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Plant-Inhabiting Ant Utilizes Chemical Cues for Host Discrimination

Tiffany L. Weir[1], Scott Newbold[2], Jorge M. Vivanco[1,6], Megan van Haren[1], Christopher Fritchman[1], Aaron T. Dossey[3], Stefan Bartram[4], Wilhelm Boland[4], Eric G. Cosio[5], and **Waltraud Kofer[5]**

[1] Department of Horticulture, Colorado State University, Fort Collins, Colorado 80523-1173, U.S.A.

[2] Shortgrass Steppe LTER, Colorado State University, Fort Collins, Colorado 80523-1499, U.S.A.

[3] USDA-ARS, 3751 SW 20th Ave APT# 1, Gainesville, Florida 32607, U.S.A.

[4] Department of Bioorganic Chemistry, Max Planck Institute for Chemical Ecology, Hans-Knöll-Strasse 8, D-07745 Jena, Germany

[5] Chemistry Section, Pontifical Catholic University of Peru, A.P. 1761, Lima 100, Peru

ABSTRACT

The Neotropical ant *Pseudomyrmex triplarinus* is involved in an obligate and complex symbiotic association with *Triplaris americana* trees. The ants inhabit trunk and branch domatia and respond aggressively to foreign invaders. Their degree of host specificity and basis for recognition of host trees has not been studied. We determined that, in contrast to *T. americana* seedlings, heterospecific seedlings set around the host trees suffered continuous pruning. Ants also removed 80–100 percent of heterospecific leaves attached to the trunk in contrast to only 10–30 percent of conspecific leaves. True species specificity was demonstrated by the selective removal of leaves from *Triplaris poeppigiana* pinned to host trees. This selectivity was also observed in a matrix-independent bioassay using leaf cuticular extracts on glass microfiber strips. Strips treated with leaf wax extracts from host trees and pinned to the trunk of host trees received only 42 percent of the number of ant visits recorded on solvent-treated controls by the end of the experiment. Strips treated with extracts of a related species, *T. poeppigiana*, received 64 percent of the number of ant visits compared with solvent-treated controls. These experiments also suggest that *P. triplarinus* recognizes surface chemicals of their host tree, independent of the texture or architecture of the carrier material; although these factors may still play some role in recognition. This is the first study that we are aware of to investigate the mechanism of host discrimination related to pruning behavior.

Abstract in Spanish is available at http://www.blackwell-synergy.com/loi/btp.

Key words: ant–plant interaction; epicuticular wax; host recognition; myrmecophytes; *Pseudomyrmex triplarinus*; Tambopata National Reserve, Peru; *Triplaris americana*.

THE FIRST COEVOLVED MUTUALISM TO BE WELL CHARACTERIZED was the symbiosis between certain tropical species of ants and their host plants (Janzen 1967). Ants involved in these interactions live in specific structures, called domatia, inside plant stems, petioles, or leaf pockets. Besides providing housing, plants often provide the ants with nutrient sources such as sugars, lipids, and protein resources in food bodies or extrafloral nectaries (Bentley 1977, O'Dowd 1982, Heil & McKey 2003). In return, the ants defend the trees against herbivores, microbial pathogens (Letourneau 1998, de la Fuente & Marquis 1999, Heil *et al.* 1999), and encroaching vegetation (Frederickson *et al.* 2005). Some plants even derive benefits from nutrients obtained from ant debris (Sagers *et al.* 2000, Solano & Dejean 2004). Although the basis by which ants recognize their host plants is unknown, evidence points to chemical cues that allow ants to distinguish between host and non-host plants (reviewed in Blatrix & Mayer 2010). For example, volatiles from *Cordia nodosa* attract the queens of an ant species that establish obligate associations with these plants (Edwards *et al.* 2006). Further, a number of studies implicate plant volatile compounds in stimulating recruitment of worker ants to damaged host tissues (Agrawal 1998, Jürgens *et al.* 2006, Mayer *et al.* 2008, Schatz *et al.* 2009). Nevertheless, the identity of the chemical cues involved in eliciting particular ant behaviors remains elusive.

We examined a common, but rarely studied, myrmecophyte, *Triplaris americana* L., and its ant mutualist, *Pseudomyrmex triplarinus* Weddell (Formicidae). Found throughout the Neotropics, the genus *Triplaris* (Polygonaceae) consists of species of medium height (5–30 m) with narrow trunks (~30 cm dbh). Twenty of the 25 named *Triplaris* species harbor ant colonies in hollow trunk and branch domatia (Wheeler 1942, Benson 1985, Brandbyge 1986). *T. americana* L., commonly known as *tangarana*, *hormigo*, *formigueiro*, and *pau-de-formiga*, are found growing in clusters within disturbed lowland forest. They are typically inhabited by colonies of aggressive *P. triplarinus*, which are obligate symbionts of *T. americana, T. surinamensis* (Oliveira *et al.* 1987), and *Triplaris filipensis* (Jaffe *et al.* 1986). Isolation studies showed that *P. triplarinus* do not leave their tree to hunt, but rather feed on coccids that co-inhabit the domatia, honeydew provided by the coccids, and nematodes that feed on ant refuse in tree internodes (Wheeler

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[6]Corresponding author; e-mail: j.vivanco@colostate.edu

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& Darlington 1930, Schremmer 1984). The ants guard the coccids and transport them to safe locations if the tree is damaged or attacked by predators. The ants react viciously toward intruders, biting and stinging anything that comes into contact with their host tree.

Vegetation surrounding *T. americana* trees is unusually sparse: heterospecific seedling establishment within 2 m of the canopy is roughly half that found beyond the canopy (Larrea-Alcazar & Simonetti 2007). This is due to pruning of heterospecific seedlings by resident *P. triplarinus* ants, which is thought to prevent bridge points between host trees and neighboring flora that might otherwise allow invading ant species access to the colony (Davidson *et al.* 1988), although even seedlings with no contact to the host plant are pruned (Larrea-Alcazar & Simonetti 2007). We hypothesized that *P. triplarinus* possessed the ability to discriminate and selectively remove nonhost plant material growing in *T. americana* stands. We also explore potential chemical mechanisms underlying host discrimination in pruning behavior.

METHODS

Study sites.—Field studies were conducted from October 2007 to October 2008 in the Tambopata National Reserve, located approximately 70 km southwest of Puerto Maldonado, Peru. The study consisted of two areas, which contained experimental plots with focal *T. americana* trees. The first study area (12°50′07″ S, 69°17′18″ W) consisted of two plots of about 5 × 5 m, each containing 15 *T. americana* trees. The area was located about 3 m away from a heavily used trail, but was clearly marked and labeled to limit human disturbance. Trees from plots in this area were used in all experiments with the exception of the ant recruitment experiments, which were carried out in a second area (12°15′11″ S, 69°17′42″ W) near the Tambopata River. The second area was about 25 × 25 m and contained six *T. americana* trees.

Seedling recognition.—We tested the reaction of *P. triplarinus* ants to non-*T. americana* plants by challenging ant colonies with host and nonhost seedlings planted around *T. americana* host trees in two experimental plots. Seven *T. americana* trees were randomly selected within each plot and served as focal trees for the experiment. Plant species used in the experiment included the host, *T. americana*, and two nonhost species that were common understory plants in the research area, *Pharus lappulaceus* Aubl. (synonym *Pharus glaber*, a grass) and *Adiantum petiolatum* Desv. (a fern). Seedlings of these species were harvested from the study area and replanted in containers near the research station 3 wk before conducting the experiment. After 3 wk, nine plants (three of each species) were transplanted and distributed randomly into pre-dug holes around each of the seven focal *T. americana* trees. After transplanting, the total number of leaves (and leaflets for *A. petiolatum*) was recorded for each plant, and pruning by ants was determined by monitoring total leaf number daily for 5 wk (33 d). This experiment was repeated in a second trial using seven different *T. americana* trees in a second experimental plot established adjacent to the first plot. Data for the second trial were collected daily for 2 wk at which point flooding of both plots resulted in termination of the experiments.

We calculated the proportion of leaves either removed or gained (final no. of leaves/initial no. of leaves) during the course of a given experimental trial for individual seedlings and then calculated the mean change in proportion of leaves per seedling species for each host tree ($N = 7$) using the three seedlings as subsamples. Proportions were used for analyses rather than raw counts to account for natural variation in the initial number of leaves present at the start of the experiment among the three seedling species (see 'Results'). We tested for differences in the change in proportion of leaves among the three seedling species using a one-way analysis of variance (ANOVA) with the Tukey multiple-comparison test. Separate analyses were conducted for each trial of the experiment (trials one and two). For all field experiments, each *T. americana* tree was assumed to harbor a single *P. triplarinus* colony; thus the individual tree (or resident ant colony) constituted our study unit.

Leaf pruning.—Ant colonies living in ten randomly chosen *T. americana* trees were evaluated for their ability to distinguish host vs. nonhost plant material attached to their host tree. The ten focal trees selected were located within the two plots in the study area. Leaves of *T. americana* (collected from nonfocal trees), *P. lappulaceus*, *A. petiolatum*, and *Cedrela odorata* L. (a tree) were collected from the study area and attached to host trees using pins. All leaves of a given species were similar in size and age, and free of any signs of herbivory or disease. One leaf from each of the four species was attached individually to a focal tree at a height of ~1.2 m and monitored for 4 d. Presence/absence data for leaves that were clearly removed by the ants (not those that appeared to have been removed by wind, rain, or other disturbance) were recorded daily. This experiment was repeated approximately 1 mo later in a second trial using the same ten focal *T. americana* trees. We tested for differences in the removal rate of leaves by ants among leaf species using an ANOVA on logit-transformed data (using PROC GLIMMIX in SAS) with the Tukey multiple-comparison test. This procedure allowed us to use data from the two sampling trials, which were treated as subsamples and averaged before analysis. Because one species (*C. odorata*) had all zeros (*i.e.*, pinned leaves were removed from all ten trees in both trials) and therefore GLIMMIX would not converge, an observation for one trial on one tree was changed from zero to one for *C. odorata*.

In a second related experiment, leaves of the closely related species *Triplaris poeppigiana*, which is also a myrmecophyte that reportedly hosts ants of the genus *Azteca* (Davidson *et al.* 2003), were pinned to ten *T. americana* trees within the study area to determine if the residing colonies could distinguish between two closely related tree species. Briefly, three leaves of *T. poeppigiana* and three leaves of *T. americana* were pinned around the trunk of each of the ten trees at a height of approximately ~1.2 m and monitored for 5 d. Percent of leaf removed was recorded on the fifth day. We calculated the mean percent of leaf removed using the three leaves per species per tree as subsamples. We conducted a one-way ANOVA comparing percent of leaves removed between the two leaf species.

ANT RECRUITMENT.—Studies to determine the chemical basis of host plant recognition by *P. triplarinus* were conducted using leaf cuticular extracts of *T. americana*. Two GF/A glass fiber filter strips (7×40 mm), control and treatment, respectively, were pinned to opposite sides of the trunk of five different *T. americana* trees. Trees used in these studies were spaced at least 3–10 m apart and were located in the second study area, close to the research station. Treated strips were soaked with leaf surface extracts of *T. americana* prepared on-site by individually submerging 12 mature leaves in 250 mL dichloromethane for 10 sec a total of three times per leaf. Control strips were also submerged in dichloromethane. All strips were allowed to air-dry before affixing them to tree trunks. Response of the ants toward the filter strips was quantified by counting the number of ants present on control and treated strips at 15-min intervals over a period of 1 h. The experiment was repeated three times ($N = 3$ trials) over the course of 2 d. Additionally, a second study was conducted using strips soaked with leaf surface extracts of *T. poeppigiana*. In this study, control and treated strips were pinned to six *T. americana* trees in a single trial.

We evaluated the influence of filter paper treatment (control vs. *Triplaris* leaf extract) and time (four observations at 15-min intervals) on ant recruitment rates using a repeated measures two-way ANOVA. For the experiment using *T. americana* leaf extract, we calculated the mean number of ants per strip for each 15-min interval for each host tree ($N = 5$) using the three trials. For the experiment using *T. poeppigiana* leaf extract, we used the one set of observations collected for each host tree ($N = 6$). Data were transformed using a square-root transformation to meet assumptions of parametric tests.

LEAF SURFACE WAX EXTRACTION.—We used a solvent-based and cryo-adhesive extraction method. Surface waxes from 150 g fresh weight of mature *T. americana* leaves were extracted by submerging each leaf two times for 10 sec in a 500 mL glass beaker containing 250 mL of high-performance liquid chromatography grade dichloromethane (CH_2Cl_2). Extracts were filtered using GF/A glass fiber filters and dried under reduced pressure, resulting in a clear yellow residue. The residue was re-dissolved in 50 mL of dichloromethane and stored at –20°C under argon before use.

Epicuticular waxes from ten *T. americana* leaves were *also* mechanically stripped using a cryo-adhesive extraction method modified from Jetter and Schäffer (2001). Briefly, 100 metal discs (23 mm diam) were used to remove a total surface area of *ca* 415 cm^2 from the adaxial surface of the leaves. Leaves were washed with 200 μL of deionized water and the stainless-steel disc was pressed onto the leaf and flash frozen in liquid nitrogen. Metal disks with the frozen water-wax layer were pooled in 50 mL CH_2Cl_2. The waxes were extracted from the surface of the metal discs by sonication for 15 min in dichloromethane. Extraction was repeated twice with 50 mL of fresh CH_2Cl_2 each and the combined organic phases were filtered through a GF/A filter. After separation from the aqueous fraction, the organic solvent was evaporated under reduced pressure, resulting in 2 mg of epicuticular waxes. The extract was then dissolved in chloroform to a final concentration of 4 mg/mL and stored at -20°C under argon.

ANT SURFACE WAX EXTRACTION.—Approximately 200–300 ants were collected from two *T. americana* trees at the study site, killed immediately by exposure to CH_2Cl_2 vapors. Cuticular extracts were made by rinsing the ants three times for 30 sec with 50 mL of CH_2Cl_2 at room temperature. The sample was filtered and concentrated to dryness, weighed, and redissolved in 2 mL dichloromethane. The sample was divided in two aliquots, which were stored under argon before GC-MS analysis. Each aliquot was analyzed independently at the University of Florida, Gainesville (UF) and the Max Planck Institute for Chemical Ecology, Jena (MPI) facilities.

CHEMICAL ANALYSES.—Wax components were analyzed both without modification, and derivatized by trimethylsilylation and analyzed by gas chromatography with mass spectrometry detection using electron-impact ionization (GC-EI-MS). Samples in the MPI (Jena) facilities were analyzed in a Thermo Scientific ThermoQuest Trace GC-MS with a Phenomenex ZB-5MS column (30 m × 0.25 mm ID, film thickness 0.25 μm) (Phenomenex Inc, Torrence, California, U.S.A.). Helium was used as carrier gas (1 mL/min). Injector and transfer lines were set at 300°C and injection was carried out in splitless, 10:1, or 20:1 modes. The temperature program consisted of a 3-min isothermal step at 50°C, followed by a linear 30°C/min gradient to 170°C, followed by a 5°C/min gradient to 300°C, a 30°C/min gradient to 320°C and a final isothermal step at 320°C for 15 min. Compounds eluting from the column underwent electron ionization (EI) at 70 eV and ions from *m/z* 40 to 700 were detected. Eluted compounds were aligned with a reference alkane series (C_8–C_{40}, Fluka Analytical, St. Gallen, Switzerland) and their spectra compared with NIST 2002 and Wiley mass spectral libraries. A parallel set of samples analyzed at UF facilities were performed with a Thermo Scientific Trace DSQ mass spectrometer, equipped with an Alltech AT™-5 ms column (60 m × 0.25mm ID, film thickness 0.25 μm) (W.R. Grace and Co, Deerfield, Illinois, U.S.A) and with helium as carrier gas (1 mL/min). The sample was injected in splitless mode. The GC oven temperature was maintained at 40°C for 2 min, and then increased at a rate of 20–320°C/min. The injector and transfer lines were set at 280°C while the ion source was at 180°C. Compounds eluting from the column underwent EI at 70 eV and ions from *m/z* 50 to 700 were detected. Acquired spectra were compared with those from the NIST/EPA/NIH Mass Spectral Library using the NIST MS Search (v.2) software (Stein *et al.* 1987–2002).

RESULTS

GENERAL OBSERVATIONS.—All *T. americana* trees examined during this study were colonized by *P. triplarinus* ants. None of them had vines or other epiphytes growing on their trunks or crowns. Signs of herbivore damage were few and undergrowth in the vicinity of *T. americana* trees was sparser than around neighboring trees of other species. *P. triplarinus* patrolled more actively in the late afternoon and early evening hours, and ant activity was minimal during rainy periods; although they still defended their host tree any time it was disturbed. We also observed *P. triplarinus* attacking leaf-cutter ants from a nearby nest when they came too close to the host tree.

RECOGNITION OF NONHOST PLANTS BY ANTS.—Upon encountering seedlings, the ants readily identified the non-*Triplaris* species and began to prune them (Video S1). The initial number of leaves varied among seedling species, with *A. petiolatum* having more leaves (10.1 ± 0.29, mean $\pm$ SE, $n = 42$) than *P. lappulaceus* (5.5 ± 0.34) and *T. americana* (3.8 ± 0.22). In the initial trial over a period of 33 d, the change in proportion of leaves differed among the three seedling species ($F_{2, 18} = 29.90$, $P < 0.0001$; Fig. 1A); ants removed significantly more leaves from ferns (71%) and grasses (68%) than from *T. americana* seedlings ($P < 0.05$, Tukey test), which lost few or no leaves to ants over the course of the trial. In contrast to the nonhost seedlings, nearly all *T. americana* seedlings grew additional leaves during the experiment (10% increase) (Fig. 1A). Similarly, in the shorter-duration, second trial, ants removed a greater proportion of leaves from ferns (62%) and grasses (52%) than from *T. americana* seedlings, which again showed an increase in leaf number (5%) ($F_{2, 18} = 13.94$, $P = 0.0002$; $P < 0.05$, Tukey test; Fig. 1B).

There was also a striking difference in the number of pinned leaves removed by ants among the host (*T. americana*) and nonhost (*P. lappulaceus*, *A. petiolatum*, and *C. odorata*) species; ants removed 80–100 percent of non-*Triplaris* leaves, but only 10–30 percent of *T. americana* leaves (Table 1). The removal rate of *T. americana* leaves by ants differed significantly from all other species (ANOVA, $F_{3, 27} = 7.52$, $P = 0.0008$; $P < 0.05$, Tukey test), while pair-wise comparisons among the three nonhost species were not significant ($P > 0.05$, Tukey test). In the study comparing a closely related species (*T. poeppigiana*) and the host tree (*T. americana*), ants removed a significantly greater percentage of *T. poeppigiana* leaves ($54 \pm 12\%$; mean $\pm$ SE, $N = 10$) compared with *T. americana* leaves ($24 \pm 8\%$) that were pinned to host *T. americana* trees during the course of the 5-d experiment (ANOVA, $F_{1, 18} = 4.43$, $P = 0.049$).

ANT RECRUITMENT TO EXTRACT-TREATED CARRIER MATERIAL.—Ants were able to distinguish between control GF/A filter strips and those treated with epicuticular wax extracts of the host tree. Figure 2 shows that in the ant recruitment experiment, control (CH_2Cl_2) strips received significantly more damage than strips that were permeated with *T. americana* leaf wax extracts. Ant attack usually continued until the filter strip was removed from the trunk. In general, strips treated with leaf wax extracts from *T. americana* received considerably fewer ant visitors than control strips ($F_{1, 28} = 124.13$, $P < 0.0001$; Fig. 2A), and the number of ants recruiting to strips generally increased over time ($F_{3, 28} = 15.93$, $P < 0.0001$; Fig. 2A). There was no interaction between treatment and time ($F_{3, 28} = 0.37$, $P = 0.78$). In a similar fashion, strips treated with leaf wax extracts from *T. poeppigiana* also attracted significantly fewer ant visitors than control strips ($F_{1, 33} = 34.58$, $P < 0.0001$; Fig. 2B). Though less pronounced than with *T. americana*, ant recruitment rates did increase with time ($F_{3, 33} = 3.76$, $P = 0.020$; Fig. 2B), and there was no interaction between treatment and time ($F_{3, 33} = 0.49$, $P = 0.69$). Severity of damage between control and treated strips could be clearly distinguished by simple visual inspection (Fig. S1A).

TABLE 1. *Total number of leaves removed by ants (out of ten) from ten host trees sampled on two occasions (trial one and two).*

	Trial	
Leaf species	1	2
Adiantum petiolatum	8	10
Cedrela odorata	10	10
Pharus lappulaceus	8	9
Triplaris americana	3	1

CHEMICAL ANALYSIS OF LEAVES.—Using GC-MS analysis, we found that dichloromethane extracts of the leaf surface of *T. americana* (Fig. S2, upper panel) were primarily comprised of linear C_{12}–C_{36} hydrocarbons, fatty acids (even-numbered, C_{12} to C_{20}), alcohols (C_{12} to C_{20}), esters of these and aldehydes along with a number of pentacyclic β-amyrin and friedelin-like triterpenes (Table S1). However, our extraction method resulted in significant damage to the leaf epicuticular surfaces; therefore, we tested an alternative, method of extraction involving mechanical removal of the

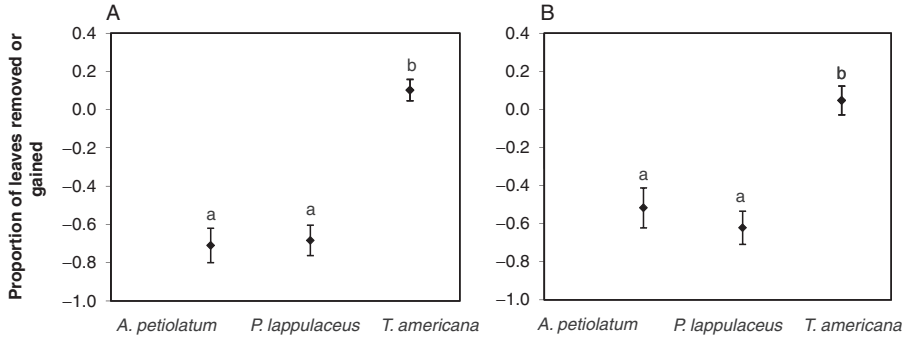

FIGURE 1. Proportion of leaves lost or gained by seedlings during (A) trial one, and (B) trial two. Negative values indicate that leaves have been removed by ants while positive values for *Triplaris americana* indicate new leaves that emerged during the experimental trial. Trial one was conducted for 33 d and trial two was conducted for 14 d, after which the experiment ended due to flooding in the study area from heavy rains. Leaf species with the same letter are not significantly different based on analysis of variance. Error bars are ± 1 SE.

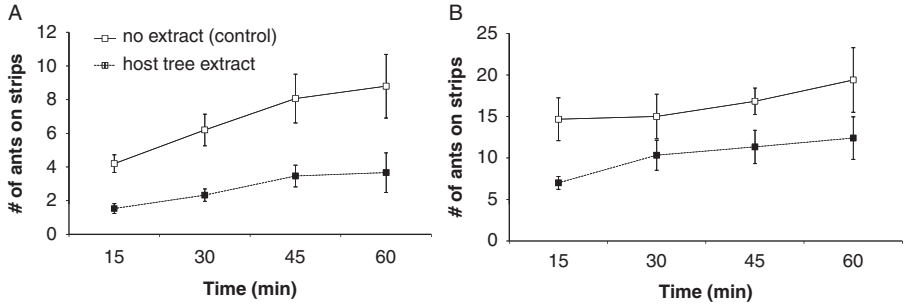

FIGURE 2. Number of ants recruiting to strips of filter paper treated with leaf wax extract from (A) *Triplaris americana*, or (B) *Triplaris poeppigiana* compared with control strips with no *Triplaris* sp. extract. Error bars are ± 1 SE.

epicuticular surface (Jetter & Schäffer 2001). Epicuticular components extracted by the cryoadhesive method had an enriched hydrocarbon component while the triterpenoids detected in the solvent-based extraction (R_t between 35 and 39 min, Fig. S2, upper panel) were essentially absent. Preliminary qualitative data from a filter strip assay using commercially purchased friedelin, a triterpenoid identified in cuticular extracts, showed that this compound did not reduce ant aggressiveness (data not shown).

CHEMICAL ANALYSIS OF ANTS.—GC-MS analysis of underivatized *P. triplarinus* surface extracts showed they contain primarily long chain even-carbon number fatty acids and their esters (C_{16}–C_{18}) with or without double bonds, simple unsaturated hydrocarbons (some branched, some not, and one small ketone [C_8]) (Table S1; Fig. S2). The epicuticular surface of *P. triplarinus* contained fewer components than the dichloromethane extracts of *T. americana* leaves (Table S1 and Fig. S2 middle and lower panels). Small amounts of free fatty acids such as palmitic and oleic acid were found in both the ant and leaf extracts. Further, small amounts of methoxydehydroabietic acid and stigmasterol derivatives were detected in the *P. triplarinus* ant extracts (Table S1).

DISCUSSION

Studies examining the pruning behavior of *P. triplarinus* have concluded that these ants attack and remove vegetation that physically encroaches on their host tree (Davidson *et al*. 1988) as well as seedlings that are growing in the vicinity of host trees (Larrea-Alcazar & Simonetti 2007). The finding that host contact was not required to trigger pruning behavior led us to speculate about the specificity and underlying mechanism of *P. triplarinus'* ability to discriminate between host and nonhost material. Consistent with published studies, we observed pruning of foliage in direct contact with the hosts and of seedlings growing near the host, but also demonstrated that *P. triplarinus* were clearly able to differentiate between host and nonhost plants and plant material. This degree of specificity has been seen in other ant–plant interactions (Renner & Ricklefs 1998, Frederickson *et al*. 2005, Fredrickson & Gordon 2007), but has not previously been explored in the *P. triplarinus*–*T. americana* symbiosis. It has been suggested that pruning behavior may be directly beneficial to the host tree by eliminating plants that would compete for light and nutrients, thereby indirectly benefitting the colony (Janzen 1967, 1969). An alternative explanation is that the colony is protecting itself by preventing foliar bridges that would allow competing ant species to access and attack the colonies in their host trees (Davidson *et al*. 1988, Stanton *et al*. 1999, Yumoto & Maruhashi 1999, Federle *et al*. 2002, Palmer *et al*. 2002). Our observation that nonhost plant material was selectively removed suggests that the pruning behavior of *P. triplarinus* may have direct benefits to the colony rather than to individual host trees. Conspecific seedlings were exclusively allowed to remain near host trees despite the fact that they would eventually compete with these trees for sparingly available resources. Because young *T. americana* trees in this area are eventually colonized by *P. triplarinus*, allowing conspecific seedlings to establish is not likely to amplify the threat of attack by other ants and would increase the number of available nest sites. Fredrickson and Gordon (2009) found that *Myrmelachista schumanni* colonies occupying more *Duroia hirsuta* trees survived longer than those occupying fewer trees. While there are no published reports and we were unable to determine whether *P. triplarinus* colonies can also inhabit multiple trees, cultivating conspecific plant material could enhance colony growth and survival or facilitate establishment of new colonies. While cultivation of new nest sites may explain why host seedlings are not removed, at least one study has shown that plant species, rather than suitability of a plant for nesting, is the basis of host discrimination. Frederickson *et al*. (2005) found that *D. hirsuta* seedlings planted close to the host tree were not attacked by *M. schumanni*, regardless of whether or not the domatia had been removed from the plants.

Many myrmecophytic species have evolved within a particular plant genus (McKey & Davidson 1993), but few ant–myrmecophyte interactions have been reported to be highly specific. We observed that *P. triplarinus*, which has been reported to form associations with *T. americana* (Ward 1999), *T. filipensis* (Jaffe *et al*. 1986), and *T. surinamensis* (Oliveira *et al*. 1987), was clearly able to distinguish between the leaves of *T. americana* and *T. poeppigiana,* two closely related species. In our experiments, *P. triplarinus* were twice as likely to remove *T. poeppigiana* leaves pinned to their host tree than to remove *T. americana* leaves collected from other, nonfocal *T. americana* trees. This suggested that the colony was able to distinguish between the two plant species, and not simply to recognize its own host plant. Similarly, foundress queens of different

Crematogaster species demonstrated a significant preference for entering the domatia of seedlings from their host species rather than other *Macaranga* seedlings (Inui *et al.* 2001). In this study, it was concluded that low volatility compounds on the plant surface were likely responsible for this specificity (Inui *et al.* 2001).

Based on several studies (Inui *et al.* 2001, Edwards *et al.* 2006, Jürgens *et al.* 2006, Grangier *et al.* 2009), and our own observations that the ants specifically identified *T. americana* leaves as host, we focused our search for chemical recognition factors on leaf surface chemicals. Ants were able to recognize cuticular extracts from leaves of host plant species obtained by solvent or cryogenic extraction. Filter papers treated with *T. americana* surface extracts received less ant attention and ultimately suffered less destruction than control papers, and papers infiltrated with extracts from *T. poeppigiana* leaves demonstrated the high degree of specificity in host recognition by *P. triplarinus*. *T. poeppigiana* leaves received 64 percent ant visits compared with controls while *T. americana* received only 42 percent ant visits compared with solvent controls. The degree of recognition, however, between *T. americana* and *T. poeppigiana* was greater when intact leaves were used rather than the extract-treated filters, suggesting that other factors such as surface architecture might also be involved in host recognition specificity.

In vivo ant recruitment experiments clearly showed that cuticular components play a key role in host recognition. In order to assess whether the triterpenes played a crucial role in ant recognition in the *Triplaris–Pseudomyrmex* interaction, preliminary experiments using friedelin, a cuticular component of *T. americana* leaves from which a number of triterpenoids are derived, were performed. Filter strips infiltrated with friedelin did not, however, deter ant attacks, suggesting that the friedelin-derived triterpenoids extracted by the solvent from the lower cuticular layer are less likely to be a signal for ants than the superficially located hydrocarbons.

There is a large body of literature implicating cuticular hydrocarbons as signals used by social insects to recognize nestmates, dictate task-related behaviors, and serve as dominance and fertility cues (reviewed in Howard & Blomquist 2005). Thus, one working hypothesis in this study is that *T. americana* evolved a chemical mimicry, hijacking existing cuticular hydrocarbon cues utilized by *P. triplarinus* before the evolution of their symbiosis. Indeed, biochemical convergence is thought to be the basis of recognition between *Pseudomyrmex ferrugineus*, their host tree, *Acacia collinsii*, and a social wasp, *Parachartergus azteca*, which is allowed by the ant colony to nest in the host tree. The cuticular hydrocarbons profiles of ant and wasp were nearly identical, while thorns of *A. collinsii* have a surface wax layer with hydrocarbons of similar chain length to those of their inhabiting insects (Espelie & Hermann 1988). Some ants have been shown to incorporate dietary hydrocarbons (Liang & Silverman 2000), and thus, shared hydrocarbons between *T. americana* and *P. triplarinus* may be a result of the complex trophic interactions between the ants, their host tree and coccids tended by the ants. Finally, the amount of specific hydrocarbons has been shown to be an important factor in their effectiveness as nestmate recognition cues, suggesting that hydrocarbon signaling relies on sensitive responses to just one or a few compounds (Martin *et al.* 2008). Colonies of *Formica exsecta* exhibited reduced aggressiveness toward glass beads coated with nestmate alkenes, but reducing the amount of Z9-alkene significantly increased aggressiveness toward the beads (Martin *et al.* 2008). Thus, the abundance of a chemical cue utilized for host recognition may be more important than its uniqueness to the host plant, and could account for the varying degrees of aggression that we observed toward filter papers impregnated with cuticular components of the two closely related *Triplaris* species.

Our analyses revealed that not only were there similar cuticular hydrocarbons, but that there were also nonhydrocarbon cuticular components shared between the ants and *T. americana* leaves that could also be important for host discrimination. Small amounts of free fatty acids such as palmitic and oleic acid were found in both the ant and leaf extracts. It has been suggested that free fatty acids serve as ubiquitous death recognition signals (Rollo *et al.* 1994), and this has been demonstrated experimentally with oleic acid in ant colonies (Wilson *et al.* 1958, Ayasse & Paxton 2002). Indeed, we found a huge oleic acid signal in a CH_2Cl_2 extract of *P. triplarinus* where the ants had been incubated only half exposed to the solvent in a vial at room temperature for several days. Fresher extracts of the ants, however, contained substantially less oleic acid, but an otherwise almost identical GC-MS profile. These compounds are also particularly abundant in elaiosomes of ant-dispersed seeds and are thought to act as an attractant that stimulate the ants to carry these seeds to the nest (Rico-Grey & Oliviera 2007 and references therein).

Small amounts of methoxydehydroabietic acid and stigmasterol derivatives were also detected in the *P. triplarinus* ant extracts. Both of these compounds are known to be only of plant, not ant, origin and are reported in the literature as being collected by insects for nutritional or protective purposes (Eisner *et al.* 1974, Castella *et al.* 2006), and are thus unlikely chemical recognition cues. Analysis of plant signals alone, however, may shed some light on the specific cues utilized in *P. triplarinus* host discrimination. Chemical analysis of *T. poeppigiana* leaves, which the ants showed some degree of recognition, could be helpful in identifying shared hydrocarbons with different relative abundances and may aid in specifically pinpointing potential hydrocarbon recognition signals. In conclusion, we believe that cuticular compounds are the primary chemical signals involved in host discrimination, with surface architecture potentially providing secondary cues, and more in-depth chemical analysis and field bioassays are underway to determine the importance of cuticular fractions and individual compounds on host discrimination by *P. triplarinus*.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

TABLE S1. *Preliminary listing of major compounds found in* Triplaris americana *leaf surface and* Pseudomyrmex triplarinus *dichloromethane extracts*.

FIGURE S1. Filter strips that were permeated with cuticular extracts of *T. american* had fewer ant visitors and showed less damage from ant attacks than filter strips treated only with solvent.

FIGURE S2. GC elution profiles of underivatized cuticular extracts of *T. americana* solvent extract, *T. americana* cryo-adhesively extracted surface and *P. triplarinus* cuticle.

VIDEO S1. *Pseudomyrmex triplarinus* ant cutting the leaves on a non-host seedling planted in the vicinity of its host tree.

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