HOST PLANT SELECTION BY AN ATTINE ANT

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SUMMARY

Although leafcutting (*Atta*) are highly polyphagous insects, many plant species readily available to the ants in nature escape ant attack nearly completely. It is reasonable to suppose that at least some of these plant species are avoided because they are chemically defended against the ants, containing substances toxic to the ants, to their food fungus, or to both. We have been studying the host plant preferences of *Atta cephalotes* in the laboratory and in nature in Santa Rosa National Park, Costa Rica. We report here on the development and use of a rapid bioassay for testing ant preferences for leaves in the field. Our results show that the ants collectively exhibit a continuously graded response when presented with a simultaneous choice of leaves from a large number of species. The results also indicate that the ants are capable of making very subtle distinctions in leaf quality, not only between species, but also between individual plants within species, and even between leaves from different parts of the same plant. We also describe a laboratory bioassay for testing the repellency of chemical extracts of plant species most often avoided by the ants. Using this laboratory bioassay, we have isolated specific ant repellents from several plant species from Santa Rosa. The first of these repellents to be completely characterized, caryophyllene exposide, has proven to be a potent fungicide, lethal in small doses to the ant's fungus.

RESUMEN

Selección de la planta huésped por una hormiga Attine

Aunque las hormigas cortadoras de hojas (*Atta*) son insectos polífagos, muchas especies de plantas facilmente disponible para ellas en el campo escapan a su ataque. Es razonable presumir que al menos unas de estas especies pueden ser evitadas por las hormigas en razón de tener algún tipo de protección química que puede ser tóxica tanto para las hormigas, como para el hongo que ellas cultivan, o para los dos.

Hemos estudiado las preferencia de *Atta cephalotes* en cuanto a la planta huésped en el laboratorio (nido en catividad) y en el campo (Parque Nacional de Santa Rosa, Costa Rica). Se relata aquí el desarrollo y uso de un rápido bioensayo para probar las preferencias de las hormigas en el campo. Los resultados indican que las hormigas tienen colectivamente una repuesta continuamente graduada cuando son presentadas con una selección simultánea de hojas de muchas especies. Los resultados del bioensayo también muestran que las hormigas tienen la capacidad de hacer distinciones muy sútiles en la calidad de las hojas, no sólo entre especies, sino también entre plantas individuales a dentro de las especies y también aún entre hojas de diferentes partes de la misma planta. Se describe además un bioensayo en el laboratorio para probar la repelencia de extractor químicos de especies de plantas normalmente evitadas. Usando este bioensayo en el laboratorio, hemos aislado, de algunas especies de plantas de Santa Rosa, repelentes específicos contra las hormigas. El primero de estos repelentes caracterizado, el epóxido de cariofilene, es un fungicida potente, letal en dosis muy pequeñas, para el hongo de las hormigas.

INTRODUCTION

Leafcutting ants of the genus *Atta* (Hymenoptera, Formicidae, Attini) are highly polyphagous herbivores, attacking a large number of plant species in a diverse array of plant families. Adult ants feed primarily on plant sap, taken while they cut and mulch leaves (Littledyke and Cherrett, 1976 ; Quinlan and Cherrett, 1979), and supplement their diet with fungus. Larvae feed exclusively on hyphal juice from a specific fungus, *Rhozites gongylophora*, cultivated in underground gardens on the collected leaves (Weber, 1972).

The ant-fungus mutualism has made attines among the most successful and abundant insect herbivores in the neotropics, elevating them to pest status in many areas. The fungus makes available a broader selection of potential host plant species by transforming plant material that would otherwise be indigestible, toxic, or simply unpalatable (Boyd and Martin, 1975a, b). A number of fungal digestive enzymes pass unaffected through the ant gut and are defecated onto the fungus garden, thereby hastening the penetration of fungal hyphae into the leaf tissue. The enzymes function in the ant gut as well ; and Martin (1979) has recently suggested that attines and many other fungus-eating insects are primarily mycophagous to acquire digestive enzymes which the insects are unable to make themselves.

The fungus may also be a superior diet than plant sap for the larvae, which require better nutrition for growth than is required for maintenance of adult ants. Fungi have the capacity to concentrate biologically important elements such as nitrogen and phosphorus from extremely dilute sources (cf. Martin, 1979) ; and leaves and other plant tissues are frequently lower in nitrogen content than fungi (Mattson, 1980). Whether *Rhozites* has higher available protein N than the bulk of the leaf material collected by *Atta* is not presently known, however.

Although the ants are highly polyphagous, it has been known for some time that the ants rarely or never cut many plant species they encounter in nature (e.g., Cherrett, 1968 ; Rockwood, 1975, 1976). A number of factors have been suggested as important in the decision by leafcutting ants to cut or reject a particular set of leaves. These include : (1) secondary compounds which are toxic to the ants, their fungus, or both ; (2) secondary compounds which possibly reduce the digestibility of the plant material by the ant or its fungus, such as tannins (Feeny, 1970) ; (3) the nutritional value, including usable protein, carbohydrate, and lipid constituents, as well as trace essential substances such as steroids (Waller, pers. comm.) ; (4) mechanical defenses of the plant, including leaf toughness, trichome density, and gluey or copious sap, especially latex saps (Stradling, 1978) ; and (5) the moisture content of leaves (Janzen, Bowers, pers. comm.). In addition, a particular colony of ants may not cut an otherwise acceptible plant species because of : (1) the availability of superior host plant species (Hubbell and Rockwood, 1983) ; (2)history effects such as where trail systems develop and ant experience with some plant species and not others ; (3) retrieval costs which are too great (e.g., plants are too distant) ; (4) competition from rival colonies in adjacent territories ; and (5) the presence of aggressive ant species (Cherrett, this volume).

We have been studying the natural foraging patterns of *Atta cephalotes* in semi-decidous forest in Santa Rosa National Park, Costa Rica (Hubbell and Rockwood, 1983 ; Hubbell, Stevens, and Wilson, unpubl.). A year-long study of foraging by 20 colonies revealed strong seasonality in cutting patterns, confirming the findings of Rockwood (1975, 1976). Two pronounced patterns attracted our attention. First, colonies exhibited graded cutting responses, such that the amount of leaf material harvested was lognormally distributed over plant species ; and it could be shown that the lognormal was not derived from plant abundances. Hubbell and Rockwood (1983) proposed that the ants have built-in graded responses to leaves based on their relative suitability as substrate for growth of their fungus.

The second pronounced pattern was the tendency for the ants to attack plants flushing new leaves, a pattern seen in both evergreen as well as deciduous species. Preference for new leaves in leafcutting ants has been reported several times (e.g., Cherrett, 1972a ; Rockwood, 1975 ; Littledyke and Cherrett, 1978), but there has been little work on the causes of this preference. Cherrett (1972b) has suggested that old leaves might accumulate ant repellent substances ; subsequently, Littledyke and Cherrett (1978) showed that lipid extracts of old leaves were more repellent than extracts of young leaves in two tropical and four temperate species.

We have begun studying why the mature leaves of many plant species at Santa Rosa are completely or nearly completely avoided by *Atta cephalotes*. Initially, we have limited our efforts to determining more precisely which species and individual plants are repellent to the ants, and when they are repellent seasonally ; and to isolating and identifying chemically extractable ant repellents if they are present, using a laboratory bioassay to guide the isolation procedure. In this paper we report on the development of a rapid bioassay of leaf preferences for use in the field, and on some results of its application in studying seasonal changes in leaf preferences in Santa Rosa *A. cephalotes* colonies. We then describe the laboratory bioassay and our initial progress in characterizing ant repellents from several species avoided by the ants.

We organize our discussion around a consideration of the following hypotheses : (1) Leafcutter ants will exhibit continuously graded leaf preferences when a large number of species is presented to them at one time for choice ; (2) Colonies will show marked seasonal changes in leaf preferences among the same set of plant species ; (3) Within plant species, the ants will prefer leaves of some individuals over others ; (4) Within individual plants, the ants will prefer some leaves over others, especially new leaves over old, and sun leaves over shade leaves ; (5) Ants will prefer species with high nitrogen content over species with low nitrogen content ; (6) Mature leaves of at least some of the avoided species are not cut by the ants because they contain chemicals repellent and/or toxic to the ants or their fungus ; and (7) Both polar (e.g., phenolics) and nonpolar (e.g., terpenoids) compounds will be isolated as potent ant repellents.

PROCEDURE

Field leaf preference tests

Leaf preference tests were carried out in Santa Rosa National Park, Guanacaste Province, Costa Rica, on colonies of *Atta cephalotes* studied previously (Hubbell and Rockwood, 1981 ; Hubbell, Stevens, and Wilson, unpublished). Tests were made on colony 5, 16 in July, 1980, and on colonies 5, 16, 6, 20, and 7, 22 in October, 1980, months which represent early-middle and late wet season, respectively. In the main set of preference tests, the ants were given a simultaneous choice of 20 species in 18 plants families (Table I), selected at random from 150 tree and shrub species known to occur in the forest. All 20 species were represented by leaves taken from a single, known individual, and the same tree or shrub in both July and October as well. An attempt was made to pick only mature, sunlit leaves for the choice tests.

Table I – Primary group of 20 species, and family ; secondary group of species, and family

Tabla I – Grupo primario de especies, y familia ; grupo secundario de especies, y familia

Primary set of 20 species	Family	Second set of species	Family
Allophylus occidentalis	Sapindaceae	*Acacia collinsii*	Mimosaceae
Annona purpurea	Annonaceae	*Alibertia edulis*	Rubiaceae
Bombacopsis quinata *	Bombacaceae	*Astronium graviolens*	Anacardiaceae
Bursera simaruba	Burseraceae	*Bombacopsis quinata* *	Bombacaceae
Calycophyllum candidissimun	Rubiaceae	*Casearia sylvestris*	Flacourtiaceae
Chlorophora tinctoria	Moraceae	*Cedrela odorata*	Meliaceae
Chomelia spinosa	Rubiaceae	*Cordia alliodora* *	Boraginaceae
Cordia alliodora *	Boraginaceae	*Euphorbia schlectendahlii*	Euphorbiaceae
Exostema mexicanum	Rubiaceae	*Ficus* sp.	Moraceae
Hemiangium excelsum	Celastraceae	*Genipa americana*	Rubiaceae
Hymenaea courbaril *	Papillionaceae	*Hymenaea courbaril* *	Papillionaceae
Licania arborea	Chrysobalanaceae	*Luehea speciosa* *	Tiliaceae
Luehea speciosa *	Tiliaceae	*Muntingia calabura*	Elaeocarpaceae
Malvaviscus arboreus	Malvaceae	*Quercus oleiodes*	Fagaceae
Manilkara zapota	Sapotaceae	*Randia lasianthea*	Rubiaceae
Ocotea veraguensis	Lauraceae	*Sapium thelocarpum*	Euphorbiaceae
Piper amalgo	Piperaceae	*Spondias mombin*	Anacardiaceae
Simarouba glauca	Simaroubaceae	*Tabebuia chrysantha*	Bignoniaceae
Spondias purpurea	Anacardiaceae	*Trichilia colimana*	Meliaceae
Trichilia cuneata	Meliaceae	*Verbesina gigantea*	Compositae
		Zanthoxylum setulosum	Rutaceae

* Species in common between the two lists

Uniform 38 m^2 leaf disks were punched from the leaves of each species. All species were offered simultaneously to the ants, one disk per species, along active foraging trails to the colony, and in a way that all disks would be encountered frequently. As soon as any disk was picked up and carried into the nest, it was recorded and replaced by a new disk of the same species. At the end of 1 to 2 hours, the number of disks of each species taken by the ants was tallied. To eliminate possible position effects, the locations of species in the line of disks was randomized every 15 minutes in the July tests ; but this practice was discontinued after no position effects could be detected. It was necessary to do these tests on trails with a relatively controlled trail activity level. In initial experiments, we discovered an inverse relationship between number of species of leaf disks picked up and the number of laden ants returning to the nest with leaf fragments (Fig. 1). It appears that workers on very active trails are already committed to particular leaf resources and are less likely to pick up the leaf disks of less preferred species. We were able to control trail activity in our choice experiments to between 150 and 250 laden ants per half hour.

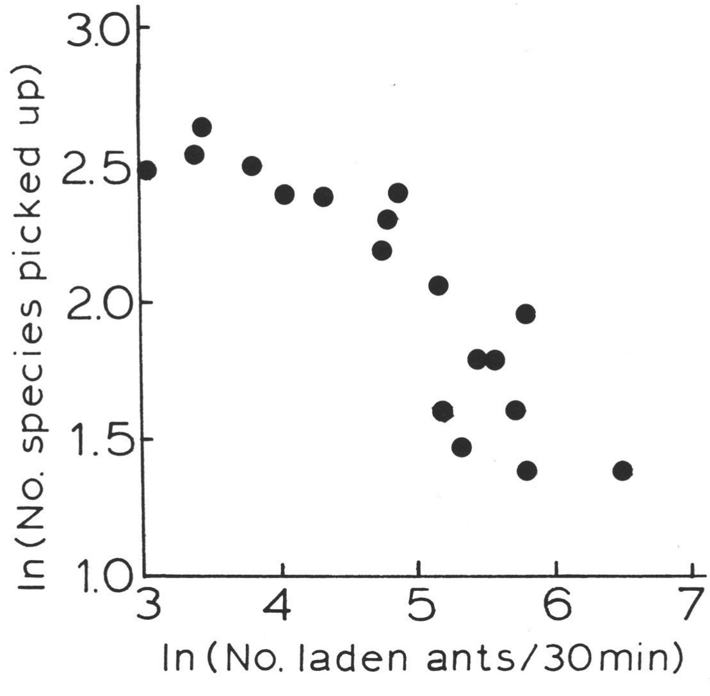


Fig. 1 – Inverse relationship between the number of species of leaf disks picked up by the ants in a given preference test, and the number of laden ants returning to the nest already carrying a leaf fragment. The axes are log transformed to reduce the graph scale.

Fig. 1 – Relación inversa entre el número de tipos de discos de hojas tomado por las hormigas en una prueba de preferencias, y el número de hormigas que regresan al nido trayendo un pedazo de hoja. Los ejes son transformados logaritmicamente para reducir la escala de la gráfica.

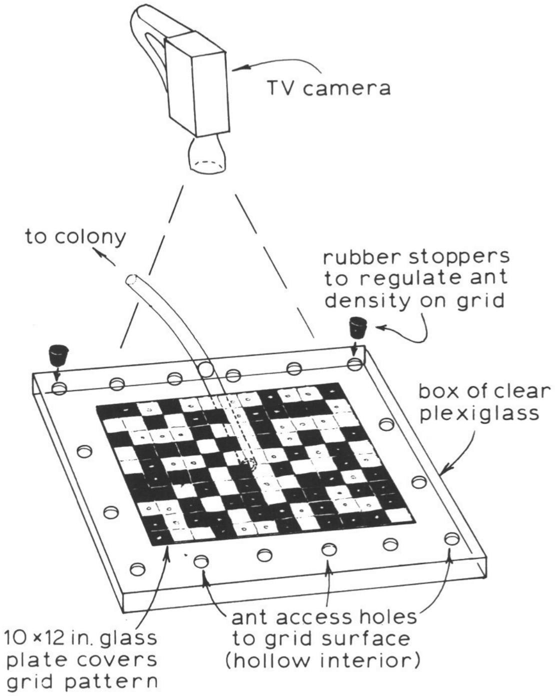
In October an additional 17 species were tested in combination with 4 species from the original list of 20 for standardization (Table I). We also tested for intraspecific and intra-plant variability in leaf palatability in 3 species in October. Comparisons were made between young versus old, and sun versus shade leaves in all combinations.

Laboratory biossay

Leaf extracts were tested for repellency in choice tests conducted on a laboratory *Atta cephalotes* colony. The colony consists of several hundred thousand workers and about 20 liters of fungus, and is maintained on a daily diet of 80 g of frozen lilac leaves. Choice tests were carried out on a foraging platform connected to the colony by a 2.5 cm diameter tube (Fig.1). The platform is a thin, hollow box of plexiglass. Ants arriving from the colony are released into the center of the box and from there disperse in all directions. Ants gain access to the test arena on the top surface via a ring of holes around the perimeter of the platform. The number of ants in the test arena and the direction of ant arrival can conveniently be regulated by opening or closing selected holes with rubber stoppers.

Fig. 2 – Hollow plexiglass foraging platform on which laboratory bioassays of leaf extracts are tested for repellency. The checkerboard pattern represents the computer-generated random grid design for locatinf control and test flakes.

Fig. 2 – Caja de plástico ahuecada usada como plataforma sobre la cual se realizaron los bioensayos en laboratorio para probar la repelencia de los extractos de hojas. El diseño en ajedrez representa el dibujo generado al azar para localizar las hojuelas control y de prueba.



Ants forage for pressed rye flakes, which serve as the carrier material for leaf extracts ; and the removal of flakes by the ants is videotaped (Fig. 2). Flakes are soaked in solvent (CH_2Cl_2) alone («control flakes») or in solvent plus leaf extract («test flakes») for 1 minute and air dried. Sixty control and 60 test flakes are then arranged according to a computer-generated random design in a 10x12 grid pattern. The printed design, which is legible on the TV monitor, is placed directly on the foraging platform and covered by a glass plate onto which the flakes are placed. A fresh plate, cleaned with acetone to prevent possible carryover of pheromone trails between trials, is used for each test. Once the flakes are in place, the ants are allowed onto the top of the platform (while setting up, all access holes are closed). During a run, the time is announced every minute and recorded on the audio channel of the videotape. On playback, the tape is stopped every minute for stop-action analysis of the number of remaining test and control flakes.

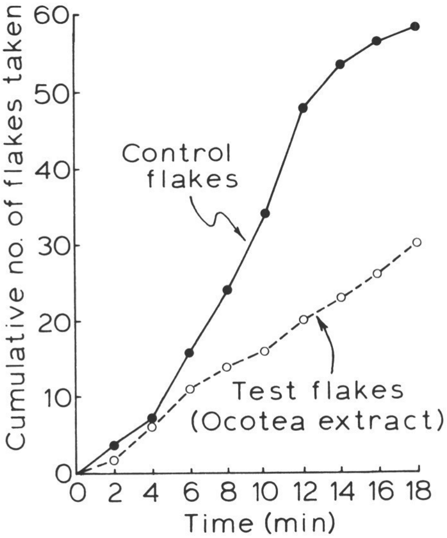


Fig. 3 – Typical bioassay test run results, in this case for the crude chloroform extract of *Ocotea veraguensis* leaves. There are 60 control and 60 test flakes containing the *Ocotea* extract. The difference is statistically significant at $p < 0.001$.

Fig. 3 – Resultados típicos de un bioensayo, en este caso para el extracto crudo en cloroformo de hojas de *Ocotea veraguensis* (60 huejelas control y 60 de prueba). La diferencia es estadisticamente significativa para $p < 0.001$.

Figure 3 shows the result of a typical bioassay, in this case of the crude chloroform extract of nature *Ocotea veraguensis* leaves. In this illustrative run, we continued the analysis until all of the control flakes had been removed (18 minutes). However, in general we need only analyze the tapes to the point at which half of the control or test flakes have been removed, whichever comes first. We then test the significance of the difference in number of control and test flakes removed using a modified binomial test. Thus, if 30 control flakes have been removed (half), and if the null hypothesis is that test flakes are no more repellent than control flakes, then we can reject the null hypothesis if the number of test flakes is : less than 23 ($p < 0.05$), less than 10 ($p < 0.01$), or less than 17 ($p < 0.001$), for a one-tailed test. Thus, the *Ocotea* chloroform extract is significantly more repellent than the control at $p < 0.001$. We have found that this method of bioassay gives very reliable and repeatable results. It is also very rapid, making feasible the analisys of large numbers of samples, such as are easily generated by column chromatography of a chemically complex leaf extract.

General chemical procedures

Although the late stages of isolation and characterization of ant repellents differ depending on the nature, novelty, and chemical complexity of the substances involved, the initial stages of extraction and partial separation do not change markedly. Using large capacity Soxhlet extractors, we extracted 100 g samples of leaves in chloroform to obtain the lipid-soluble, nonpolar substances, and in ethanol to obtain the polar substances. The solvent are evaporated, and the extracted material is weighed and then redissolved in a much smaller volume of solvent (usually methylene chloride). Samples of the redissolved extract are then bioassayed for repellency. Typically, one but not both fractions is active (more often the chloroform fraction). The remaining sample is washed with 1.0 HCl to remove alkaloids, and 1.0 N NaOH to remove acids. These acid-soluble, neutral, and base-soluble fractions are then bioassayed. One or more of these fractions may be active. Each active fraction is further fractionated by column chromatography using gradient elution procedures. A series of 20 or more fractions is then obtained. These can often be reduced to a smaller set of samples by combining fractions judjed similar on the basis of thin layer and gas chromatographic analysis. Each of these fractions is then bioassayed individually for repellency. At this stage of separation, the activity is usually localized in one or at most a few samples, each of which contains a small number of compounds. The fractionation-bioassay cycle is repeated until pure compounds are isolated.

Thereafter we attempt to determine the structure of the ant repellent compounds using standard physical and chemical methods, including combined gas chromatography-mass spectrometry (GC-MS), proton and carbon nuclear magnetic resonance spectroscopy (NMR), and infrared (IR) and ultraviolet (UV) spectroscopy as needed. If spectral data do not provide sufficient information to characterize a compound, further structural information is sought through simple chemical transformations or degradative experiments. Reactions which can be conducted on microgram samples, such as ozonolysis, catalytic hydrogenation, acylation, oxidation and reduction, etc., often can confirm the presence of functional groups suggested but not proved by spectroscopic data. In certain cases, it is possible to prove a proposed structure by direct comparison with an authentic sample of the compound, or by partial synthesis from a known natural product.

It should be emphasized that, because there are thousands of compounds in a typical leaf, the bioassay for repellency to the leafcutting ants is critical to the success of each step in the isolation procedure. By the same token, we are assured by the bioassay that every compound so isolated will possess ant repellent properties. Once the compound or compounds are identified, it must still be demonstrated that naturally occuring concentrations of these compounds are repellent to the ants since it is expected that their degree of repellency will be concentration-dependent. It is therefore necessary to estimate the natural concentration of these compounds in the leaf.

Finally, we performed an elemental nitrogen and carbon analysis on leaf tissue samples from the same batches of leaves used in the field leaf preference tests. Assuming that total leaf nitrogen is correlated with total leaf protein as a first order approximation, we can make a preliminary test of the hypothesis that the ants prefer plant species with high-N leaves.

RESULTS

Hypothesis 1 was confirmed : the ants did exhibit continuously graded preferences when presented with a large number of species to choose among. Figure 4 shows the pickup responses of ants from colony 5,16 to leaf disks of 37 plant species in 26 families. Of special interest is that there is a nearly

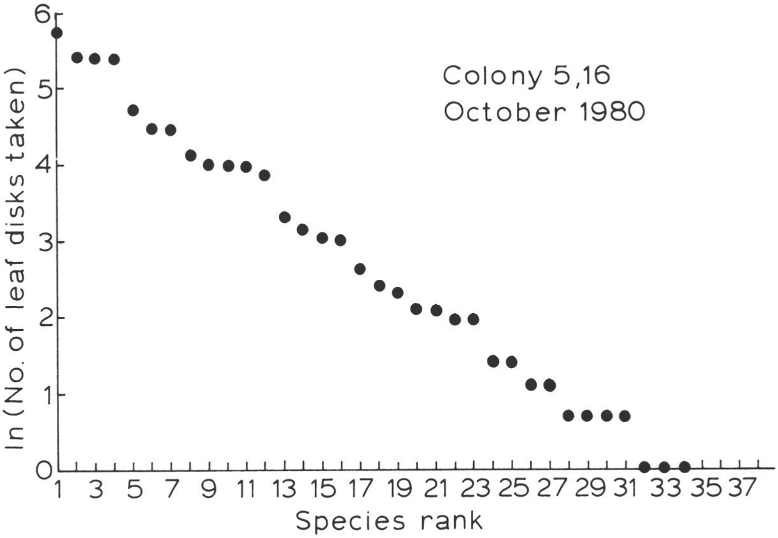


Fig. 4 – Geometric progression of number of leaf disks picked up by colony 5,16 October 1980. Species are ordered in rank from most picked up on the left to least picked up on the right. Species 35-37 were not picked up at all.

Fig. 4 – Progesión geométrica del número de discos de hojas recolectados por la colonia 5,16 en octubre de 1980. Las especies son dispuestas en orden decreciente según como fueron preferidas para la recolección de izquierda a derecha). Ningún disco de las especies 35-37 fué escogido.

perfect geometric series in the relationship between the number of leaf disks taken by the ants and the rank of the species, ordered from most (rank 1) to least (rank 37) preferred. These results show that it is not possible to make a simple partition of plant species into those which are acceptable and those which are unacceptable to the ants, and suggest that there are multiple factors influencing the decision of individual workers to pick up or leave a particular leaf disk.

Hypothesis 2 was partially confirmed : approximately half (11) of the 20 principal test species showed significant seasonal change in acceptability to the ants between July and October (Table 2). Most of the preferred species showed significant seasonal changes in acceptability. A particularly striking case was *Simarouba glauca* which, although almost completely avoided in July, became the fourth most preferred species in October. As a qualitative result, however, most of the preferred species in July remained preferred in October, in spite of quantitative shifts in relative palatabilities. The three preference-test replicates performed on colony 5,16 in October (Table 2) show that, while there is some sample variation, such variation does not obscure the basic pattern of preferences.

Table II – Field leaf preference tests on colony 5,16. Counts are numbers of leaf disks picked up during 1-hour tests in October or July. Three different trails were used for the tests in October, and one trail was used in July. Percentages by species of disks picked up, and binomial contrasts between October and July results, are also shown.

Tabla II – Ensayos de preferencia en el campo sobre la colonia 5,16. Los datos representan el número de discos de hojas colectados durante una hora en octubre o julio. Tres pistas diferentes fueron utilizadas en octubre y una en julio. Son también indicados los porcentajes de discos colectados por especie y los contrastes binomiales entre los resultados de octubre y julio.

		October				July		October - July
Species	1	2	3	Total	%	1	%	Contrasts (p <)
Spondias purpurea	138	96	86	320	36.8	51	23.0	.001
Bombacopsis quinata	84	64	76	224	25.8	71	32.0	.05
Bursera simaruba	66	21	24	111	12.8	15	6.8	.01
Simarouba glauca	60	13	14	87	10.0	1	0.5	.0001
Hymenaea courbaril	30	8	15	53	6.1	20	9.0	n.s.
Licania arborea	11	0	3	14	1.6	6	2.7	n.s.
Chlrophora tinctoria	9	14	0	23	2.6	13	5.9	.05
Calycophyllum candidissimum	7	1	0	8	0.9	4	1.8	n.s.
Allophylus occidentalis	6	1	3	10	1.2	2	0.9	n.s.
Exostema mexicanum	5	0	2	7	0.8	1	0.5	n.s.
Hemiangium excelsum	2	0	1	3	0.4	4	1.8	.05
Chomelia spinosa	2	0	0	2	0.2	4	1.8	.01
Annona reticulata	2	0	0	2	0.2	10	4.5	.001
Trichilia cuneata	1	1	0	2	0.2	2	0.9	n.s.
Cordia alliodora	0	0	0	0	0.0	1	0.4	n.s.
Ocotea veraguensis	0	0	1	1	0.2	0	0.0.	n.s.
Manilkara zapota	0	0	0	0	0.0	7	3.1	.001
Piper amalgo	0	0	0	0	0.0	5	2.2.	.001
Malvaviscus arboreus	0	0	2	2	0.2	2	0.9	n.s.
Luehea speciosa	0	0	1	1	0.1	3	1.3	.01

There is also relatively good agreement between different colonies regarding leaf acceptability. Figure 5 compares the October preferences of 3 colonies for the same set of 20 species and individual plants. The species listed across the bottom are ranked from most to least preferred, using colony 5,16 to generate the rankings. Down the right margin of the graph is the cumulative percent of all leaf disks picked up, from highest to lowest ranked species. The top 6 species accounted for 90 % of the leaf disks taken (species above the dotted line) ; and there is nearly perfect agreement between the colonies on the ranking of these species. For the species having preferences below the dotted line, differences between colonies sometimes appear large but for the most part are not statistically significant. Note that these species collectively account for less than 10 % of the leaf disks picked up by the ants.

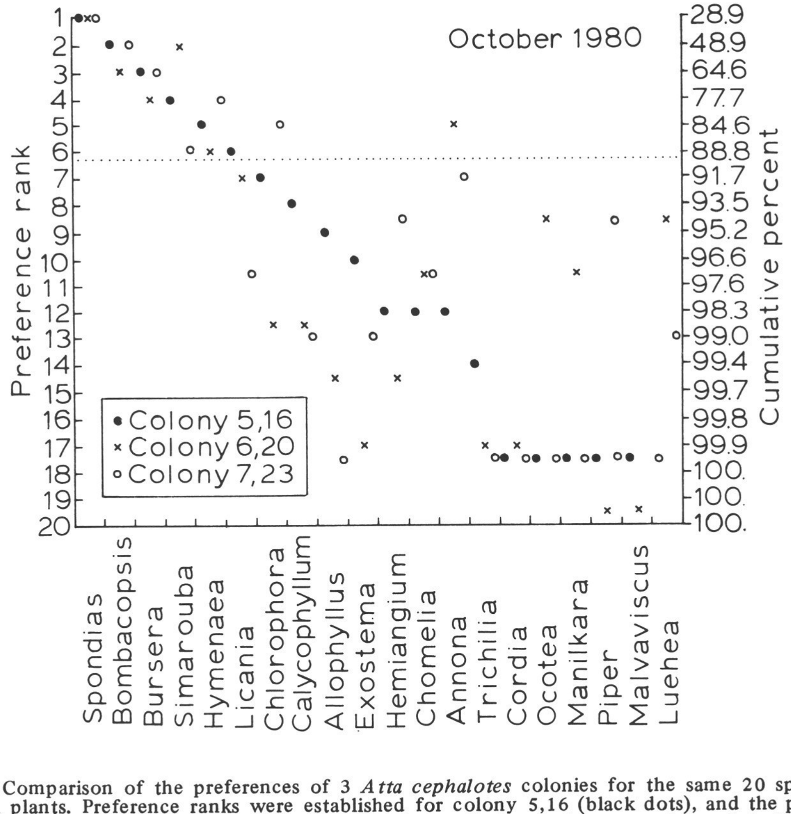


Fig. 5 – Comparison of the preferences of 3 *Atta cephalotes* colonies for the same 20 species and individual plants. Preference ranks were established for colony 5,16 (black dots), and the preference ranks of colonies 6,20 (x's) and 7,23 (open circles) are displayed for comparison of similarities and differences. Along the right margin is shown the cumulative percent of all leaf disks taken, cumulated down from the top-ranked species. Tied preference ranks are shown on the same line.

Fig. 5 – Comparación de las preferencias de 3 colonias de *Atta cephalotes* para las mismas 20 especies o plantas individuales. El grado de preferencia fué establecido para la colonia, 5,16 (puntos negros), y los grados de preferencia de las colonias 6,20 (x's) y 7,23 (circulos abiertos) estan presentados para permitir la comparación de semejanzas y diferencias. A lo largo de la margen derecha se muestran los porcentajes cumulativos de todos los discos de hojas colectados, a partir de la especie de hoja preferida. Los parámetros de preferencia idénticos aparecen en la misma línea.

There are, however, a few significant colony differences for some species (e.g., *Annona purpurea* and *Chlorophora tinctoria*). Since all colonies were offered disks punched from the same leaf samples from the same trees, these differences must be attributed to historical factors, such as the cutting history of the colony. Elsewhere we show that ant preferences can be shifted at least to a modest extent by altering the previous cutting experience of the ants (Hubbell, Stevens, and Wilson, unpubl.).

The preceding results argue that species differences in leaf palatability exist, but it now appears likely that at least some of the observed differences are more of an individual plant effect than a species effect. This conclusion is unavoidable because hypotheses 3 and 4 were also confirmed, namely that the ants can and do discriminate between individual trees, and between leaves from different parts of the same tree. We examined 4 individual > 10 cm in diameter of each of 3 tree species, *Cochlospermum vitifolium* (Cochlospermaceae), *Calycophyllum candidissimum* (Rubiaceae), and *Bursera simaruba* (Burseraceae). The species were chosen because they are known to be attacked by the ants at least for part of the year, and because it was possible to find new and old leaves, and sun and shade leaves, in all combinations on the same tree at one time. Leaf disks of all 4 combinations of young and old, sun and shade leaves of each tree were offered in a simultaneous choice test to the ants. The results for all 3 species were that the leaves of some trees were completely avoided, regardless of the age or light treatment (Table III). If any leaves were taken, in general sunlit leaves were preferred over shade leaves. To

Table III – Preference tests of leaf disks from 4 individuals of 3 tree species. Number of leaf disks picked up in a 1-hour test with simultaneous presentation of each leaf type.

Tabla III – Ensayos de preferencia para discos de hojas de 4 individuos de cada 3 especies de árboles. Número de discos de hojas colectados en 1 hora de bio-ensayo con presentación simultánea de cada tipo de hoja.

Species	Ind.	Young leaves sun	Young leaves shade	Old leaves sun	Old leaves shade	Ind. totals
Cochlospermum	A	0	0	0	0	0
	B	2	0	4	0	6
	C	12	7	5	6	30
	D	0	0	0	0	0
Calycophyllum	A	0	0	0	0	0
	B	3	0	1	0	4
	C	0	1	0	0	1
	D	5	1	2	3	11
Bursera	A	35	4	22	13	74
	B	0	0	0	0	0
	C	2	0	0	0	2
	D	7	7	14	6	34
TOTALS		66	20	48	28	

our surprise, the effect of light treatment appeared to be much stronger than the effect of leaf age.

Hypothesis 5 was not confirmed : the ants do not appear to be selecting leaves which are especially high in nitrogen content. We tested the remainder of the leaf samples which were used in the July field choice tests for total N. Figure 6 shows the lack of relationship between percent nitrogen and the number of leaf disks picked up by ants of colony 5,16 in July. Nitrogen values range from a low of 0,87 % in *Manilkara zapota* to a high of 3,34 % in *Exostema mexicanum*. However, the two species most preferred by the ants, *Spondias purpurea* and *Bombacopsis quinata*, had nitrogen values of 2,30 % and 1,46 %, respectively, right in the middle of the distribution. Although it is still possible that these figures do not accurately reflect the usable protein available to the ants, these results suggest that relative protein content is unlikely to be important in host plant discrimination by these ants. Indeed, a more reasonable hypothesis might be that the ants avoid leaves of either

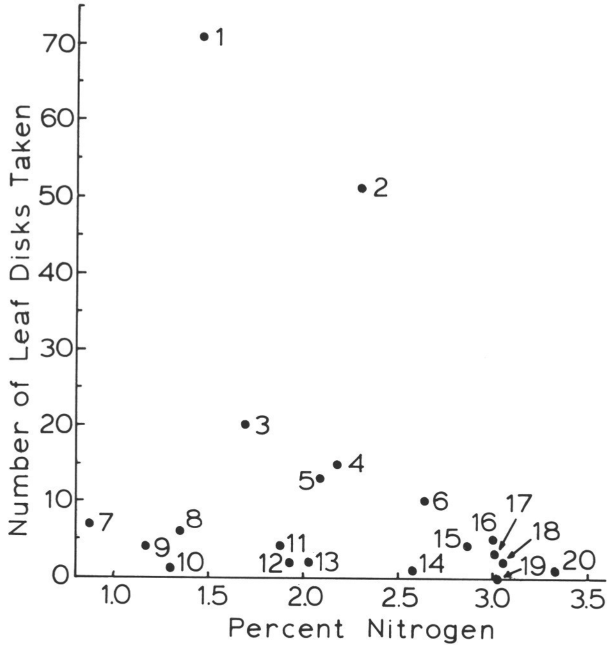


Fig. 6 – Lack of relationship between ant preference and percent nitrogen in leaves of the 20 primary test species. Species 1 is *Bombacopsis* ; species 2 is *Spondias*.

Fig. 6 – Ausencia de relación entre la preferencia de las hormigas y el porcentaje de nitrógeno crudo contenido en las hojas de 20 especies estudiadas. La especie 1 es *Bombacopsis* y la especie 2 es *Spondias*.

extreme : very high or very low N. Resolution of these questions will require study of a much larger number of species, and more accurate assessment of usable leaf protein. As a final comment, we also found no relationship between percent carbon and leaf preference by the ants.

We turn now to our attempts to extract repellent chemicals from the mature leaves of Santa Rosa species avoided by the ants. To date wa have found extractable repellency in 12 out of 18 species we have tested (Table 4). Of particular interest is that most of the chloroform extracts were repellent, whereas most of the ethanol extracts were not. This strongly suggests that most of the ant repellents in these species are nonpolar, lipid soluble molecules such as terpenoids, and not polar compounds such as low molecular weight phenols. Whether this trend is generalizable can only be ascertained from a larger sample of species.

Table IV – Eighteen plant species tested for chemically extractable ant repellency. A (+) indicates significant repellency of crude extracts ($P < 0.05$).

Tabla IV – Diez y ocho especies de plantas probadas para la repelencia de sus extractos. A (+) indica una repelencia significativa de los extractos crudos ($P < 0.05$).

Species	$CHCl_3$	ETOH
Alibertia edulis	–	–
Allophylus occidentalis	+	+
Casearia sylvestris	+	+
Cornutia grandiflora	+	+
Erythroxylon havanense	–	+
Eupatorium morifolium	+	+
Euphorbia schlectendahlii	–	–
Guazuma ulmifolia	–	–
Hemiangium excelsum	–	–
Hymenaea excelsum	+	–
Lasianthea fruticosa	+	–
Luehea speciosa	+	–
Malvaviscus arboreus	+	–
Ocotea veraguensis	+	–
Passiflora slotta	+	+
Pithecollobium saman	–	–
Verbesina gigantea	+	–
Zuelania guidonia	–	–

Using our laboratory bioassay, we have thus far isolated specific ant-repellent compounds from three unrelated species, *Hymenaea courbaril* (Papillionaceae), *Lasianthea fruticosa* (Compositae), and *Ocotea veraguensis* (Lauraceae). We have gone the farthest in the case of *Hymenaea* in that we have demonstrated that the isolated repellent actually functions to repel leafcutting ants in nature. Although the procedures used in isolating and characterizing the repellent in *Hymenaea* leaves are discussed in detail elsewhere (Hubbell, Wiemer, and Adejare, 1983), it is useful to summarize our findings here.

An initial chloroform extract of the leaves was highly repellent, whereas the ethanol extract showed no activity. The activity remained associated with the chloroform solution when this solution was washed with 1.0 N HCl to remove alkaloids and 1.0 N NaOH to remove acids. The material remaining in the neutral fractions was further fractionated by column chromatography, eventually yielding a set of 18 fractions. However, only one of these fractions showed any significant repellency, and that fraction consisted of an essentially pure compound. The ant repellent was identified by GC-MS and ^1H and ^{13}C NMR spectroscopy. The spectral data were most consistent with an epoxide derivative of caryophyllene. We confirmed the structure to be caryophyllene epoxide (Fig. 7) by comparison with an authentic sample prepared by partial synthesis.

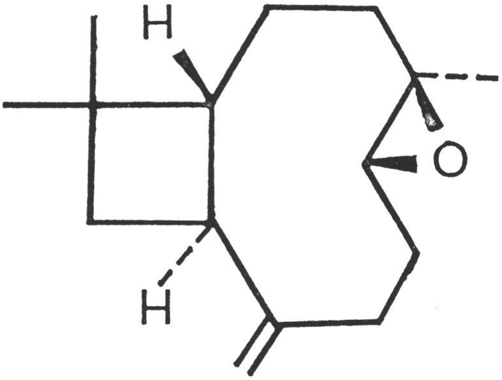


Fig. 7 – Structure of caryophyllene epoxide.

Fig. 7 – Estructura del epóxido de cariofileno.

We estimate the natural concentration of caryophyllene epoxide in *Hymenaea* leaves at approximatively 8 mg/g of leaves (Hubbell, Wiemer, and Adejare, 1983). The laboratory bioassay was then used to measure the repellency of pressed rye flakes treated with caryophyllene epoxide to reflect natural concentrations. Flakes so treated were highly repellent ($p < 0.01$). In contrast, flakes treated with caryophyllene, the precursor to the epoxide, were not significantly more repellent than control flakes, even when tested at concentrations higher than natural levels in *Hymenaea* leaves.

We have also tested the repellency of caryophyllene epoxide under field conditions in Costa Rica. Using the leaf preference bioassay, we demonstrated that leaves of a species normally quite palatable to the ants, *Spondias purpurea*, become nearly as unpalatable as *Hymenaea* leaves when treated with caryophyllene epoxide at the concentration characteristic of *Hymenaea* (Fig. 8). *Spondias* leaves treated with solvent alone were only slightly more repellent than untreated control *Spondias* leaves, so that the effect could clearly be attributed to the caryophyllene epoxide. If the concentration of the epoxide was increased above natural levels, the treated *Spondias* leaves became even more repellent than *Hymenaea*. At concentrations 10 times normal, contact with the chemical produced dramatic changes in ant behavior : the ants jumped or reared back and began intense grooming. These results are convincing evidence that caryophyllene epoxide can and does function in nature as a potent deterrent to leafcutting ant attack in *Hymenaea courbaril*.

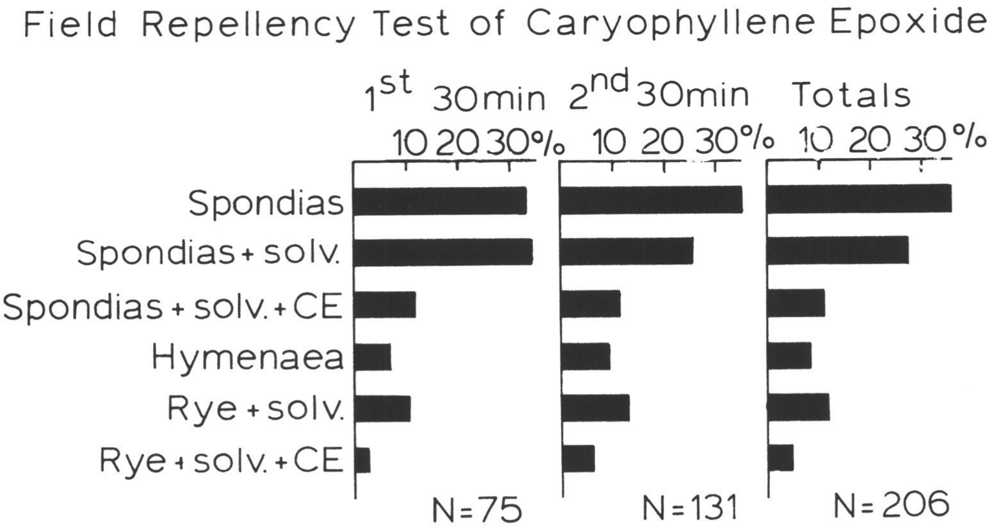


Fig. 8 – Field bioassay of repellency of caryophyllene epoxide (CE). Percentages are the fractions of leaf disks of each treatment picked up in the indicated period.

Fig. 8 – Bioensayo de campo sobre la repelencia del expóxido de cariofileno (CE). Los porcentajes indican las fracciones de discos de hojas recolectados durante el período de tiempo indicado para cada tratamiento.

DISCUSSION

This study has shown that leafcutter ants, when faced with a choice of leaves, are capable of quite finely tuned discrimination of differences in leaf quality. This discrimination operates not only between species, but between individual plants within species, and even between leaves from different parts of individual plants. This may explain why trees in a closed-canopy forest are rarely completely defoliated, and why ants tend to cut only the uppermost, sunlit branches, which presumably have leaves of higher quality.

Because the ants are not required to cut the leaf disks out of leaves in our field leaf preference tests, the ants in making their choices probably rely to a much greater extent on purely chemical cues, rather than on a mixture of chemical and physical cues as would normally be the case. Before picking up a leaf disk, ants always antennated, and nearly always bit, the cut edge of the disk, in behavior most readily interpreted as «tasting». However, this tasting behavior does not put the ant in contact with the chemicals in the leaf for nearly as long as it would take the ant to cut out the disk. If there are slow-acting repellents which only work on relatively prolonged contact, or if the repellents are at lower concentrations where their activity takes more exposure to be expressed, then the pickup bioassay would be expected to overestimate the true palatability of less acceptable species. For example, we suspect that this may have happened in the case of *Hymenaea* in our preference tests : in spite of the presence of known ant repellents, leaf disks of *Hymenaea* were overall sixth in preference. Nonetheless, for our purpose the pickup bioassay is a good conservative screen for finding species which are likely to contain potent ant repellents.

The geometric progression of species ranked by preference was also found by Hubbell and Rockwood (1983) for the leaf material harvested for a month-long period by each of the 20 colonies of *Atta cephalotes* at Santa Rosa. They suggested that the geometric series was actually a small-sample artifact, and showed that if the foraging of a colony were cumulated for an entire year, the distribution became lognormal. They suggested a rather complex model for colony organization wherein such a lognormal could arise, which supposed a colony composed of loosely federated suburbs with semi-autonomous foraging by trail-faithful workers, and a feedback between fungus growth rate and foraging rate for particular plant species.

The present results suggest that a much simpler model of foraging behavior will suffice. These data show that workers on a single trail can also collectively generate the geometric series, and would probably also generate the lognormal if their choices for all 150 + species in the forest were tested. If the same pattern can be seen in a single trail of a colony and does not require pooling over all trails of the colony, then maybe the same behavior is resident in each worker ant as well. Presumably natural selection has fashioned ants capable of discriminating good leaf material from bad as potential substrate for the growth of their fungus.

It should be noted that normally workers do not have a smorgasbord of choices laid out for their choice. The usual situation is that they have walked some tens of meters out from the nest, and a few more tens of meters to the top of a tree, at which point they must make a decision whether to cut or not cut the species, plant, and particular leaf they have encountered. If they choose not to cut, they are forced to search for a longer time to find something acceptable.

It is reasonable to suppose that, as time passes without finding a leaf to cut, the ant's willingness to accept a less palatable species increases. Given the cost of a foraging trip, it would seem better to take some leaf back to the colony, even one of rather inferior quality, than no leaf at all. According to this hypothesis, the distribution of pickup responses in our leaf preference bioassay will be a reflection of the distribution of failure times to find an acceptable leaf to cut. This hypothesis is attractive because it explains the observation in Figure 1, wherein there is a negative relationship between the number of species of leaf disks picked up and trail activity as measured by number of ants returning laden with leaves. It also predicts the observation that the few species which are picked up when there are many laden ants are only those of the highest acceptibility.

We were somewhat surprised to find that the ants paid little attention to the nitrogen content of their leaves. One might have excepted that the ants would be nitrogen (protein) limited and to prefer leaves with high protein content. This may still be true because an elemental nitrogen analysis is only a crude measure of relative protein content. On the other hand, it may also be true that the fungus is sufficiently good at concentrating nitrogen from plant materials that ant choice of species with especially proteinacious leaves is unnecessary. Among fungi, the ant fungus *Rhozites* is very slow growing, suggesting that it may spend considerable metabolic energy in making especially rich dietary substances for the ant larvae.

We were also somewhat surprised at the infrequency which which polar compounds such as small molecular weight phenols have turned up in our bioassay. Because our chemical search for ant repellents is directed by the ants' behavior, there is no particular bias toward finding one particular class of repellent compounds over another. Among students of plant-animal interactions, there is a widespread belief that generalized antimetabolites, such as tannins, are also important anti-herbivore plant defensive compounds (Feeny, 1976 ; Rhoades and Cates, 1976). Data on leaf tannin contents of tree species found in two African forests circumstantially supports this hypothesis (McKey et al., 1978). In one forest growing on nutrient poor soils where leaf production is expensive to the plant, tannin contents are high ; in the other forest, growing on nutrient-rich soils, leaf tannin levels are much lower. Nevertheless, for whatever reason, relatively few tannins have resulted from searches for specific insect antifeedants. In part this may reflect the unwillingness or inability of chemists to characterize these complex polymeric substances. However, in our study to date there has been a genuinely limited role of tannins in the defense of plants against leafcutter ant attack compared to terpenoids. Whether this trend will continue remains to be seen.

One of the most exciting post-result discoveries of this study was the finding that caryophyllene epoxide is a potent fungicide. Not only is it extremely toxic to the ant's own fungus (Hubbell, Wiemer, and Adejare, 1983), but it also kills nearly half of a set of 45 human and plant pathogenic fungi maintened in culture by the Department of Microbiology at the University of Iowa (Cazin, personal communication). Agar media containing as little as 100 μg/ml of caryophyllene epoxide killed 80 % of our *Rhozites* cultures within 48 hours – a concentration only a little more than 1 % of the concentration of caryophyllene epoxide in *Hymenaea* leaves.

Whether plants such as *Hymenaea* have compounds such as caryophyllene epoxide primarily as defenses against fungi or leafcutter ants is not yet known. However, we favor the hypothesis that evergreen species such as *Hymenaea* have probably had a more persistent battle with fungi over their evolutionary history than with leafcutting ants, and that these compounds only incidentally protect *Hymenaea* from ant attack. This hypothesis is supported by the observation that caryophyllene epoxide does not appear to be toxic, or at least strongly toxic, directly to the ants. A test of this hypothesis would be to measure the caryophyllene epoxide concentration of leaves from *Hymenaea* trees growing in Puerto Rico, which has no leafcutting ants.

Further research will reveal whether *Atta cephalotes* has the ability to detect other broad-spectrum fungicides in plants, or wheter the caryophyllene epoxide result was pure coincidence. We strongly suspect, however, that additional ant repellents which we isolate will frequently turn out to have antifungal properties as well.

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References

BOYD N.D., MARTIN M.M., 1975a. – Faecal proteinases of the fungus-growing ant, *Atta texana*, properties, significance, and possible origin. *Insect Biochem., 5*, 619-635.

BOYD N.D., MARTIN M.M., 1975b. – Faecal proteinases of the fungus-growing ant, *Atta texana* : their fungal origin and ecological significance. *J. Insect Physiol., 21*, 1815-1820.

CHERRETT J.M., 1968. – The foraging of *Atta cephalotes* L. (Hymenoptera, Formicidae). Foraging patterns and plant species attacked in a tropical rain forest. *J. Anim. Ecol., 37*, 387-403.

CHERRETT J.M., 1972a. – Some factors involved in the selection of vegetable substrate by *Atta cephalotes* (L.) (Hymenoptera : Formicidae) in tropical rain forest. *J. Anim. Ecol., 41*, 647-660.

CHERRETT J.M., 1972b. – Chemical aspects of plant attack by leafcutting ants. pp. 13-24, *in* Harbourne J.B., ed., «Phytochemical Ecology». Academic Press, New York.

FEENY P., 1970. – Seasonal changes in oak leaf tannins and nutrients as a cause of spring feeding by winter moth caterpillars. *Ecology, 51*, 565-581.

FEENY P., 1976. – Plant apparency and chemical defense, pp. 1-40, *in* Wallace J.W., Mansell R., eds., «Recent Advances in Phytochemistry», Plenum Press, New York.

HUBBELL S.P., ROCKWOOD L.L., 1983. – A hypothesis concerning the foraging strategy of a tropical leafcutting ant. *Biotropica* (in press).

HUBBELL S.P., WIEMER D.F., ADEJARE A., 1983. An antifungal terpenoid defends a Neotropical tree (*Hymenaea*) against attack by fungus-growing ants (*Atta*).*Oecologia* (in press).

LITTLEDYKE M., CHERRETT J.M., 1975. – Variability in the selection of substrate by the leafcutting ants *Atta cephalotes* (L.) and *Acromyrmex octospinosus* (Reich) (Formicidae, Attini). *Bull. ent. Res., 65*, 33-47.

LITTLEDYKE M., CHERRETT J.M., 1978. – Defense mechanisms in young and old leaves against cutting by the leafcutting ants *Atta cephalotes* (L.) and *Acromyrmex octospinosus* (Reich) (Formicidae, Attini). *Bull. ent. Res., 68*, 263-271.

MARTIN M.M., 1979. – Biochemical implications of insect mycophagy. *Biol. Rev., 54*, 1-21.

MATTSON W.J., Jr., 1980. – Herbivory in relation to plant nitrogen content. *Ann. Rev. Ecol. Syst., 11*, 119-162.

McKEY D., WATERMAN P.G., MBI C.N., GARTLAN J.S., STRUHSAKER T.T., 1978. – Phenolic content of vegetation in two African rain forests : ecological implications. *Science, 202*, 61-6.

QUINLAN R.J., CHERRETT J.M., 1979. – The role of fungus in the diet of the leaf-cutting ant, *Atta cephalotes* (L.). *Ecol. Entomol., 4*, 151-160.

RHOADES D.F., CATES R.G., 1976. – Toward a general theory of plant anti-herbivore chemistry, pp. 168-213, *in* Wallace J.W., Mansell R., eds. «Recent Advances in Phytochemistry», Plenum Press, New York.

ROCKWOOD L.L., 1975. – The effects of seasonality on foraging in two species of leaf-cutting ants (*Atta*) in Guanacaste Province, Costa Rica. *Biotropica, 7*, 176-193.

ROCKWOOD L.L., 1976. – Plant selection and foraging patterns in two species of leaf-cutting ants (*Atta*). *Ecology, 57*, 48-61.

STRADLING D.J., 1978. – The influence of size on foraging in the ant *Atta cephalotes*, and the effect of some plant defense mechanisms. *J. Anim. Ecol., 47*, 173-188.

WEBER N.A., 1972. – Gardening ants, the attines. *Mem. Amer. Phil. Soc., 92*, 1-146.