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RESEARCH

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ABSTRACT. Recent studies have shown an insecticidal effect of *Tithonia diversifolia* (Hemsl.) Gray (Asterales: Asteraceae) foliage on workers of *Atta cephalotes* L. and inhibitory effects of this plant on the growth of the symbiotic fungus *Leucoagaricus gongylophorus* (A. Müller) Singer. To evaluate the potential of *T. diversifolia* as a biological control treatment of this important pest, we assessed the effect of green manure (mulch) of this plant on natural nests of *A. cephalotes*, in Cali, Colombia. Three treatments were randomly assigned to 30 nests: 1) green mulch of *T. diversifolia*, 2) green mulch of *Miconia* sp., Ruiz & Pav. and 3) unmulched control. Every 2 wk for 6 mo, the surface of the nests was completely covered with leaves. Physical and chemical parameters of nest soil were assessed before the first and after the last application of the mulch. Ant foraging in *T. diversifolia*-treated nests decreased by 60% after the initial applications of the mulch, while nest surface area decreased by 40%. When the nests covered with *T. diversifolia* were opened, it was observed that the superficial fungus chambers had been relocated at a greater depth. In addition, microbial activity and soil pH increased by 84% and 12%, respectively, in nests covered with plant residues. In conclusion, the continued use of *T. diversifolia* mulch reduces foraging activity and negatively affects the internal conditions of the colonies, thereby inducing the ants to relocate the fungus chambers within the nests.

Key Words: green manure, biological control, leaf-cutting ant, *Atta cephalotes*

Leaf-cutting ants of the genera *Atta* and *Acromyrmex* (Hymenoptera: Formicidae) harvest leaf biomass from a large variety of plant species to the extent of being considered among the main herbivores of the tropics and subtropics (Della-Lucia 2011, Hölldobler and Wilson 2011). The uncontrolled proliferation of *Atta cephalotes* L. is a result of unsustainable land use practices and environmental degradation (Montoya-Lerma et al. 2011). This species causes significant economic damage by defoliating crops, ornamental plants, grasslands, and forestry species (Fernández and Jaffé 1995, Forti et al. 2000). Organophosphates, pyrethroids sulfuramides, and other chemical insecticides used in controlling leaf-cutting ants (Godoy et al. 2005) cause serious pollution and have toxic effects on nontarget species (Godoy et al. 2005, Caffarini et al. 2008, Della-Lucia 2011). The need to substitute these risky chemicals has motivated an active search for effective and environmentally safe control methods (Santos-Oliveira et al. 2006, De Melo Cazal et al. 2009).

Leaf-cutter ants have an obligate mutualism with *Leucoagaricus gongylophorus* (A. Möler) Singer (Agaricales: Agaricaceae), in which the ants supply substrates for fungal growth (Martínez and Servín-Montoya 2002, Della-Lucia 2003) and the basidiomycete fungus feeds ant colonies (Silva et al. 2003, Valmir et al. 2004). As some plant substrates are unsuitable for the fungus, the ants display foraging preferences (Rockwood 1976, Hubbell and Howard 1984, Rodríguez et al. 2008) influenced by factors such as water content, nutritional quality, hardness, repellence, or toxic secondary metabolites (Hubbell and Howard 1984, Berish 1986, Barone and Coley 2002, Mundim et al. 2009). Various plant species with secondary metabolites that repel ant foraging, disrupt ant behavior, or have toxic effects on the ants or their symbiotic fungus have been studied as alternatives to chemical control (Caffarini et al. 2008, De Melo Cazal et al. 2009). This is the case of the Mexican sunflower (*Tithonia diversifolia* (Hemsl.) Gray), which has metabolites such as sterols, terpenes, coumarins, and lactone compounds (García and Delgado 2006, Ambrósio et al. 2008, Medina et al. 2009, Chagas-Paula et al. 2012).

In laboratory colonies fed with *T. diversifolia*, workers of *A. cephalotes* rejected this plant as a forage resource (Rodríguez et al. 2008), and the plant caused a 100% size reduction and up to 83.3% mortality of the symbiotic fungus (Valderrama et al. 2009). Castaño et al. (2013) observed that an ethanol extract of dry *T. diversifolia* leaves supplied as part of an artificial diet or applied directly on the cuticle caused 100% mortality of workers within 8 d.

Giraldo (2005) found that *Montanoa quadrangularis* Sch. (Asterales: Asteraceae) trees in a young plantation were less prone to the attack of leaf-cutting ants when fertilized with *T. diversifolia* green manure than when chemically fertilized. Considering that nest construction depends on soil pH, porosity, and infiltration capacity (Cammeraat et al. 2002, Moutinho et al. 2003), it is likely that the green manure of *T. diversifolia* not only alters fertility through the incorporation of nutrients from decomposition (Ikerra et al. 2006, Crespo et al. 2011) but also releases secondary metabolites that deter ant colonization. If so, this foliage could be applied deliberately to control *A. cephalotes* by altering the chemical and biological environment of the nests.

Green manure or mulch provides a natural cover with multiple benefits: it inhibits weed development (Burkhard et al. 2009) while reducing nutrient leaching, increases soil organic matter (Kuepper and Diver 2010) and cation exchange capacity (Proyecto Checua 2000), and creates a favorable environment for natural enemies of agricultural pests (Hartwig and Ammon 2002) thus enhancing biological pest control (Hooks et al. 1998, Frank and Liburd 2005, Prasifka et al. 2006, Pullaro et al. 2006). Studies of arthropod activity on mulch-covered soil are scant. For instance, not much information exists on how arthropods are affected by plant residues on the soil surface. The main objective of this study was to evaluate the physical, chemical, and biological impacts of *Tithonia* mulch on field colonies of *A. cephalotes*. We hypothesized that the *Tithonia* mulch would affect foraging and nest construction and that the decomposition of the green manure on top of the ant nests would alter soil physical-chemical properties and directly affect the development of the symbiotic fungus inside the nest.

Materials and Methods

Study Area. Field trials were conducted at two farms (Entre Quebradas 3° 24' 58.78" N; 76° 35' 30.69" W and Corral de Piedra 3° 24' 51.84" N; 76° 35' 36.39" W) located at 1,350 m above sea level in the rural area of Cali, Colombia, with 80% average relative humidity and 23°C average temperature. Conventional small-scale pastures for cattle grazing occupied 90% of the area, covered by Bahia grass (*Paspalum notatum* Alain ex Flügge [Poales: Poaceae]), small *Miconia* spp. trees and a few large *Inga* Mill (Fabales: Fabaceae) and *Ficus* L. (Rosales: Moraceae) trees. The remaining 10% of the area was covered by riparian vegetation dominated by bamboo *Guadua angustifolia* Kunth (Poales: Poaceae) and *Ficus* sp (Cali: Colombia).

In 2009, a crop of Mexican sunflower was planted at the Universidad del Valle campus (3° 22' 23.07" N; 76° 31' 50.69" O). The plants were pruned in April 2011, and 2 mo later, the stems were cut and transported to the study area. Additional *Tithonia* biomass was collected along the Pance River, where this plant grows spontaneously.

Selection of *A. cephalotes* Nests and Application of Green Manure. An intensive search for *A. cephalotes* nests was conducted in the study area between May and June 2011. Nests with a surface area $\leq 35 \text{ m}^2$ were selected and georeferenced with a portable GPS after verifying the absence of pest control treatments.

Selected *A. cephalotes* nests were randomly assigned to the three treatments: *T. diversifolia* and *Miconia* mulches and control (no mulch). Between July and December 2011, stems and leaves of both plant species were applied biweekly covering the entire surface of 20 nests (10 nests for each mulch treatment), until completing 12 applications. Plant residues from previous mulching were not removed. The amount of green manure varied with nest size (10–30 kg per nest). A group of 10 nests without green manure was used as an unmulched control. No insecticides were applied throughout the experiment.

Characterization of the Nests. A biological and physical characterization of all nests was done before and after applying the treatments, taking into account the following variables, validated in previous studies (Montoya-Lerma et al. 2006, López 2008): 1) total number of entrances: all nest mound openings, including those used for forage entrance, ventilation, and excavation; 2) total nest area (m^2): estimated as the product of the N-S and E-W distances between the most remote mound openings. 3) foraging activity: average number of foraging ants within a 20 cm radius from the main mound opening, assessed with a hand counter. Observations were made during 1-min bouts, and the average of three counts was recorded. 4) excavation activity: the number of ants observed removing nest materials at the entrance of the mound opening with the most active excavation. This was counted for 1 min, only once.

Throughout the study, the sequence of nests was randomized, ensuring that the variables were always evaluated at different times between 0600 and 0800 hours, before applying the plant material and then 1, 3, 5, and 10 d after each application.

To estimate soil microbial activity, soil samples (collected at depths of 0, 10, and 20 cm) were taken randomly from four nests per treatment. The samples were analyzed by modifying the method proposed by Lagomarsino et al. (2011). For the physical characterization, soil samples were taken randomly from 12 nests (four per treatment) using a drill with a 10 cm diameter. The drill was introduced in the central area of each nest to depths of 0, 10 cm, and 20 cm. Soil samples collected at the same depths 5 m away from each nest were used as references. Porosity and pH were analyzed in the soil samples to assess permeability and acidity, respectively.

Samples for evaluating microbial activity and physical properties of the nests were taken before applying the treatments and 10 d after the last application of green mulch (only for pH and microbial activity). The analyses were carried out at the Soil Laboratory (Universidad del Valle, Cali).

Once data collection ended, all nests were excavated, and the symbiotic fungus chambers were located. The volume of the symbiotic fungus, number of empty chambers, and depth were recorded.

Data Analysis. Repeated-measures analysis of variance (ANOVA) and post-ANOVA (Von Ende 1993) were done after verifying compliance with the assumptions of homogeneity of variance and normal distribution. Data were analyzed for differences between treatments in the foraging and excavation activities, the number of entrances, and the surface area of the nests. Pre- and posttreatment microbial activity and pH were compared with a Student's *t*-test for dependent samples. Nest soil density and porosity, as well as depth of the chambers, were analyzed with a Student's *t*-test for independent samples. All analyses were performed using STATISTICA software (Statsoft 2007).

Results

Approximately 1.3 and 1.1 tons of *T. diversifolia* and *Miconia* sp. foliage were used throughout the study, respectively. *Miconia* sp. mulch, but not *Tithonia*, was partially foraged by ant workers. *T. diversifolia* mulch decayed rapidly, forming a thick layer on top of the treated nests. In contrast, *Miconia* mulch dried and hardened, forming a loose cover. The mulch-treated area varied throughout the experiment in nests covered with *T. diversifolia* mulch, due mainly to the obstruction of the nest entrances located underneath the mulch and the opening of new ones around it. Ant activity declined more than 50% between the fourth and eighth weeks. In most cases, the mound openings were closed by the ants, thus reducing the external area of the nests. However, after the 12th week, the opening of new entrances in the periphery was concomitant with an increase in the surface area of the nests. The observed interruption of nest activity, the closing of some entrances, and the excavation of new ones outside the mulch between the 4th and 12th weeks reflected an internal modification of nest structure. This relocation behavior was observed exclusively in the *T. diversifolia* mulch treatment.

Nest area varied significantly between treatments at the end of the study ($F(10,115)$: 1.7858; $P = 0.0486$). In those treated with *T. diversifolia* mulch, nest area decreased by 40% after the fourth application, reaching a significantly smaller size than control or *Miconia* mulched nests ($F(2,23)$: 6.4740; $P = 0.0059$). However, at the end of the study, the average size reduction of the nests treated with *T. diversifolia* mulch was only 6%. Final area increments of 24% and 47.4% were recorded on unmulched control and *Miconia* sp. mulch treatments, respectively.

The treatments did not affect the number of excavation, foraging, or ventilation mound openings. However, changes in the activities carried out in these openings, such as the simultaneous use of a single mound for removing soil particles and entering cut material, were noted in all treatments.

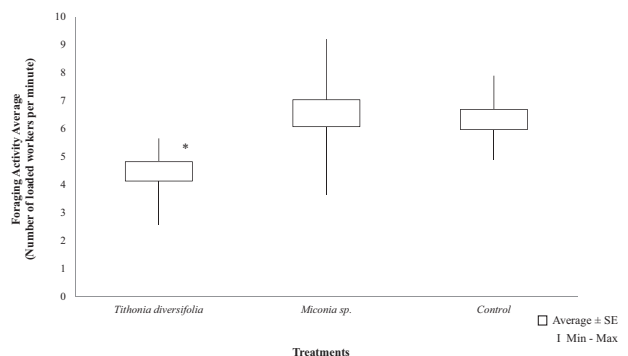


Fig. 1. Average foraging activity of ant workers in the three treatments. For each treatment, box limits show standard error and bars show minimum and maximum values. *Tukey's test: $P < 0.05$.

Foraging activity showed significant differences between treatments ($F(2,24)$: 7.2992; $P = 0.0033$) (Fig. 1) and throughout the experiment ($F(28,672)$: 1.5531; $P = 0.0352$). Ant foraging declined significantly in the *T. diversifolia* mulch nests (less than two loaded workers per minute) between the fourth and eighth weeks of the study. In contrast, excavation activity showed no significant differences between treatments ($F(2,24)$: 0.5041; $P = 0.6103$) or weeks ($F(28,672)$: 0.5856 $P = 0.9576$) even though a peak in excavation activity was observed in *T. diversifolia* mulch nests between the fourth and ninth weeks.

No statistically significant differences in soil microporosity were found between ant nests and the surrounding soil or at different depths. However, macroporosity was significantly higher in nest soil between 0 and 10 cm ($t_{(9)}$: -3.3278; $P = 0.0235$) and 10 to 20 cm depth ($t_{(9)}$: -2.9080; $P = 0.0173$) compared with surrounding soil; no differences were observed between 20 and 30 cm ($t_{(9)}$: -0.6095; $P = 0.5557$). Soil pH increased significantly after all applications on the surface of the *T. diversifolia* mulch ($t_{(3)}$: -3.3142; $P = 0.0452$) and *Miconia* sp. mulch ($t_{(3)}$: -4.5033; $P = 0.0204$) nests. However, differences disappeared at 20 and 30 cm depths. Unmulched control nests showed no significant variations in pH (Table 1). Microbial activity increased by 84% ($t_{(3)}$: -3.3379; $P = 0.0444$) on the surface of the *T. diversifolia* mulch nests and decreased by 22% and 8% at 20 and 30 cm, respectively. In the *Miconia* sp. mulch and unmulched control nests, microbial activity decreased at all depths sampled and was significantly lower at 10–20 cm ($t_{(3)}$: 6.2316; $P = 0.008$) in the *Miconia* sp. mulch treatment (Table 1).

During nest excavation, chambers were found to be empty or with soil and symbiotic fungus in all treatments (Table 2). There were no significant differences between treatments in the number of chambers; however, nests covered with *T. diversifolia* mulch had a higher percentage of empty chambers (Fig. 2). At the end of the study, the most superficial chambers containing symbiotic fungus had a significantly deeper location in the *T. diversifolia* mulch nests ($t_{(16)}$: 2.6836; $P = 0.0163$) (Fig. 3). However, in the *T. diversifolia* mulch nests, the culture chambers excavated on soil that had not been covered with the green manure were found at a similar depth to the other treatments.

New entrances excavated in the *T. diversifolia* mulch nests led to new culture chambers; only two empty chambers were found in the expansion area of the nests. The majority (87%) of the empty chambers were located underneath the foliage-covered surface, 76% of them between 0 and 80 cm. These observations, coupled with the presence of new openings, suggest the relocation of the nests from *T. diversifolia* mulch to mulch-free areas. This behavior was not observed in the other treatments.

Discussion

In this study, *T. diversifolia* mulch decomposed forming a dense layer with effects on the studied nests of *A. cephalotes*. It also induced a

partial or total reduction of foraging activity. Additionally, ants relocated the fungus chambers to areas not affected by the mulch. In some instances, we observed the complete abandonment of the nest areas that had been covered with this plant. Soil pH and microbial activity increased in the nests covered with *T. diversifolia* mulch, whereas soil porosity remained unchanged. We propose that changes in soil chemistry altered the microhabitat inducing the ants to modify nest structure and the workers to reduce their activity outside of the nests. Other studies have shown effects of plant mulches on ants. Meissner and Silverman (2001) observed that the mulch of *Juniperus virginiana* L. (Cupressales: Cupressaceae) is toxic to the ants *Tapinoma sessile* Say and *Linepithema humile* Mayr (Hymenoptera: Formicidae) under laboratory conditions and deters field colonization of the latter species (Meissner and Silverman 2003). Pullaro et al. (2006) assessed *Brassica oleracea* L. (Brassicales: Brassicaceae) and *Capsicum annuum* L. (Solanales: Solanaceae) plantations covered with mulch of *Vicia sativa* L. (Fabales: Fabaceae) and *Secale cereale* L. (Poales: Poaceae) and observed a higher predation of weed seeds and insect pests by *Solenopsis invicta* Buren (Hymenoptera: Formicidae) ant than in control lots (covered by plastic).

Aluminium, nitrogen, phosphorus, calcium, and magnesium released during the decomposition of *T. diversifolia* biomass (Jama et al. 2000, Kwabiah et al. 2003, Partey et al. 2011) can alter soil acidity (Frouz et al. 2003), likely increasing soil pH underneath the green manure (Ikerra et al. 2006). Boaretto et al. (1999) and Loeck et al. (2004) have shown that the elevation of pH can affect the fungal symbionts of different ant species. The internal modification of the *T. diversifolia*-treated nests in our study was probably related to higher soil pH and microbial activity, which induced the workers to build new chambers and tunnels, resulting in the observed reduction of the external activity of ant workers. This lower foraging activity in *T. diversifolia* mulch nests suggests a negative effect of the treatment on ant colonies, a pattern comparable to those observed in studies of *Atta* spp. control with *Metarhizium anisopliae* and *Trichoderma viride* (López and Orduz 2003); compost made with animal manure, plant residues, molasses, yeast, and agricultural lime (Chaves 2006); grain formulations incorporating plant and fungi extracts (Herrera-Salazar 2009); and the control of *Acromyrmex* spp. with homeopathic preparations (Giesel et al. 2012).

Leaf-cutting ants display activity and behavioral changes when control measures are applied in their nests. In our study, ants reacted to disturbance by opening mound entrances outside the mulch-covered area. The same behavior was observed in partially excavated nests (Montoya-Correa et al. 2007) and those covered with organic compost (Chaves 2006). However, the opening of new entrances around the mulch could also be a reaction to the blocking of communication with the exterior. Different studies have shown that ant nests exchange gases through the entrances (Roces and Kleineidam 2000, Kleineidam et al. 2001, Bollazzi et al. 2012), and as the *T. diversifolia* mulch formed a

Table 1. Physicochemical properties of nests under three treatments

Treatment	0–10 cm (depth)		10–20 cm (depth)		20–30 cm (depth)	
	Microbial activity (kg C-CO ₂ ha ⁻¹ d ⁻¹)	pH	Microbial activity (kg C-CO ₂ ha ⁻¹ d ⁻¹)	pH	Microbial activity (kg C-CO ₂ ha ⁻¹ d ⁻¹)	pH
TdM						
0 mo	9.90 ± 1.40*	4.28 ± 0.12 *	11.28 ± 2.48	4.45 ± 0.08	10.67 ± 1.77	4.37 ± 0.12
6 mo	18.20 ± 1.16*	4.80 ± 0.15 *	8.69 ± 0.57	4.66 ± 0.11	9.72 ± 1.27	4.49 ± 0.36
MM						
0 mo	13.12 ± 1.92	4.78 ± 0.21	21.99 ± 1.61 *	4.71 ± 0.24	16.74 ± 1.97	4.65 ± 0.09
6 mo	12.72 ± 2.14	4.88 ± 0.16	17.34 ± 1.24 *	4.90 ± 0.15	14.59 ± 2.11	4.71 ± 0.16
UC						
0 mo	14.37 ± 1.86	4.67 ± 0.20	12.78 ± 2.13	4.79 ± 0.21	14.54 ± 1.66	4.87 ± 0.28
6 mo	12.05 ± 3.83	4.99 ± 0.14	9.81 ± 2.27	4.68 ± 0.07	12.93 ± 2.58	4.93 ± 0.31

TdM, *T. diversifolia*; MM, *Miconia*; UC, control (mean values ± standard error; four samples per treatment).

*Significant differences between 0 and 6 mo ($P < 0.05$).

Table 2. Number and state of internal chambers in ant nests with different treatments (TdM, Mulche of *T. diversifolia*; MM, Mulche of *Miconia* sp.; UC, unmulched control)

Treatment	Nest	Empty chambers	Chambers with soil	Symbiotic fungus culture chambers	Number of chambers	Volume of symbiotic fungus (l)
TdM	3	10	0	27	37	124
	5	5	2	13	20	67
	8	9	5	41	55	191
	10	55	5	201	261	897
	12	20	0	0	20	0
	20	4	0	24	28	71
	23	3	0	121	124	291
	25	5	4	27	36	75
	30	6	4	2	12	1
		Total	117	20	456	593
	Average (%)	19.74	3.37	76.89	100	
MM	1	3	3	7	13	17
	9	5	0	77	82	148
	13	2	0	144	146	283
	14	16	6	7	29	19
	16	2	0	13	15	41
	19	5	0	5	10	8
	22	1	5	9	15	12
	26	2	0	6	8	11
	Total	36	14	268	318	539
	Average (%)	11.33	4.40	84.27	100	
UC	4	15	4	139	158	339
	6	4	0	8	12	26
	11	4	0	54	58	178
	15	5	8	16	29	76
	17	0	0	7	7	18
	18	3	0	5	8	13
	21	6	7	11	24	50
	24	2	2	29	33	96
	29	0	2	17	19	23
		Total	39	23	286	348
	Average (%)	11.20	6.61	82.19	100	

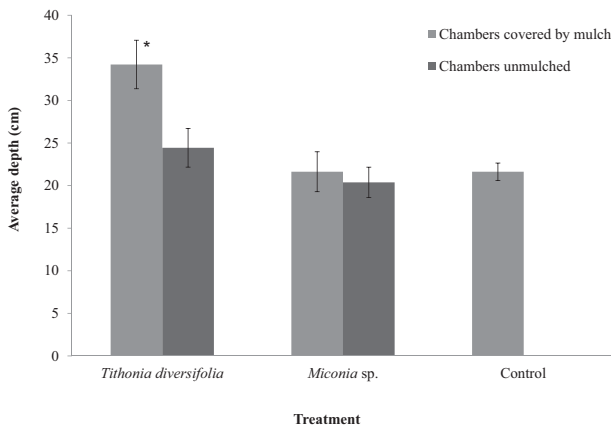


Fig. 2. Distribution (%) of chamber contents inside the excavated nests. Small fragments of the symbiotic fungus were found in some of the empty chambers of *T. diversifolia* mulch nests.

thick layer on top of the treated nests, it might have affected nest ventilation. More studies are needed to determine whether the blocking of entrances triggers the modification of nest structure (Jonkman 1980, Horstmann and Schmid 1986, Bollazzi and Roces 2007).

Nearly 150 chemical compounds have been found in *T. diversifolia* including sesquiterpenes, lactones, diterpenes, and flavonoids (Chagas-Paula et al. 2012). With so many secondary metabolites, it is not surprising that this plant has diverse applications in medicine and agricultural production. Some of its documented effects are the inhibition of *Sarcoptes scabiei* De Geer (Astigmata: Sarcoptidae) parasitism on

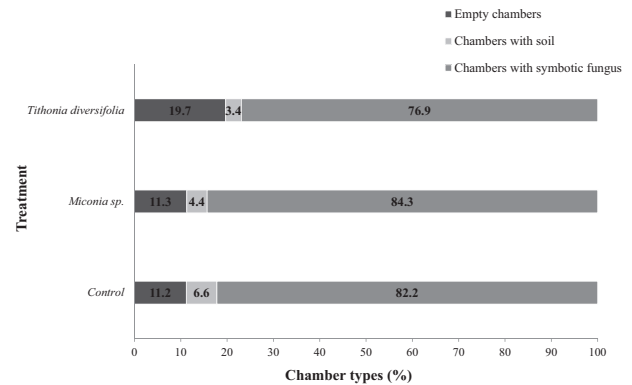


Fig. 3. Average depth of the most superficial fungus culture chambers. Bars show standard error. ($t_{(16)}: 2.6836; P = 0.0163$). The largest depth was recorded under the area initially covered by the mulch that remained covered throughout the study.

rabbits (Thu Hang et al. 2012), a phago-deterrent activity in the whitefly *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) (Bagnarello et al. 2009), a repellent effect on mosquitoes (Oyewole et al. 2008), and insecticidal properties on termites (Adoyo et al. 1997) and *Callosobrochus maculatus* F. (Coleoptera: Bruchidae) (Adedire and Akinneye 2003, Kolawole et al. 2011).

In Colombia, Giraldo (2005) found reduced herbivory of *A. cephalotes* on *M. quadrangularis* when young trees were planted together and fertilized with green *T. diversifolia* foliage. In addition, Valderrama et al. (2009) found an antifungal effect of the Mexican sunflower on the symbiotic fungus *L. gongylophorus* associated with *A. cephalotes*.

Moreover, Castaño et al. (2013) showed an insecticidal effect of this plant on leaf-cutting ant workers reared in the laboratory. Recent research has shown that leaf-cutting ants have symbiotic associations with specialized microorganisms, mostly bacteria and yeasts, which contribute to the proper functioning of the colonies and are involved in ant protection and decomposition of plant biomass (Santos et al. 2004, Rodrigues et al. 2005, Abril and Bucher 2007, Rodrigues et al. 2009, Rengifo-Ruiz 2012, Ortiz 2012). For this reason, the negative effect of Mexican sunflower mulch on *A. cephalotes* nests and the emergence of culture chambers outside the central conglomerate may be related to the antifungal, bactericidal, or insecticidal properties of this plant, all of which can threaten the stability and functioning of *A. cephalotes* colonies by disrupting the growth of the fungus, the symbiotic microorganisms and the ant workers.

In summary, the degradation of *T. diversifolia* green mulch induced the ants to relocate the symbiotic fungus into new chambers in response to the stressful conditions of the nest interior. External activity of the colonies declined 2 mo after mulch applications, as the ants built new chambers and tunnels inside the nests. The use of mulch is an economic and practical method that does not require specialized equipment. *T. diversifolia* can be planted close to ant nests and can be used by farmers as a live fence, fodder bank, or soil enhancer in alley cropping systems (Ikerra et al. 2006, Partey et al. 2011). Additionally, some studies report positive effects of *Tithonia* mulch on soil fertility and crop production; soils fallowed with Mexican sunflower have higher organic matter (Agbede and Afolabi 2014), and the mulch *Tithonia* improves growth and development of beans (*Phaseolus vulgaris* L.) (Mustonen et al. 2014) and white yam *Dioscorea rotundata* Poir (Agbede et al. 2014). In turn, the Mexican sunflower increases the susceptibility of nests to complementary pest control measures. To halt nest expansion, we recommend supplementing the application of *T. diversifolia* mulch with other control measures, as soon as nest excavation and other external activities start to decline. More research is needed to understand how microenvironmental and physicochemical conditions inside the nests, especially around the culture chambers, affect the development of the symbiotic fungus. It is also important to understand how the modifications made by the ant workers in the nests counteract the effects of the control measures.

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