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Research Article

Action mode of fipronil and sulfluramid in baits on *Acromyrmex crassispinus* (Forel, 1909) (Hymenoptera: Formicidae) in laboratory conditions

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Abstract. The leaf-cutting ants (LCAs) of the genus *Atta* Fabricius, 1804 and *Acromyrmex* Mayr, 1865 (Hymenoptera: Formicidae) are major pests in forest plantations, agriculture and livestock. Toxic baits with the active ingredients fipronil and sulfluramid are the main method to manage these insects. The internal hygiene of the ant colony needs to be considered to carry out chemical control of LCA. The baits must act as a true 'Trojan horse' deceiving the toxic action during the moment of transport. The objective was to evaluate fipronil and sulfluramid in toxic baits against *Acromyrmex crassispinus* (Forel, 1909) (Hymenoptera: Formicidae) in the laboratory. The action was observed in the first hours after application. The pick-up of the bait with fipronil was more heterogeneous and more random than that with the sulfluramid. Individuals of *A. crassispinus* stopped cutting leaves after four and seven days after application (DAA) of fipronil and sulfluramid baits, respectively. The foraging activity tended to zero over time with the sulfluramid bait, but a low carry of leaves was observed on the 11th DAA with the fipronil bait. The fipronil and sulfluramid baits, in laboratory conditions, were toxic and efficient to the ant *A. crassispinus*. The lethal action of fipronil was faster, an undesired aspect in the management of social insects. A control method that simulates the mythical 'Trojan horse' on LCAs nests is desirable.

Keywords: chemical control, leaf-cutting ants, pest control.

Introduction

Leaf-cutting ants (LCAs) are major pests in several cultures in the Neotropical region, due to its behavior of cutting leaves to cultivate its mutualistic symbiotic fungus *Leucocoprinus gongylophorus* (Heim) Moeller, the colony food source (Hölldobler & Wilson 1990). These insects choose plants nutritionally adequate for its fungus without potentially harmful substances (Britto et al. 2016). The LCAs are truly social insects presenting social organization, foraging, fungus cultivation, hygiene and complex nest structure, what difficult their control (Della Lucia et al. 2014). Ever-increasing restrictive requirements by forest certification besides lack of control agents and few techniques to keep these ant pests below economic thresholds difficult the management of these pests in forest companies (Isenring & Neumeister 2010).

The internal hygiene of the ant colony needs to be considered to carry out chemical control of LCA. These insects have associate hygienic strategies with social complexity involving: 1) mixed chemicals from exocrine glands, mainly the metapleural ones, 2) antimicrobials from mutualistic bacteria cultured on the ant's exoskeleton, as observed in *Acromyrmex* Mayr, 1865 (Hymenoptera: Formicidae), 3) waste disposal compartmentalization and management, and 4) communication and worker recognition (Della Lucia et al. 2014). Toxic baits are effective (Britto et al. 2016) because they partially circumvent this complex hygienic structure, deceiving the mutualism between the ant and the fungus. The baits must act as a true 'Trojan horse' deceiving the toxic action during the moment of transport.

The LCAs are mainly managed in planted forests with chemical insecticides applied as fumigation, powder, thermal fogging and, mainly, as toxic granulated baits (Britto et al. 2016). The fipronil and sulfluramid in granulated baits (Zanetti et al. 2014) are the most used, viable and successful method to mange LCA in commercial forest plantations (Britto et al. 2016; Zanuncio et al. 2016) and their mechanisms to suppress ant colonies need further study (Antunes et al. 2000; Montoya-Lerma et al. 2012). Fipronil acts in the central nervous system, specifically the GABA (gamma-aminobutyric acid), and the sulfluramid is broken and transformed into DESFA (perfluorooctanesulfonamide) in the ant body, acting in the oxidative phosphorylation process, interrupting ATP production in the mitochondria and leading the insect to death (Schnellman & Manning 1990).

The insecticides fipronil and sulfluramid are not specific and they may cause undesirable toxic effects on non-target species and contamination of water sources and soil (Ying & Kookana 2006). These active principles are a few used in granulated baits to manage LCAs because they suppress the ant colony after the baits are collected from the trails and taken to the nest. These granulated baits should not kill instantaneously the ant workers because the rapid death of these insects is an undesirable trait for toxic baits (Guedes et al. 2016). Ant colonies are suppressed either by directly compromising the minor workers, the fungus garden or the ant queen (Gandra et al. 2016).

The objective was to evaluate the action of fipronil and sulfluramid in toxic baits against *Acromyrmex crassispinus* (Forel, 1909) (Hymenoptera: Formicidae) in the laboratory.

Material and Methods

The assay consisted of three stages between October and December 2019: a) collection of the ants in the field, b) maintenance in the laboratory and application of the baits, and c) evaluation of the variables.

The ant *A. crassispinus* was selected from its visible nests above the surface covered with remains of dead plants on the top of the area where its fungus is cultivated, in the field in the localities of Diamante, Hasenkamp and Oro Verde, Entre Rios province, Argentina) (Fig. 1). The waste zone of these ant colonies is found outside and adjacent to the nest, with decomposed plant material with a light brown color (Fig. 1). One liter of fungus containing about 6,500 to 8,000 ants at all stages (eggs, larvae, pupae, smaller and larger workers in full foraging activity) was extracted from each ant colony and deposited per two-liter plastic containers (Gandra et al. 2016).

Figure 1. Nest of *Acromyrmex crassispinus* (Hymenoptera: Formicidae) in the Green Gold Botanical Garden in Entre Rios, Argentina (LS 31°50'2.8", LW 60°31'35").

Sixty-six colonies of *A. crassispinus* were conditioned in the laboratory with a maximum of 33 colonies per period in the containers. Twenty-two colonies were used per treatment: a) control, b) fipronil, c) sulfluramid. These containers were disinfected with 95% ethyl alcohol before replacing the colonies and inert liquid petroleum jelly placed on its sides to prevent the ants from leaving them. Each colony received pellets of baits without insecticide (placebo bait) within 24 hours of starting the experiment as a preparation for bait acceptance, thus avoiding its rejection. In addition, each colony received daily *ad libitum* green plant material composed mainly of clover until the bait application.

Three grams of the commercial baits with fipronil (0.003%) or sulfluramid (0.3%) were applied per ant colony three days after their acclimatization per period. Control colonies received daily green plant material *ad libitum* without commercial baits. The baits remained available for collection for 24 h, after which the remaining pellets were recovered and weighed to estimate the effective quantity transported by the ants. Five grams of fresh leaves were placed, daily, after the application of the bait until the eventual suppression of the colonies, that is, between seven and 11 days.

The mass of leaves remaining uncut by the ants per colony was evaluated after each application of the baits. Mortality of ant workers (mass of dead workers) was assessed daily by inspecting the waste area and those dead were recovered and weighed on a balance (0.1 mg). The count of each dead ant was not possible due to their large number and because they become entangled to each other. Six evaluations were carried out per colony, at one, three, four, five, seven, and 11 days after each bait application (DAA).

Data on the percentage of effective bait carried those for the fipronil (Shapiro-Wilk test, p=0.0027) and sulfluramid (Shapiro-Wilk test, p= 0.0359) baits did not fit the normality and thus they were evaluated using Mann Whitney U test. The weight of dead ants and of the remaining material were transformed (Templenton 2011) because they did not present a normal distribution (Supplementary Data). This method consists of transforming the variable into a percentile rank resulting in uniformly distributed probabilities and applying the inverse-normal transformation to the results as the first step to form a variable consisting of normally distributed z-scores. Levene's variance homogeneity test was used prior to performing the ANOVA. Subsequently, a one-way ANOVA was performed for both variables to establish the significant differences among treatments per day. Post-hoc comparisons using least significant difference (LSD) among treatments were made and a Student's t-test of paired data was used to establish differences among days per treatment.

Results

The ant *A. crassispinus* transported the baits with fipronil and sulfluramid to the interior of its colonies within 12 hours. The pick-up of the bait with fipronil was more heterogeneous and randomly than that with the sulfluramid. The effective carry rate was higher for the sulfluramid (78 ± 16%) compared to the fipronil (69 ± 30%) (W= 471.5, df= 43, p= 0.58, Mann Whitney U Test) baits. The mass of dead ants differed among treatments and evaluations (Supplementary data). The mass of dead ants was greater with fipronil than with the sulfluramid baits (Tab. 1).

Individuals of *A. crassispinus* stopped cutting leaves before four and seven days after the fipronil and sulfluramid baits application, respectively. The remaining green weight of the leaves in the treatment with the sulfluramid bait was greater than that with the fipronil bait, a symptom of almost total death of the colony with the first bait, from the 7th DAA until the end of test. The foraging activity tended to zero over time with the sulfluramid bait, but a low carry of leaves was observed in the 11th DAA with the fipronil bait (Fig. 2).

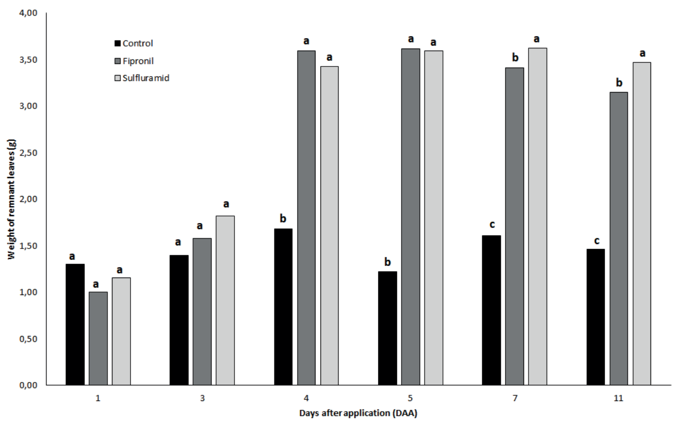

Figure 2. Weight of remnant of leaves by *Acromyrmex crassispinus* (Hymenoptera: Formicidae) after application of toxic baits (fipronil and sulfluramid) and control (without baits). Means with different letters are compared on each day by treatment, they are significantly different according to one-way ANOVA with LSD Post-hoc test (1DAA p=0.770; 3DAA p= 0.165; and the days after $p < 0.0001$, see Supplementary Material).

The mass of dead ants in the waste dump area was greater from the 1st DAA with the fipronil bait than with the sulfluramid one (Tab. 1).

Table 1. Compared mean (± SD) mass of dead ant workers (g) of *Acromyrmex crassispinus* (Hymenoptera: Formicidae) per treatment and day of evaluation in the control and after the application of the baits with fipronil and sulfluramid. Means with different letters are compared on each day by treatment, they are significantly different according to one-way ANOVA with LSD Post-hoc test (p < 0.0001 in all days, see Supplementary Material).

Treatments	Days after application (DAA)					
	1	3	4	5	7	11
Control	0.18±0.04ac	0.17±0.05ac	0.11±0.02a	0.14±0.01a	0.19±0.02a	0.23±0.06a
Fipronil	0.39±0.29b	1.05±0.59b	1.43±0.63b	1.90±0.98b	2.35±1.15b	4.60±1.37b
Sulfluramid	0.11±0.09c	0.44±0.25c	0.88±0.39c	1.70±0.85b	2.31±0.68b	3.05±0.75c

These masses of dead ants increased over time until the 3rd DAA when it was higher and decreased after this day. The leaf remnants were similar in the nests treated with sulfluramid and fipronil baits especially at 11 DAA (Fig. 2). The minor workers of *A. crassispinus* dispersed the toxic baits within the fungus garden (Fig. 3). A parasitic fungus invaded the colony from the 4th DAA and completely colonized and inhibited the growth of the symbiotic fungus *L. gongylophorus* of this ant at the end of the trial.

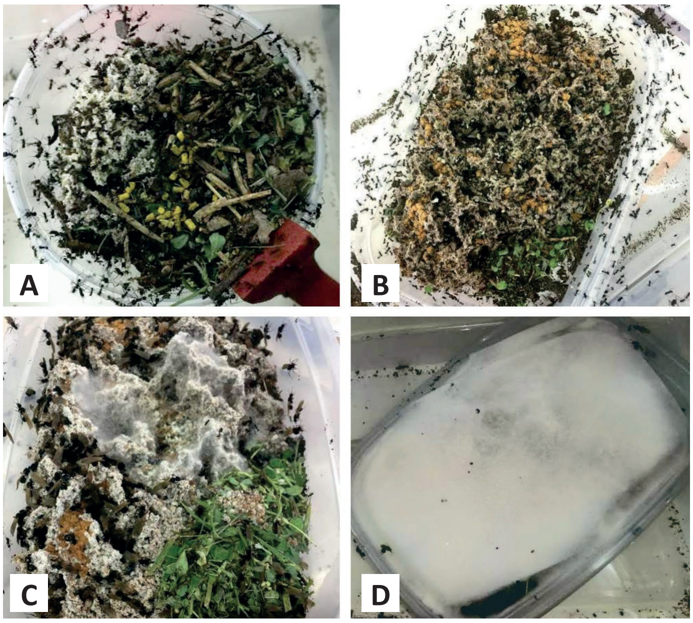

Figure 3. *Acromyrmex crassispinus* (Hymenoptera: Formicidae) workers brought and deposit the baits on the top of the fungus garden (A). The bait was moistened with the internal humidity of the colony and disintegrated (B). The ants deposited the cut leaves material on the fungus hyphae. The invasion of a parasitic fungus was observed from the 4th day after application (DAA) (C) and inhibited the growth of the symbiotic fungus *Leucocoprinus gongylophorus* at the 11th DAA (D). Numerous dead ants were observed in the container.

Discussion

The ant *A. crassispinus* transported the fipronil and sulfluramid baits before 12 hours of their supply, a transport period normal under laboratory but higher than that for *Acromyrmex subterraneus subterraneus* (Forel, 1893) (Hymenoptera: Formicidae) that only transports 66 ± 17% of a fipronil bait in the first 24 hours, as reported by Gandra et al. (2016). The LCA *A. crassispinus* picked-up and introduced the bait granules with fipronil and sulfluramid into the nest at the same rate as the placebo ones (Gandra et al. 2016). Therefore, no repellence or direct short-term acute mortality was detected in the foraging ants and the well acceptance of these baits into the colony is a pivotal trait for the success of suppressing colonies of LCAs (Della Lucía & Vilela 1993).

The heterogeneity of the transport of baits with fipronil compared to that with sulfluramid confirms that the latter is more accepted and transported. This was observed for workers of the ants *Atta bisphaerica* (Forel, 1908), *Atta capiguara* (Gonçalves, 1944), *Atta cephalotes* (Linnaeus, 1758), *Atta laevigata* (Smith F., 1858) and *Atta sexdens rubropilosa* (Forel, 1908) (Hymenoptera: Formicidae), which cut dicotyledons and grasses, with a control efficiency from 90 to 100% of their colonies (Zanuncio et al. 1992; 1993; Laranjeiro & Zanuncio 1995; Forti et al. 2003), and of *Acromyrmex* spp. (Sabattini et al. 2022). However, the similar carry rates of fipronil and sulfluramid baits in field studies were, possibly due to the high-quality physical properties of both baits (Sabattini et al. 2022). The percentage of bait carried also depends on its attractants, individual weight and mass and physical structure (Rudolph & Loudon 1986; Wetterer 1994; Roschard & Roces 2003) with this third parameter affecting transport speed. The load transported by the ant workers maximizes this parameter, but it is well below that which would maximize the individual transport rate per ant worker (Roschard & Roces 2003).

The variations in the efficiency of fipronil is due to its heterogeneity in the transport (Sabattini et al. 2022), even in controlled experimental conditions and this would bring problems in operational conditions of management programs of LCAs in forest plantations (Sabattini 2018). The low transport rate will increase the exposure to the bait in the field and thus increasing the impact on non-target organisms. Consequently, it would generate inconsistencies and complications from the environmental point of view, reducing the possibilities of using fipronil in granulated baits as an alternative to managing LCAs compared to sulfluramid, with a more homogeneous transport rate. Bait transport rate is the main factor determining the control efficiency of LCAs because no effect on the colony will occur without or with deficient bait transport. The higher transport of sulfluramid baits compared to that with fipronil by *Acromyrmex* spp. is, possibly, due to the heterogeneity among species of this genus (Nagamoto & Forti 1999; Forti et al. 2003). However, this compound is highly efficient against other insects such as cockroaches due to its rapid action (Buczkowski & Schal 2001), but it is not ideal for social or semi-social insects, as reported for *Acromyrmex lundii* (Guérin-Méneville, 1838) (Hymenoptera: Formicidae) (Ríos de Saluso 2010).

The greater weight of dead ants with the fipronil bait than with sulfluramid is due to the more rapid action of the first insecticide and the slower action of the latter even with no ants alive in the 11th day of evaluation. The sulfluramid increases the spread of this active ingredient by grooming and allogrooming among ants and thus benefiting the ant control (Mota Filho et al. 2021). The two modes of action, contact and ingestion, of the insecticide fipronil (Tomlin 2000) cause a knock down effect, with hyperactivity, inability to move and death in a short period of time of ants contaminated by this compound. This effect is because fipronil belongs to the phenyl pyrazole chemical group, inhibiting the neurotransmitter GABA and, consequently, blocking the transmission of signals from nerve cells (Cole et al. 1993; Tomlin 2000).

The perception that fipronil affects the fungus garden of LCAs is unlikely because no fungicidal activity has been found for this compound but it favored the contamination by *Escovopsis* sp., a specialized fungus, accelerating the eventual suppression of the ant symbiotic fungus garden. The insecticide activity on the minor workers manipulating the fungus garden and in contact with this active principle for a long time explains this effect.

The paralysis in the cutting and transporting of leaves after 24 hours of both baits application differed from longer periods for sulfluramid baits but confirmed the common symptoms in LCAs by this active ingredient. Symptoms of intoxication by sulfluramid, *i.e.*, in ant workers include slow movement and decreased aggressiveness due to their energy drop until interrupting ant metabolism and causing their death (Britto et al. 2016). This condition would generate greater effectiveness in the control of ant colonies and these results support the hypothesis that the baits should have low toxicity, *i.e.*, less than 15% mortality after 24 hours exposure and more than 89% mortality at 20 days (Stringer et al. 1964). Social interactions increasing insecticide dispersion among LCA workers is a disadvantage because the distribution of a toxin throughout the entire colony (Hooper-Bui & Rust 2000) stops as soon the warning signals are triggered. For this reason, as mentioned by Britto et al. (2016), the "ideal" bait must be: a) attractive and composed of some sugary or protein food to maximize its transport, b) size and shape suitable to facilitate its collection, c) easy to break in aqueous conditions, d) delayed toxic action without repellence and effective at low dosage range, and e) formulated with waterproofing agents to reduce effects of environmental conditions on its properties.

Chemical control with baits, formulated with active ingredients at very low concentration (sulfluramid at 0.3%) is still the only available technology with technical, economic and operational feasibility to manage the LCAs of the genera *Atta* Fabricius, 1804 and *Acromyrmex*. This formulation presents low cost, great efficiency and low risks to man and the environment. Sulfluramid is the only active ingredient with adequate characteristics for toxic baits and the only effective option to manage these insects (Forti et al. 2007; Nagamoto et al. 2007; Della Lucia et al. 2014). Therefore, the maintenance of this active ingredient is very important, and its discontinuation may cause a setback in the management of LCAs, such as an increase in the population of this pest and greater losses for the agribusiness.

The fipronil and sulfluramid baits, in laboratory conditions, were toxic and efficient to *A. crassispinus*. The lethal action of fipronil is faster, an undesired effect in the management and control of social insects. On the other hand, lethal action of the sulfluramid is delayed that would be a priori to increase the toxic effects within the colony. This work provides new results to confirm the hypotheses about the difficulty in controlling LCAs.

A control method that simulates the mythical 'Trojan horse' on LCA nests is necessary. This will provoke unexpected toxic effect with the longest possible period to expand the lethal action on the colonies avoiding detectable damage on these insects before their death. This search must be based on the basic pillars of environmental sustainability, minimizing risks to human health and maximizing the economic benefits by reducing damage by these insects on, for example, forest plantations.

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Authors' Contributions

JS conceived the research, conducted statistical analyses and experiments, and wrote and discussed the manuscript; JZ and RC tabulated the data, discussed statistical analyses and wrote the manuscript; LCF analyzed and discussed the manuscript. All authors read and approved the manuscript.

Conflict of Interest Statement

The authors declare no conflicts of interest.

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