Sociobiology
An international journal on social insects

RESEARCH ARTICLE - ANTS

Sustainable Management of *Acromyrmex octospinosus* (Reich): How Botanical Extracts Should Promote an Ecofriendly Control Strategy

I Boulogne[1,2,3]*, L Desfontaines[2], H Ozier-Lafontaine[2], G Loranger-Merciris[1,2]

[1] Université des Antilles et de la Guyane, UFR Sciences Exactes et Naturelles, Pointe-à-Pitre Cedex (Guadeloupe), France.

[2] INRA, UR1321, ASTRO Agrosystèmes tropicaux, 97170, Petit-Bourg (Guadeloupe), France.

[3] Normandie Université, UNIROUEN, Laboratoire Glyco-MEV EA 4358, Fédération de Recherche Normandie Végétal FED 4277, F-76821 Mont Saint Aignan, France.

Article History

Edited by
Gilberto M. M. Santos - UEFS, Brazil
Received 04 April 2017
Initial acceptance 28 March 2018
Final acceptance 07 August 2018
Publication date 01 October 2018

Keywords
Attini, *Mammea americana*, chemical analysis, lethal concentrations, lethal doses, lethal times.

Corresponding author
Isabelle Boulogne
Normandie Université, UNIROUEN
Laboratoire Glyco-MEV EA 4358
Fédération de Recherche Normandie Végétal FED 4277, F-76821 Mont Saint Aignan, France
Email: isabelle.boulogne@univ-rouen.fr

Abstract

The leaf-cutting ant *Acromyrmex octospinosus* (Reich) causes serious damage to crops and protected areas due to its foraging activity. The main method of control of this species consists of the use of synthetic insecticides that can lead to environmental damage and negative side effects on human health. Consequently, alternative strategies, such as biopesticides, are needed. Insecticide evaluation by ingestion assays was performed using *A. octospinosus in vitro* bioassay and laboratory nests. Chemical analyses were also performed to know the contents of plant extracts. This study showed that *Mammea americana* L. is the most promising insecticidal plant extract in the control of *A. octospinosus.* Indeed, the lethal concentrations (LC_{50} and LC_{99}) an the lethal dose (LD_{99}) of the *M. americana* extract (51.31 $mg.mL^{-1}$, 131.92 $mg.mL^{-1}$, and 17.36 mg/g of ant respectively) were the closest to those of Fipronil, 0.03 g/kg, the commercial insecticide used as positive control.

Introduction

The synthetic pesticides used to manage insect pests cause damage to ecosystems, enhance resistance to insecticides in agricultural pests, adversely affect non-target organisms, cause environmental pollution, and have negative side effects on human health. These facts suggest a clear need for alternatives and have led to a renewed interest in biopesticides. Botanical insecticides represent an ecofriendly alternative for pest management because of their biodegradability, which results from the choice of solvent such as water (Boulogne *et al* 2012c), and their potential to reduce the evolution of resistance because plant extracts that contain a mixture of active phytochemicals should reduce the rate of evolution of resistance compared to the selective pressure exerted by single pure toxins (Arnason *et al* 1993).

The leaf-cutting ants [genera *Atta* and *Acromyrmex* (Hymenoptera: Formicidae: Attini)] in general and *Acromyrmex octospinosus* (Reich) in particular, cause serious damage on fields crops, pastures and plantations due to their foraging activities for its symbiotic fungus cultivation (Pérez *et al* 2011). Estimated damage was, for example, several million dollars per year in USA and Brazil (Cameron & Riggs 1985). *A. octospinosus* is native to South and Central America and exotic to Guadeloupe. This species was introduced in Guadeloupe in 1954 and progressively colonized the entire territory (Boulogne *et al* 2014). Tremendous losses have also been observed in agricultural and protected areas. Indeed, a Regional Federation of Defense against Pests (FREDON) survey carried out in 2008 indicated that vegetable and fruit crops account for 90.9 % of attacks. The 1995 cyclone favored ant invasion in natural areas where some plant species, such as the arborescent ferns

of the genus *Cyathea*, are now threatened and might completely disappear due to their endemism (Boulogne *et al* 2014). The United States Department of Agriculture (USDA) classifies this ant among the most serious pests of tropical and subtropical America (Pollard 1982). The main leaf-cutting ant control method is the application of granulated toxic baits, which are basically attractive citric matrices that contain a synthetic active ingredient that exerts a delayed action on workers (i.e., dechlorane, fipronil, sulfluramid GX071HB, or sulfluramid GX439) (Nagamoto *et al* 2007).

Previous studies explored the activities of active ingredients (allelochemicals) from plants. These studies have shown that plant extracts cited by TRAMIL ethnopharmacological surveys have insecticidal potential for the control of the leaf-cutting ant *A. octospinosus.* Specifically, four plant extracts have ingestion toxicity [*Mammea americana* L. (Calophyllaceae), *Nicotiana tabacum* L. (Solanaceae)*,* and two extracts of *Nerium oleander* L. (Apocynaceae)] (Boulogne *et al* 2012a). Some others have fungicidal potential to control the symbiotic fungus *Leucoagaricus gongylophorus* (Singer) Möller (Agaricales, Basidiomycota), particularly a foliage extract of *Senna alata* (L.) Roxb. (Fabaceae) (Boulogne *et al* 2012b). An exhaustive literature search was conducted to identify the published papers related to insecticidal and fungicidal chemical compounds that stem from plant species. This meta-analysis revealed that alkaloids, phenolics, and terpenoids are the three main chemical classes that are most often cited for insecticidal and fungicidal activities (Boulogne *et al* 2012c). To date, very few studies have used artificial nest bioassays (Hebling *et al* 1996, 2000) or described the lethal doses, concentrations and times of botanical pesticides that are used for the control of leaf-cutting ants.

Therefore, the aims of this study were i) to determine the lethal doses, concentrations and times of four preselected insecticidal plant extracts, ii) to manage a preliminary artificial nest bioassay, and iii) to characterize their biochemical contents in terms of alkaloids, phenolics, and terpenoids, in order to determine the most promising insecticidal and fungicidal plant extracts for use in controlling entire colonies.

Material and Methods

Rearing conditions

Two adult *A. octospinosus* nests (all casts, queen and symbiotic fungi) were collected from the field in different locations in Guadeloupe (French West Indies) and were bred in the laboratory by housing each colony in an artificial nest. The colonies were maintained for one month in the lab after collection before the initiation of the experiments and were supplied with flowers, leaves, sugarcane, corn flakes, and water daily. The workers used in the ingestion assays were collected from these laboratory nests. Some worker vouchers were deposited in the ASTRO Lab at the National Institute of Agronomic Research (INRA) of the French West Indies and Guiana.

Extracts: source and preparation

The plants used for extract preparation were collected in different locations in Guadeloupe FWI (16°22’44.36”N -61°29’14.60”W, 16°12’25.01”N-61°29’49.42”W, 16°16’48.41”N-61°30’13.30”W, 16°11’20.65”N-61°35’39.54W, 15°59’53.35”N-61°43’33.71”W), identified by voucher number (Boulogne,Gd,1,UAG/INRA; Boulogne,Gd,2,UAG/INRA; Boulogne,Gd,3,UAG/INRA), deposited at the herbarium of the Santo Domingo Botanical Garden and identified by a botanist of this herbarium, Mr. Brigido Peguero.

The *M. americana* seed maceration, the two extracts with leaves of *N. oleander* and the decoction of dried leaves of *N. tabacum* were prepared as previously described in Boulogne *et al* (2012a). All the aqueous plant extracts obtained and fresh leaves of *S. alata* were freeze dried, ground with a coffee mill, and sieved at 0.5 mm. The residues represented 99, 139, 56, 216 and 295 grams per kilogram of fresh plants of *M. americana, N. oleander* (crushed), *N. oleander* (decoction), *N. tabacum*, and *S. alata*, respectively.

Statistical analyses

Lethal concentrations (LC_{50} and LC_{99}), lethal dose values (LD_{50} and LD_{99}) and lethal times (LT_{50} and LT_{99}) (concentrations, dose and time that killed 50% and 99% of ants) and their 95% confidence intervals were calculated with logistic regression by Probit analysis. For *in vitro* insecticidal and preliminary artificial nests bioassays (records three times daily during 28 days for each nest), non-parametric analyses were performed with the Friedman test with multiple comparison method of Nemenyi. For chemical analysis, non-parametric analysis was performed with the Kruskal-Wallis test, multiple comparisons were performed with the Dunn method, and Bonferroni corrections. All these tests were made with XLSTAT® software.

Ingestion bioassay

Regarding control methods used against leaf-cutting ants, our objective was to find a toxic and attractive extract that exerted a delayed action on the workers. With this objective, ingestion bioassays were performed with groups of ten ant workers, which belong to the same colony (the bioassays were then repeated once again with an other colony). The ants were placed in 30 jam bottles (volume 324 ml, diameter 82 mm). Six bottles were used for each of the concentrations of the lyophilized plant extracts (i.e., 1, 5 and 10 $mg.mL^{-1}$), 6 bottles were negative controls, and 6 bottles containing Blitz® commercial insecticide (Granular bait, Fipronil 0.03 g/kg, 06/08/2001, 9800377) were used as positive controls. The ants were fed daily over a period of 21 days with an autoclaved artificial diet placed in plastic caps. This artificial diet was composed of glucose (50 $mg.mL^{-1}$), peptone (10 $mg.mL^{-1}$),

yeast extract (1 mg.mL^{-1}), agar (15 mg.mL^{-1}) (Bigi *et al* 2004), and the freeze-dried plant extracts, distilled water (negative control), or fipronil (0.03 g/kg) q.s. to 1 L. The experiments were performed at 25 °C and 70-80% relative humidity on a 12:12 h light:dark photoperiod. Each day, the number of dead ants in each jam bottle was recorded, the caps were removed and weighed, and new caps with fresh artificial diet were offered.

The amounts of food eaten daily in g were corrected for the numbers of live ants. Mortality was analyzed on the based on the percentages of dead ants corrected by means of Abbott formula. In all insecticidal bioassays, the concentrations used for concentration response estimates were 1, 5 and 10 mg.mL^{-1}. The delayed action of an extract was defined according to Camargo *et al* (2006) as follows: a weak action occurred when 50% of the ants died before 48 h, a medium action when 50% of ants died between 48 h and 72 h, and a high action when 50% of ants died after 72 h.

Results

The most toxic lyophilized extract was that of the *N. tabacum* dried leaf decoction with an LC_{50} = 1.33 mg.mL^{-1} and an LC_{99} = 3.93 mg.mL^{-1} after 24 h. The lethal concentrations (LC_{50} and LC_{99}) of the *M. americana* extract (51.31 mg.mL^{-1} and 131.92 mg.mL^{-1}, respectively) were the closest to those of Fipronil, 0.03 g/kg (78.85 mg.mL^{-1} and 196.95 mg.mL^{-1}, respectively, Table 1).

The LD_{50} of the *N. tabacum* extract (0.87 mg/g of ant) was the closest to that of Fipronil, 0.03 g/kg (1.48 mg/g of ant), whereas the LD_{99} of the *M. americana* extract (17.36 mg/g of ant) was the closest to that of Fipronil, 0.03 g/kg (5.05 mg/g of ant, Table 1).

The LT_{50} of the *N. oleander* extracts at 10 mg.mL^{-1} (47.2 and 69.98 hours) were the closest to those of Fipronil, 0.03 g/kg at 5 and 10 mg.mL^{-1}(52.78 and 62.8 hours), whereas the LT_{50} of the *M. americana* extract at 5 and 10 mg.mL^{-1} (102.21 and 122.95 hours) were the closest to that of Fipronil, 0.03 g/kg at 1 mg.mL^{-1} (111.94 hours) (Table 2). The LT_{99} of the *M. americana* extract at 10 mg.mL^{-1} (311.96 hours) were the closest to that of Fipronil, 0.03 g/kg at 1 mg.mL^{-1} (220.31 hours) (Table 2).

Moreover, linear regressions (with R^2 and equations) between mortalities of the ants and concentrations of the plant extracts and the positive control at 24h, 48h and 72h, (Figure 1) indicate a high delayed action for the *M. americana* extracts, a weak delayed action for most of the *N. oleander* extracts, a weak delayed action for *N. tabacum* and a "medium to high" delayed action for Fipronil, 0.03 g/kg (positive control).

The quantities of artificial food eaten each day by the ants during the insecticidal bioassays with all of the extracts and the positive control (Fipronil 0.03 g/kg, Blitz©) at 10 mg.mL^{-1} and most of extracts at 5 mg.mL^{-1} did not differ from the quantities consumed in the negative control condition (without extracts) with values between 0.2 and 0.3 g. The extracts at 1 mg.mL^{-1} were more attractive than the control with values between 0.4 and 0.45 g (Figure 2).

Data on Table 3 demontrate that the toxicity/appetency and delayed action characteristics of the *M. americana* seed extract were the most similar to those of Fipronil, 0.03 g/kg. This extract was thus chosen for the preliminary artificial nest bioassay. Although the extract of *N. tabacum* seemed to have interesting characteristics, its lack of delayed action and some toxic properties on mammals do not lead us to choose it. Indeed, its well known alkaloid (nicotine) is known for its high level of toxicity in mammals in miming the activity of acetylcholine and binding to post-synaptic receptors to cause stimulation and subsequent depression of the central nervous system, autonomic nervous system, and muscular nerve endings (Philogène *et al* 2008).

Table 1- Lethal concentrations (LC50 and LC99) and lethal doses (LD50 and LD99) after 24 h with number of ants exposed, the 95% confidence intervals, likehood ratio Chi^2, associated P values and degrees of freedom in the laboratory insecticidal bioassays of the *Mammea americana* extract, crushed extract of Nerium oleander dried leaves, decoction of *N. oleander* fresh leaves, decoction of *Nicotiana tabacum* dried leaves and the positive control (Blitz©).

	n	LC_{50} (mg.mL^{-1})	95% Confidence intervals	LC_{99} (mg.mL^{-1})	95% Confidence intervals	Chi^2 LR (P value)
Mammea americana	60	5.13	1.07 – 7.09	13.19	12.97 – 17.83	1.32 (<0.0001)
Nerium oleander (crushed)	60	1.72	1.23 – 3.59	4.43	2.95 – 10.29	1.26 (<0.0001)
Nerium oleander (decoction)	60	2.78	1.62 – 9.49	6.58	3.57 – 25.19	0.96 (<0.0001)
Nicotiana tabacum	60	1.33	0.99 – 2.26	3.93	2.75 – 7.549	1.45 (<0.0001)
Fipronil, 0.03 g/kg	60	7.88	4.74 – 10.12	19.69	10.81 – 24.86	1.38 (<0.0001)
	n	**LD_{50} (mg/g)**	**95% Confidence intervals**	**LD_{99} (mg/g)**	**95% Confidence intervals**	**Chi^2 LR (P value)**
Mammea americana	60	4.03	3.24 – 5.54	17.36	15.24 – 20.49	1.32 (<0.0001)
Nerium oleander (crushed)	60	3.66	1.32 – 5.54	40.2	34.82 – 48.15	1.26 (<0.0001)
Nerium oleander (decoction)	60	8.47	7.41 – 9.52	30.8	27.58 – 35.2	0.96 (<0.0001)
Nicotiana tabacum	60	0.87	0.77 – 2.47	25.19	20.98 – 32.24	1.45 (<0.0001)
Fipronil, 0.03 g/kg	60	1.48	1.14 – 1.82	5.05	4.12 – 6.88	1.38 (<0.0001)

Table 2- Lethal times (LT50 and LT99) with number of ants exposed, the 95% confidence intervals, likehood ratio Chi², associated P values and degrees of freedom in the laboratory insecticidal bioassays of the Mammea americana extract, crushed extract of Nerium oleander dried leaves, decoction of N. oleander fresh leaves, decoction of Nicotiana tabacum dried leaves and the positive control (Blitz©).

	n	LT50 (hours)	95% Confidence intervals	Chi² LR (P value)
Mammea americana 1mg.mL^{-1}	60	396.58	370.92 - 427.85	1.74 (<0.0001)
Mammea americana 5mg.mL^{-1}	60	122.95	115.67 - 129.89	1.13 (< 0.0001)
Mammea americana 10mg.mL^{-1}	60	102.21	87.52 - 115.28	0.58 (< 0.0001)
Nerium oleander (crushed) 1mg.mL^{-1}	60	778.72	573.30 - 1580.29	0.10 (0.001)
Nerium oleander (crushed) 5mg.mL^{-1}	60	82.39	36.99 - 116.88	1.26 (< 0.0001)
Nerium oleander (crushed) 10mg.mL^{-1}	60	47.20	22.04 - 68.31	0.36 (< 0.0001)
Nerium oleander (decoction) 1mg.mL^{-1}	60	938.23	770.46 - 1266.19	0.35 (< 0.0001)
Nerium oleander (decoction) 5mg.mL^{-1}	60	261.57	233.78 - 289.15	0.10 (< 0.0001)
Nerium oleander (decoction) 10mg.mL^{-1}	60	69.98	47.43 - 89.20	0.38 (< 0.0001)
Nicotiana tabacum 1mg.mL^{-1}	60	172.57	124.81 - 209.42	0.66 (< 0.0001)
Nicotiana tabacum 5mg.mL^{-1}	60	30.40	26.96 - 72.18	0.11 (< 0.0001)
Nicotiana tabacum 10mg.mL^{-1}	60	33.88	27.94 - 66.29	0.15 (< 0.0001)
Fipronil, 0.03 g/kg 1mg.mL^{-1}	60	111.94	106.97 - 116.91	1.43 (< 0.0001)
Fipronil, 0.03 g/kg 5mg.mL^{-1}	60	62.80	59.34 - 66.24	1.04 (< 0.0001)
Fipronil, 0.03 g/kg 10mg.mL^{-1}	60	52.78	49.79 - 55.76	0.93 (< 0.0001)
	n	**LT99 (hours)**	**95% Confidence intervals**	**Chi² LR (P value)**
Mammea americana 1mg.mL^{-1}	60	1283.84	1150.01 - 1464.90	0.17 (<0.0001)
Mammea americana 5mg.mL^{-1}	60	479.16	451.70 - 512.00	0.58 (< 0.0001)
Mammea americana 10mg.mL^{-1}	60	311.96	296.46 - 330.28	1.13 (< 0.0001)
Nerium oleander (crushed) 1mg.mL^{-1}	60	4513.30	2908.31 - 11067.41	0.10 (0.001)
Nerium oleander (crushed) 5mg.mL^{-1}	60	1124.38	990.54 - 1315.08	0.12 (< 0.0001)
Nerium oleander (crushed) 10mg.mL^{-1}	60	557.48	519.13 - 604.91	0.36 (< 0.0001)
Nerium oleander (decoction) 1mg.mL^{-1}	60	2704.99	2102.63 - 3899.17	0.35 (< 0.0001)
Nerium oleander (decoction) 5mg.mL^{-1}	60	1423.15	1236.86 - 1695.78	0.11 (< 0.0001)
Nerium oleander (decoction) 10mg.mL^{-1}	60	576.69	538.19 - 624.03	0.38 (< 0.0001)
Nicotiana tabacum 1mg.mL^{-1}	60	1660.83	1386.44 - 2109.04	0.66 (< 0.0001)
Nicotiana tabacum 5mg.mL^{-1}	60	1041.08	907.23 - 1237.89	0.10 (< 0.0001)
Nicotiana tabacum 10mg.mL^{-1}	60	657.88	589.80 - 751.42	0.15 (< 0.0001)
Fipronil, 0.03 g/kg 1mg.mL^{-1}	60	220.31	209.28 - 233.57	1.43 (< 0.0001)
Fipronil, 0.03 g/kg 5mg.mL^{-1}	60	115.30	107.99 - 124.83	1.04 (< 0.0001)
Fipronil, 0.03 g/kg 10mg.mL^{-1}	60	91.70	85.72 - 99.68	0.93 (< 0.0001)

Table 3 - Summary table showing lethal concentrations, lethal doses, appetencies and delayed actions of Mammea americana extract, crushed extract of Nerium oleander dried leaves, decoction of N. oleander fresh leaves, decoction of Nicotiana tabacum dried leaves and the positive control (Blitz©). The lethal concentrations (LC50) are indicated as follows: +, LC50 > 50 mg.mL-1; ++, LC50 between 50 mg.mL-1 and 25 mg.mL-1; and +++, LC50 < 25 mg.mL-1. The lethal concentration (LC99) are indicated as follows: +, LC99 > 100 mg.mL-1; ++, LC99 between 100 mg.mL-1 and 50 mg.mL-1; and +++, CL99 < 50 mg.mL-1. The lethal doses (LD50) are indicated as follows: +, LD50 > 5 mg/g; ++, LD50 between 5 mg/g and 2 mg/g; and +++, DL50 < 2 mg/g. The lethal doses (LD99) are indicated as follows: +, LD99 > 30 mg/g; ++, LD99 between 20 mg/g and 30 mg/g; and +++, LD99 < 20 mg/g. The appetencies are indicated as follows: +, daily consumed quantity < 0.20 g; and ++, daily consumed quantity > 0.20 g. The delayed actions are indicated as follows: +, 50% of the ants died before 48 h; ++, 50% of ants died between 48 h and 72 h; and +++, 50% of ants died after 72 h for the highest concentration (according to LT50 showed in Table 2).

	LC_{50} and LC_{99}	LD_{50}	LD_{99}	Appetency	Delayed action
Mammea americana	+	++	+++	++	+++
Nerium oleander (crushed)	+++	++	+	++	++
Nerium oleander (decoction)	++	+	++	+	++
Nicotiana tabacum	+++	+++	++	++	+
Fipronil, 0.03 g/kg	+	+++	+++	++	++

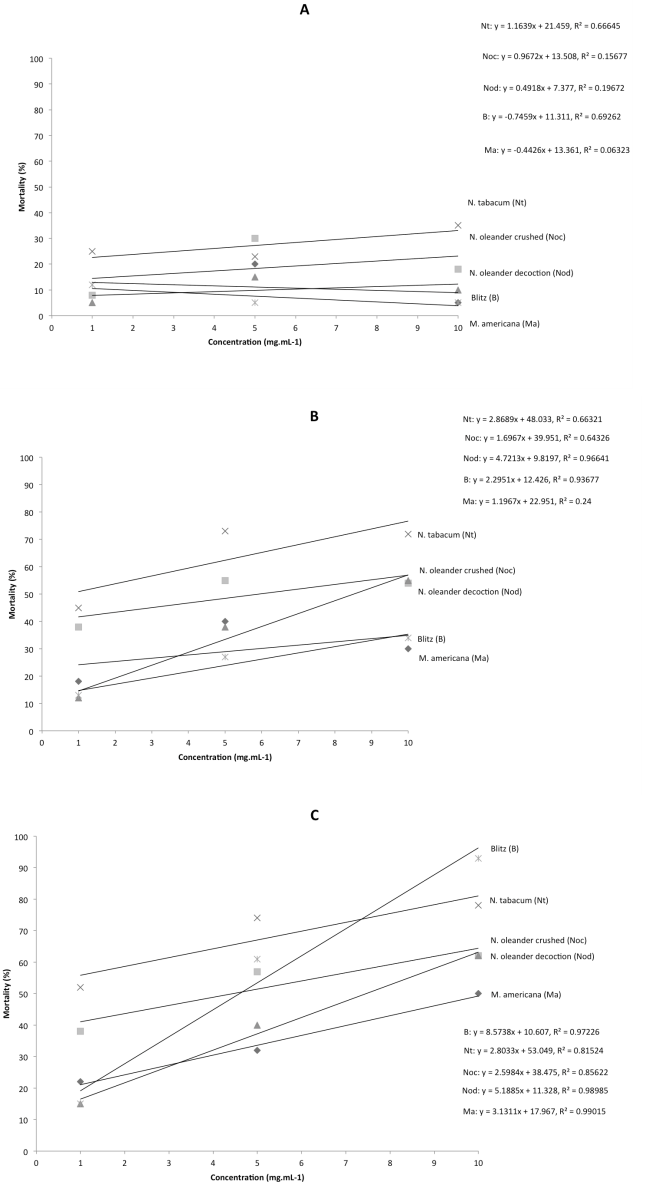

Figure 1 - Linear regressions (with R^2 and equations) between mortalities of the ants (%) and concentrations (mg.mL-1) in the insecticidal bioassays of the Mammea americana seed extract, crushed extract of Nerium oleander dried leaves, decoction of N. oleander fresh leaves, decoction of Nicotiana tabacum dried leaves, and the positive control (Blitz©) at 24h (A), 48h (B) and 72h (C).

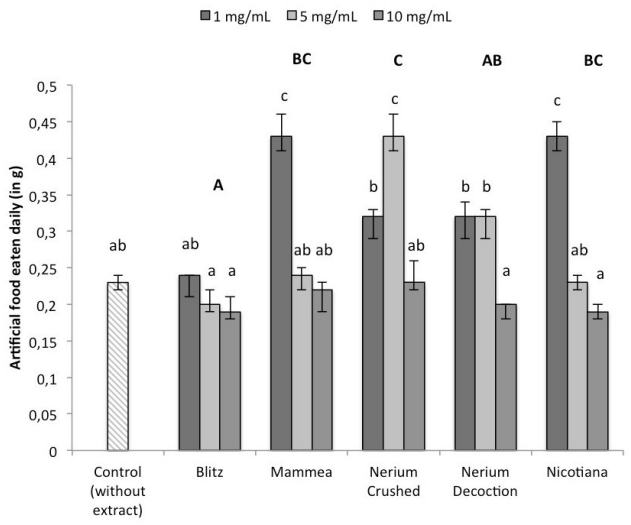

Figure 2 - Quantities of artificial food eaten by the ants daily during the laboratory insecticidal bioassays of the Mammea americana extract, crushed extract of Nerium oleander dried leaves, decoction of N. oleander fresh leaves, decoction of Nicotiana tabacum dried leaves, the positive control (Fipronil, 0.03 g/kg) at 1, 5 and 10 mg.mL^{-1} and the negative control (no extracts). The bars represent the medians, and the error bars are the 25% and 75% quartiles. The treatments without common letters differed significantly based on Friedman test with multiple comparison method of Nemenyi. The comparisons between specific concentrations are reported with small letters, and the comparisons between all treatments are reported with capital letters.

Preliminary artificial nest bioassay

To determine whether the effects of our selected extracts could be efficient against entire colonies, preliminary artificial nest bioassays were conducted. For this purpose, ten colonies were observed in field conditions in order to select the 5 most active extracts for use in the preliminary artificial nest bioassays. These colonies were placed in artificial nests and supplied with flowers, leaves, sugarcane, and corn flakes daily prior to the initiation of the bioassays. Each artificial nest was composed of 3 plastic boxes (60 ´ 40 ´ 40 cm) that were linked together by short hoses. The central box was non-transparent and hence suitable for the placement of the colony. The other two boxes on either side were transparent to simulate the outdoor environment, and the daily rations were placed in these boxes.

The nests were exposed to each of the following treatments prepared in artificial diet (one nest by treatment): *M. americana* extract at 10 mg.mL^{-1}, *S. alata* extract at 2 mg.mL^{-1}, *M. americana* extract combined with *S. alata* extract at 10 and 2 mg.mL^{-1}, respectively, a positive control (Fipronil, 0.03 g/kg), and a negative control (no extracts).

The artificial diet was the same as that used in the laboratory ingestion bioassays with the addition of the lyophilized plant extracts, distilled water (negative control) or Fipronil, 0.03 g/kg. Dried *Citrus sinensis* (L.) Osbeck (Rutaceae) peels and *Dioscorea alata* L. (Dioscoreaceae) leaves (ground with a coffee mill and sieved at 0.5 mm) were added to this artificial food at 10 g.L^{-1} because of the natural appetency and attractiveness of these components to leaf-cutting ants (Verza *et al* 2006). This attractive mixture has previously been tested, and its lack of insecticidal and fungicidal activities has been verified according to established protocols of ingestion bioassay and antifungal test (Boulogne *et al* 2012b). The diets were placed in plastic caps and offered to the ants daily. Before and after the treatments, the nests were supplied daily with flowers and leaves for 7 days. During the treatments, the nests were supplied daily for 14 days exclusively with plastic caps containing the artificial diet with or without the active extracts. The foraging activities (number

of ants incoming the nest with plant material or artificial diet during ten minutes) and quantities of food eaten were recorded three times daily at the same time for each nest before, during, and after the treatments (Lopez & Orduz 2003).

Only the nests treated with *M. americana* extract and Fipronil, 0.03 g/kg exhibited significant reductions in foraging activity (by minute) after the treatments. Similarly, only these nests exhibited significant reductions in the quantities of artificial food eaten daily in grams after the treatments. The quantities of artificial food eaten daily by these ants were significantly lower during the treatments than before or after the treatments with the exception of the nest treated with *S. alata* (Figures 3-A and B). Notably, 6 weeks after the treatment, all of the nests died out with the exception of the negative control nest (which lasted more than 2 months after this last observation). The nests treated with Fipronil, 0.03 g/kg and the *S. alata* extract contained no surviving ants or fungus gardens. The nest treated with the *M. americana* extract exhibited no surviving ants, and its fungus garden was infested with fungal competitors. The nest treated with the *M. americana* extract combined with the *S. alata* extract contained no ants and no fungus garden and was infested with fungal competitors (Figure 3-C).

Chemical analysis

The extracts were submitted to phytochemical analyses for plant secondary metabolites that are known for their potential insecticidal activities, i.e., alkaloids, phenolic

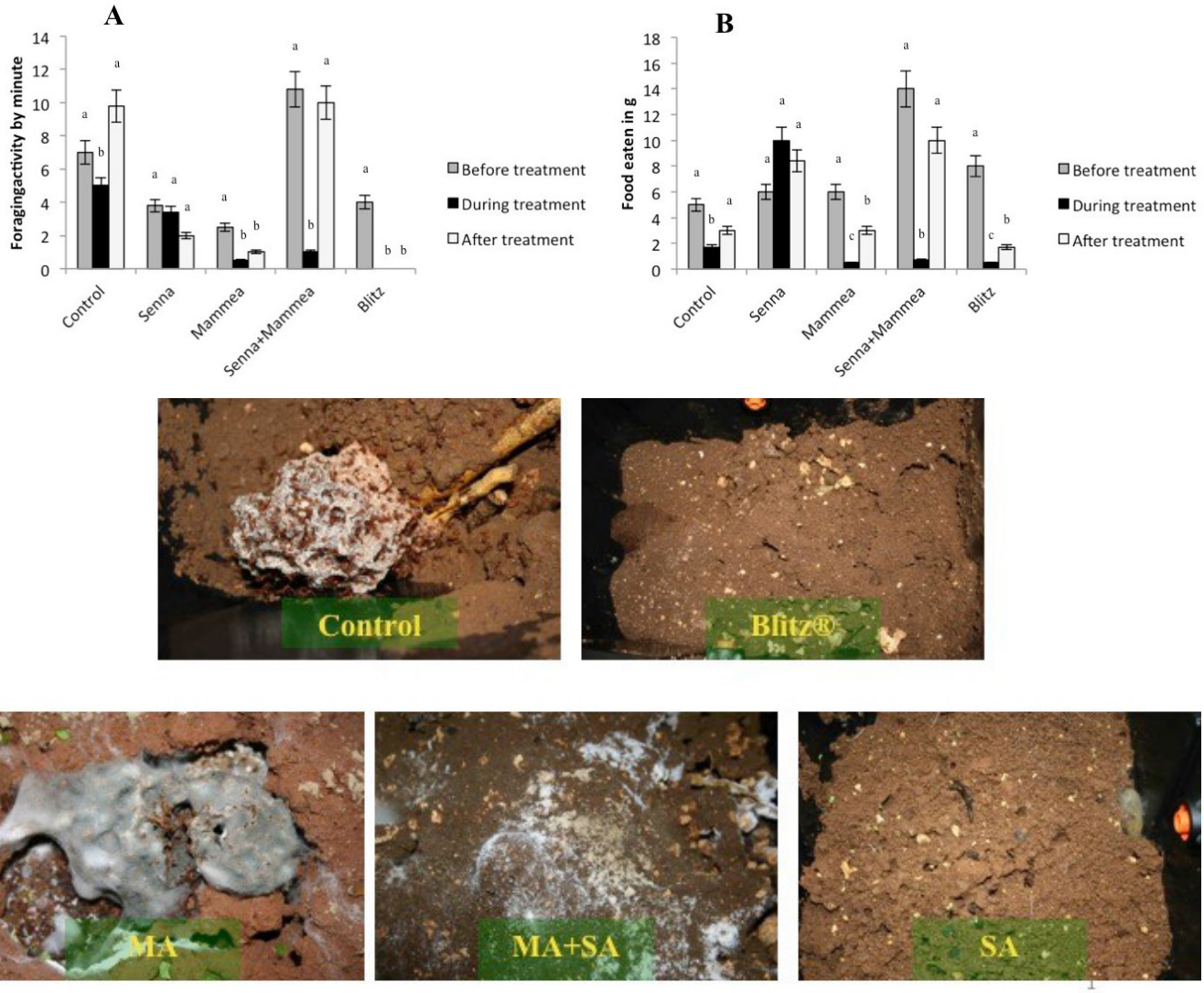

Figure 3 - Foraging activity by minute (A) and quantities of artificial food eaten (in g) daily by ants (B) in artificial before, during and after treatments (records three times daily during 28 days) of Mammea americana extract (MA), Senna alata extract (SA), M. americana extract combinated with S. alata extract, positive control (Fipronil, 0.03 g/kg) and negative control (without extracts). Treatments without common letters differ significantly based Friedman test with multiple comparison method of Nemenyi. The bars represent the medians, and the error bars are the 25% and 75% quartiles. (C) Acromyrmex octospinosus artificial nests 6 weeks after treatments with Mammea americana extract (MA), Senna alata extract (SA), Mammea americana extract combinated with Senna alata extract (MA+SA), Blitz© (positive control) and control (without extracts).

compounds, and terpenoids. The quantity of each compound was determined using a spectrophotometer according to the following quantitative methods: alkaloids were determined using Marquis's reagent with a colorimetric method (Szabo *et al* 2003), total phenolic compounds were determined using the Folin-Ciocalteau colorimetric method (Heilerová *et al* 2003), and terpenoids were determined using the iron (III) chloride-o-phosphoric acid-sulfuric acid colorimetric method (Zak *et al* 1956). All values are expressed in micrograms of standard per g of freeze-dried fresh plant sample and three replications were performed.

The *N. oleander* extracts contained the greatest quantities of total alkaloids, the *N. tabacum* and *N. oleander* decoctions contained the greatest quantities of total phenolic compounds and the *N. oleander* extracts contained the most total terpenoids. Terpenoids were the most abundant compounds in all of the extracts (between 550 and 1400 mg of standard/g of freeze-dried plant sample) (Figure 4).

M. americana

We showed that the *M. americana* seed lyophilized extract induced insecticidal toxicity following ingestion. There are few data available regarding the toxicities of *M. americana*, on Attini (Boulogne *et al* 2012a), but seeds of this plant showed insecticidal effects in other studies. Indeed, *M. americana* seeds exhibited activity against *Cerotoma ruficornis* (Oliver) (Coleoptera: Chrysomelidae) (Dev & Koul 1997) and *Aedes aegypti* (L.) (Diptera: Culicidae) (Sievers *et al* 1949). These seeds are also larvicidal against *Laphygma frugiperda* (Smith and Abbot) (Lepidoptera: Noctuidae) and *Plutella maculipennis* (Curt.) (Lepidoptera: Acrolepiidae) (Plank 1944).

Our preliminary chemical analysis revealed that the *M. Americana* seed extract contained alkaloids, phenolics and terpenoids. Analysis of the literature revealed that the compounds responsible for the insecticidal activities of *M. americana* are well-known phenolic compounds such as coumarins and particularly mammein (Duke 1989). Methanol and ethyl acetate extractions and HPLC or LC-MS analyses have revealed 4.8 mg of coumarins per gram of seed (Yang *et al* 2006).

The *M. americana* seed extract exhibited an LC_{50} of 51.31 $mg.mL^{-1}$ and an LD_{50} of 706.65 mg/g of insect. To compare our results with existing data we found a study of the toxicities of aqueous and ethanolic extracts of *M. americana* on *Artemia salina* L. (Anostraca: Artemiidae). This study revealed an LC_{50} greater than 10 $mg.mL^{-1}$ for an aqueous extract and an LC_{50} of 197 µg/mL for the ethanolic extract (Bussmann *et al* 2011). Another study of coumarins in *M. americana* revealed an LD_{50} of 4 µg/insect against *Phaedon cochleariae* Fab. (Coleoptera; Chrysomelidae) (Perez *et al* 2010). These results might suggest that ethanolic extracts and bioguided fractionations of *M. americana* seed extracts might increase the toxicity (coumarins are less miscible in water than in ethanol).

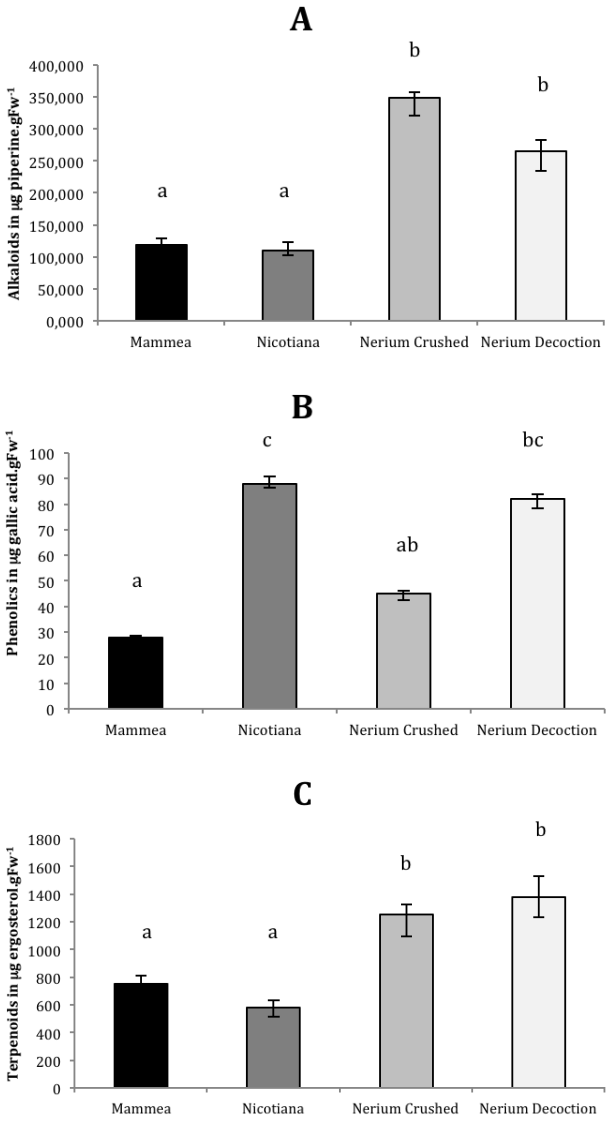

Figure 4 - Quantitation of the alkaloid (A), phenolic compound (B), and terpenoid (C) contents (milligrams of standard per gram of freeze-dried plant material) of the Mammea americana extract, crushed extract of Nerium oleander dried leaves, decoction of N. oleander fresh leaves, and decoction of Nicotiana tabacum dried leaves. The quantities without common letters differed significantly based on Kruskal-Wallis tests with Dunn's multiple comparison tests and Bonferroni corrections (n=6 and df=3). The bars represent the medians, and the error bars are the 25% and 75% quartiles.

S. alata

The nests treated with the *S. alata* extract contained no fungus garden after 6 weeks of treatment. This effect was consistent with our previous study, which showed that the *S. alata* foliar extract was fungicidal against *L. gongylophorus* (Boulogne *et al* 2012b). We also reported a preliminary chemical analysis, which revealed that *S. alata* foliage contains alkaloids, phenolic compounds and terpenoids. Previous studies exhibited fungicidal activity against several other fungi and showed that the fungicidal activity of *S. alata*

foliar extracts can be attributed to phenolic compounds such as anthraquinones (Somchit *et al* 2003) like chrysophanol (Palanichamy & Nagarajan 1990).

Artificial nests bioassay

We were aware that our artificial nest bioassay was really preliminary and all results of differences among extracts should be treated as preliminary and with caution. Indeed, the experimental design of the test had no replicates to the treatments and it could bias the results (differences might be attributed to differences in nests themselves and not to treatments). However, this preliminary assay is crucial and important to this study line since they show that these extracts are attractive to ants and consumed by them.

Concerning the length of treatment, similar bioassays revealed that *Atta sexdens rubropilosa* nests that are supplied daily with a diet containing *Ricinus communis* leaves exhibit gradual decreases in fungus gardens and substantial worker mortality after 6 weeks of treatment (Hebling *et al* 1996). Another study of *Atta sexdens* nests that were fed daily with a diet containing *Canavalia ensiformis* L. (Leguminosae) leaves reported complete nest extinction after 11 weeks of treatments (Hebling *et al* 2000).

Ant baits in field conditions

Regarding delayed action, the seed of *M. americana* seemed to naturally possess this property. It would be interesting to increase this delayed action of the substance with a digestible polymer using microencapsulation techniques (Benita 2005). Microcapsules could be made to contain a freeze-dried ethanolic or aqueous extracts of *M. americana* seeds and placed in a mix of *Citrus* sp. pulp and dried *Dioscorea alata* leaves to increase the appetency based on our laboratory results and existing data (Verza *et al* 2006). These granular baits (microcapsules and attractant mixtures compressed in granular form easy to apply) might be protected in biodegradable and compostable plastics to preserve them against sunlight and adverse weather conditions. Thus, like others well known commercial formulation containing botanical extracts (e.g. pyrethrins, rotenone, sabadilla, ryania, nicotine, azadirachtins or limonene) (Weinzierl 2000), a field study should be conducted with these kinds of baits to improve our work in field conditions. However compared to these previous studies, the force of our argument is to use mixtures of active phytochemicals to reduce conventional resistance compared with the selection pressure exerted by single pure molecule (Arnason *et al* 1993) and make the choice of extraction type and solvent with the greatest sustainability (water or eventually ethanol extractions) (Boulogne *et al* 2012c). As the example of *M. americana* extract, toxicity may be increased and transformed in granular baits (microcapsules and attractant mixture) protected in a plastic. As the same way, to improve our fungicidal results (*in vitro* and with artificial nest) in field conditions, *S. alata* foliar extracts may also be transformed in granular baits.

Our results might be useful in the control of *A. octospinosus* and might be applicable to the control of other leaf-cutting ant species. However, we need to keep in mind that, in Guadeloupe (FWI), this species is exotic. This ant might behave different in its exotic range than in its native range (e.g. the case of *Linepithema humile* (Human & Gordon 1996), and the results found might only apply for non-native populations. Thus, these results should be treated with caution before their generalization.

Our study is the first report of the toxicities of *M. Americana* seed, *N. oleander* leaf, and *N. tabacum* leaf freeze-dried extracts due to ingestion by Attini. It allowed us to determine the lethal doses, concentrations and times of four insecticidal plant extracts that were selected for examination based on previous studies. This study revealed that the *M. americana* seed extract was the most similar in terms of toxicity/appetency and delayed action to the commercial bait Fipronil, 0.03 g/kg. Our analyses also revealed that these extracts contained alkaloids, phenolics, and terpenoids. The preliminary artificial nest bioassays showed that the most promising insecticidal (*M. americana*) and fungicidal (*S. alata*) plant extracts might be useful in the control of *A. octospinosus* in Guadeloupe and where the species is exotic.

Further studies should be conducted to optimize the toxicities of these extracts against *A. octospinosus,* to verify that they lack toxicity against non-targeted organisms and to confirm their activities in entire nests in natural conditions.

Acknowledgements

The authors thank Cécilia Delag, Fred Burner, Andève Mulciba, Guy Gougougnan, Michèle Salles, and Hervé Mauléon for their technical assistance.

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