Trends in Entomology
Vol. 13, 2017

Original Communication

Combined biological and chemical control of Neotropical leaf-cutting ants (*Acromyrmex* spp.) under field conditions

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ABSTRACT

Acromyrmex crassispinus and *Acromyrmex heyerii* are two of the leaf-cutting ants found in Uruguay. These ants are dominant herbivores in the Neotropics and constitute an economically important pest in agriculture and forestry, causing severe defoliations in very young plants. Although *Metarhizium anisopliae* and *Beauveria bassiana* are well-known entomopathogens, the main aim of this study was to evaluate the use of these fungi together with lower doses of chemical products for insect control. This combined application of entomopathogenic fungi with insecticides for reducing ant populations under field conditions could be a more environment-friendly solution. The effect of different commercially available insecticides on conidia viability *in vitro* was also evaluated. Under field conditions spores of *B. bassiana* and *M. anisopliae* were assayed in ant nests with a previous application of fipronil and sulphluramid. The loss of nest activity after 1 or 2 weeks showed the effectivity of the conidia formulation and the fipronil and sulphluramid doses in *Eucalyptus* plantation.

KEYWORDS: leaf-cutting ants, *Eucalyptus*, ant baits, *Acromyrmex*

INTRODUCTION

Acromyrmex (Hymenoptera: Formicidae: Attini) is a genus of New World ants. This genus of leaf-cutting ants is found in South America and parts of Central America and the Caribbean Islands, and it contains 31 known species. Leaf-cutting ants of *Acromyrmex* spp. are the main pests found in *Pinus* and *Eucalyptus* plantations [1, 2, 3, 4, 5]. A single leaf-cutting ant colony per hectare of forest can reduce the annual tree growth by 5% in *Eucalyptus* and by 10% in *Pinus* [6]. These estimates were based on the relationship between the leaves correlating to the fungus mass used as ant food and the quantity of waste produced during the same time period [3]. However, they are ecologically important herbivores in the Neotropics since they harvest large quantities of vegetation that they masticate and use as substrate for the fungal symbiont that form the sole food of the queen and other colony members [7]. The activity of the ants as soil modifiers is much appreciated because nest building alters the chemical and physical properties of the soil, promoting the cycling of nutrients [8]. *Acromyrmex crassispinus* and *Acromyrmex heyerii* are two of the species of leaf-cutting ants found in Uruguay [9]. *A. crassispinus* "black ant" and *A. heyeri*, "red ant" build their nests with plant materials and mud arranged in layers. The chamber where mushroom is cultivated (garden) can be seen at soil level or slightly below. The nest can reach up to one meter diameter depending on the age of the colony [10, 11]. Damages caused by these ants result in important production losses in agriculture [12].

Chemical pesticides fipronil and sulphluramid are registered for several uses including the control of termites and ants. Fipronil belongs to the phenylpyrazole chemical family and sulphluramid to fluorinated sulphluramid chemical family.

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The prohibition on these and other pesticides used for insect control was temporarily derogated by the Forest Stewardship Council® (FSC) recently [13]. These derogations have been individually approved by the FSC Pesticides Committee that includes Uruguay. Certification is important for sustainable forest management, and it may also permit access to new markets. In addition, certification ensures compliance of forest management activities with local social and environmental laws, especially those that relate to protected areas and conservation estates [14]. The FSC "Principles and Criteria" includes standards for forest certification, such as the "Pesticide Policy," which describes the relevant requirements of FSC with regard to the use of pesticides for forest management.

To date several mycopesticides such as *Metarhizium* and *Beauveria* have been developed and used; however entomopathogens remain a small percentage of the total insecticides. The main reason for the reduced interest of fungi as insecticides is their slow killing rate and an increase is directly related to the killing speed [15]. A large number of studies have been conducted to improve and potentiate the virulence of entomopathogenic fungi. About 33.9% of mycoinsecticides is based on *Beauveria bassiana* and *Metarhizium anisopliae*, followed by *Isaria fumorosea* (5.8%) and *Beauveria brongiartii* (4.1%) [16, 17]. *Beauveria bassiana* and *Metarhizium anisopliae* have been evidenced to be effective fungi for *Acromyrmex* spp. control in *Eucalyptus* plantations [18, 19, 20]. There are several advantages of using fungi as insecticide; for e.g. they can reduce the hazardous effects on the environment and the health of mammals and can avoid the development of insect resistance as it usually occurs in the case with chemical insecticides. Moreover, some have endophytic ability, thereby playing important roles in plant activation of immune system. Thus, the aim of this work was to evaluate the effectivity of combined control using fipronil and sulphluramid and the entomopathogenic fungi, *Metarhizium anisopliae* and *Beauveria bassiana* to reduce the incidence of *Acromyrmex* spp. in *Eucalyptus* plantations.

MATERIALS AND METHODS

M. anisopliae FI 2411 was isolated as an endophyte from the stem xylem *of Eugenia uruguayensis*, a native Myrtaceae, located at the southeast of Uruguay, and *B. bassiana* FI 2121 was isolated from dead ants. Identification of both species was done based on the micro-morphological characteristics and confirmed by sequencing of ITS 1, 5.8S, ITS 2rDNA region and comparing with DNA sequences from GenBank.

To assess the germination on fipronil (Fiproon® 20% Mercomar, Uruguay), 500 µL of conidial water suspension with 0.02% Tween 80 (10^5 conidia/mL) was spread on malt extract agar (MEA) with 100, 200, 300, 400, 500, 600 and 1000 ppm of fipronil. Five replicates were performed.

To assess the germination on sulphluramid (Agrimex-S® 0.3%, Agritec S.A., Uruguay), 500 µL of conidial water suspension with 0.02% Tween 80 (10^5 conidia/mL) was spread on MEA with 90, 180, 360, 720 and 1500 ppm of sulphluramid. Five replicates were performed. As the sulphluramid has a very low water solubility it was diluted with acetone and mixed with MEA. Plates were placed at security chamber for acetone evaporation. At 24hs, 300 conidia of each fungus were counted for each concentration. The percentage of conidia germination was calculated. The conidia were considered viable if the long of germ tube was the double of the spore size.

The combined control with insecticides and entomopathogenic fungi was conducted at La Curva plantation route 8, Km 104, Department of Lavalleja in October 2014. Trials were performed with Lampo (fipronil 0.003%) and Agrimex-S (sulphluramid 0.3%) baits and with the entomopathogenic fungi *M. anisopliae* FI 2411 and *B. bassiana* FI 2121.

A total of 63 *A. crassispinus* nests were used, out of which 9 served as controls and of the remaining 54 nests, 2 g each of Lampo (fipronil) was placed in 27 nests and 1 g each of Agrimex (sulphluramid) was placed in the other 27 nests. After 7 days of these treatments, 2 L of *M. anisopliae* conidia suspension (10^7 conidia/mL) was added in 18 nests and 2 L of *B. bassiana* conidia suspension (10^7 conidia/mL) was added in other 18 nests. Nine nests remained as treated only with 1 g of sulphluramid and nine remained as treated only with 2 g of fipronil (Table 1).

The same trials were conducted in the selected nests of *A.heyeri* (Table 1). The percentage of completely inactive nests was calculated at 1 and 2 weeks.

Data of conidial germination were transformed as arcsen√x. Dunnett's test for multiple comparisons versus control group was performed to evaluate the effect of both insecticides on the percentage of germinated conidia of *B. bassiana* and *M. anisopliae*.

Fisher contingency tables were prepared to evaluate if significant differences existed among nests treated with fipronil, sulphluramid and both insecticide with the entomopathogenic fungi in relation to the controls.

RESULTS

The percentage of *M. anisopliae* conidia germination with fipronil revealed that it was slightly more susceptive than *Beauveria* (Figure 1).

B. bassiana and *M. anisopliae* conidia germination were completely inhibited at 1000 ppm of fipronil concentration *in vitro*.

According to the Dunnett's test, significant differences existed between the percentage of *M. anisopliae*

Table 1. Treatments performed in *A. crassispinus* and *A. heyeri* nests.

Time (days)	Treatments	*A. crassispinus*							*A. heyeri*						
		F	FM	FB	S	SM	SB	C	F	FM	FB	S	SM	SB	C
1	Pesticide application (g/nest)	2	2	2	1	1	1	_	2	2	2	1	1	1	_
7	Fungal application [10^7 conidia/mL] (L/nest)	_	2	2	_	2	2	_	_	2	2	_	2	2	_
Number of nests		9	9	9	9	9	9	9	9	9	9	9	9	9	9

F: fipronil; FM: fipronil and *M. anisopliae*; FB: fipronil and *B. bassiana*; S: sulphluramid; SM: sulphluramid and *M. anisopliae*; SB: sulphluramid and *B. bassiana*; C: control.

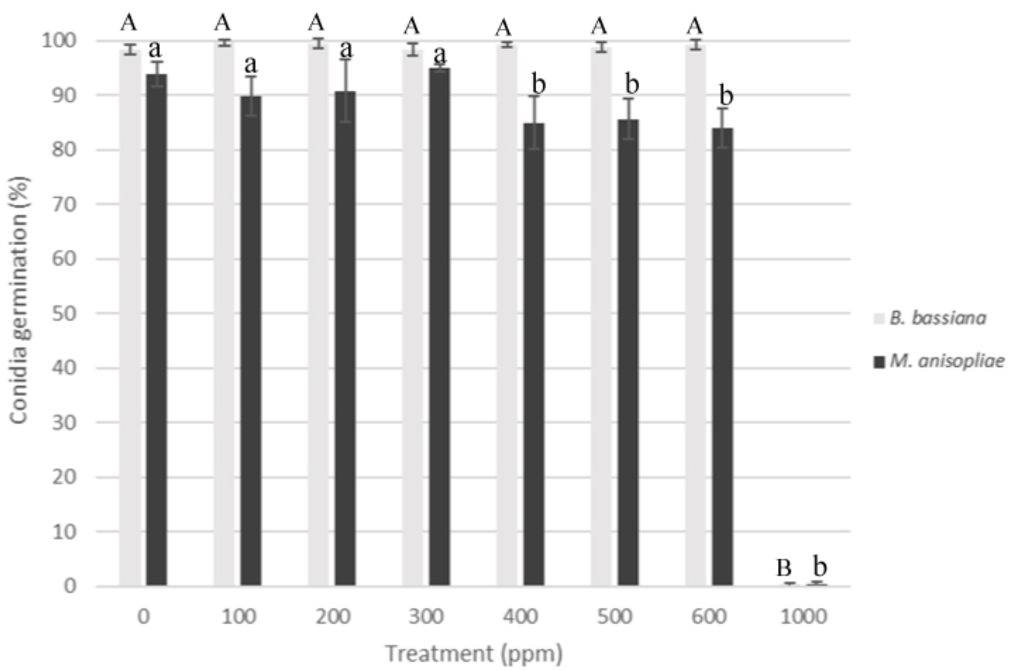

Figure 1. Effect of fipronil on *B. bassiana* and *M. anisopliae* conidia germination.
Averages followed by the different upper case alphabets for *Beauveria*, and lower case alphabets for *Metarhizium*, differ significantly (p ≤ 0.05).

conidia germination and the control. Significant differences were also evident between *B. bassiana* and *M. anisopliae* at fipronil concentrations above 400 ppm.

Metarhizium was more susceptive to the effect of sulphluramid on conidia germination than *Beauveria*. *Beauveria* and *Metarhizium* conidia germination showed significant differences at 720 and 1500 ppm of sulphuramid concentration with respect to the control (Figure 2).

Trials performed under field conditions with 2 g of fipronil at first and second weeks showed that 38% of *A. crassispinus* nests were inactive. Significant differences ($p \leq 0.05$) existed between control nests and those treated with fipronil. In the case of the nests treated with fipronil combined with *M. anisopliae*, 83% were inactivated at the second week and in the case of those treated with fipronil and *B. bassiana* 86% were inactivated reflecting significant differences with the control ($p \leq 0.05$).

In trials with 1 g of sulphluramid 22% of nests were inactivated at the first and second weeks showing significant differences with the control ($p \leq 0.05$). Trials with 1 g of sulphluramid combined with *M. anisopliae* showed that 63% and 87% of nests were inactivated at the first and second weeks, respectively, showing significant differences with the control ($p \leq 0.05$). Both fungi were equally efficient for ant control (Table 2).

The same treatments were performed on *A. heyeri* ant nests. Trials with 2 g of fipronil showed that 12% of treated nests were inactive at the first week and 19% at the second. Significant differences ($p \leq 0.05$) existed between the control nests and those treated with fipronil. Fipronil combined with *M. anisopliae* inactivated 100% of nests and when combined with *B. bassiana* it inactivated 60% at the first week and 100% at the second week. Significant difference existed between the percentage of inactive control nests and those treated with fipronil combined with *B. bassiana* or *M. anisopliae* ($p \leq 0.05$).

In trials with 1 g of sulphluramid 10% of nests were inactivated at the first week and 50% at the second showing significant differences with control ($p \leq 0.05$). Sulphluramid combined with *M. anisopliae* inactivated 100% of nests at the first week and when combined with *B. bassiana* it inactivated 100% of nests at the second week. Significant differences were evidenced with control nests ($p \leq 0.05$).

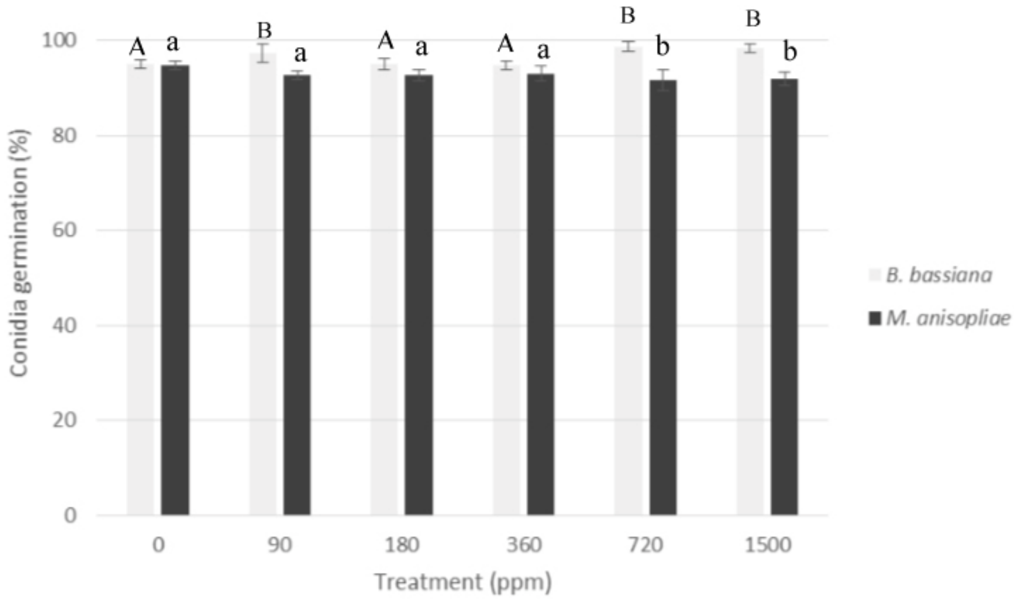

Figure 2. Effect of sulphluramid on *B. bassiana* and *M. anisopliae* conidia germination. Averages followed by the different upper case alphabets for *Beauveria*, and lower case alphabets for *Metarhizium* differ significantly ($p \leq 0.05$).

Table 2. Nests inactivated with each treatment.

Treatments	F	FM	FB	S	SM	SB	C	F	FM	FB	S	SM	SB	C
Week after fungal application	**% of inactive nest (*A. crassispinus*)**							**% of inactive nest (*A. heyeri*)**						
1	38[a]	50[a]	71[b]	22[a]	63[b]	0[a]	0[a]	12[a]	100[b]	60[b]	10[a]	100[b]	89[b]	0[a]
2	38[a]	83[b]	86[b]	22[a]	87[b]	50[a]	20[a]	19[a]		100[b]	50[a]		100[b]	20[a]

F: fipronil; FM: fipronil and *M. anisopliae*; FB: fipronil and *B. bassiana*; S: sulphluramid; SM: sulphluramid and *M. anisopliae*; SB: sulphluramid and *B. bassiana*; C: control. Different superscripted alphabets between columns of each ant species indicate significant differences, $p \leq 0.05$.

DISCUSSION

Conidia germination was inhibited with pesticides at doses that were higher than those recommended for commercial use, revealing significant differences between *M. anisopliae* and *B. bassiana* conidial germination at some of the concentrations of fipronil and sulphluramid. Germination is a crucial step for entomopathogenic fungi since enough penetration sites assure successful infection in ants. Schumacher & Phoeling found [21] that *M. anisopliae* conidia germination was inhibited at a fipronil concentration lower than what was used in this study but the contact between the insecticide and fungal conidia never occur for a long period under field conditions [22, 23, 24]. Sublethal chemical pesticides may be able to break defense mechanisms, and the entomopathogen may induce an epizootic within the ant nest [25, 26].

Neither fipronil nor sulphluramid was effective for nest inhibition of both ant species since the doses used were lower than those commercially recommended. On the other hand, entomopathogenic fungi as mycoinsecticides have the inconvenience of its slow killing rate, and several rounds of treatments are necessary to be effective [20]. It must be taken into account that the increase in the commercial use of entomopathogens is directly related to the killing speed [15].

All *A. heyeri* nests were inactivated with only one treatment of *M. anisopliae* combined with fipronil and sulphluramid. Nearly 85% of *A. crassispinus* nests were inactivated at the second week with one treatment with *B. bassiana* or *M. anisopliae* combined with fipronil. Sulphluramid combined with *M. anisopliae* also exhibited nearly 85% of ant nest inhibition but with *B. bassiana* the efficiency was reduced to 50%. However, the percentage of conidia germination of *B. bassiana* and *M. anisopliae* did not evidence significant differences probably due a higher resistance of *A. crassispinus* to *B. bassiana* combined with sulphluramid. The treatment only with *Metarhizium* or *Beauveria* must to be repeated three times to be an effective control strategy [20]. Combined treatments were more successful since one treatment could control 100% of ant nests in one or two weeks. These laboratory and field studies on combined applications of pesticides with entomopathogenic fungi might provide a useful background for a more friendly strategy for handling the control of leaf-cutting ants.

CONCLUSION

M. anisopliae conidia germination is more susceptive to fipronil and sulphluramid than *B. bassiana*. The combined treatments are efficient for ant control under field conditions, being more effective for *A. heyeri* nest inhibition than for *A. crassispinus*.

M. anisopliae combined with fipronil or sulphluramid can inactive all *A. heyeri* nests one week before than when used with *B. bassiana*.

Combined treatments of *M. anisopliae* with either fipronil or sulpluramid could be a good strategy for *Acromyrmex* control since 100% of ant nests are inactivated in one or two weeks with a lower insecticide concentration than that is commercially recommended. Ant control is speedier with combined treatments than treatment only with entomopathogens.

These field studies on combined applications of pesticides with entomopathogenic fungi could be a useful background for the development of friendly strategies for the control of leaf-cutting ants.

ACKNOWLEDGEMENTS

The authors acknowledge the financial support of Agencia Nacional de Investigación e Innovación and Forest Producers Association.

CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

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