are more attractive than those from other environments is appealing.

Acknowledgments

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