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Neotropical leaf-cutting ants (*Acromyrmex* spp.): biological control under laboratory and field conditions

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ABSTRACT

Acromyrmex lundi and *Acromyrmex heyeri* are some 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. As *Metarhizium anisopliae* is a well known entomopathogen, the main aim of this study was to evaluate the ability of this fungus for reducing ant populations under field conditions. *Metarhizium* conidia infect through the cuticle. During the process of infection ants secrete chitinase to digest the cuticle and the hemocele is invaded by the mycelium. When the insect is dead the fungus grows saprophytically and sporulate on the ant body. The median lethal dose of conidia was determined and the successful control of *A. lundi* and *A. heyeri* with *M. anisopliae* under laboratory conditions was achieved. Under field conditions different conidia concentrations of *M. anisopliae* were assayed and the volume was adjusted according to the nest size, shape and localization over or under ground. The loss of nest activity after weekly application, during 3 to 4 weeks showed the effectivity of the conidia formulation. As chemical pesticides commonly used for the control of ants will probably be eliminated in the medium term, a transient solution could be the use of conidia and compatible pesticides.

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KEYWORDS: *Metarhizium anisopliae*, cutting ants, ant hill, forest and agricultural soils

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. They are social insects having an obligatory mutualism with a fungal symbiont. The ants actively cultivate their fungus on a medium of masticated leaf tissue. This is the sole food of the queen and other colony members that remain in the nest. The fungus generally exhausts the substrate within 3-4 months after planting [1]. At this point the cultivar is moved by ants to a dump. Before leaving her nest, the queen stores a small pellet of the fungus at the bottom of her mouth. She carries this inoculum during the mating flight and then expels it to start a garden after locating and building nest in a suitable site [2]. The type of nutrition and the longevity of colonies explain why these ants are dominant in the neotropic regions [1, 3]. However, they are ecologically important herbivore since they harvest large quantities of vegetation they use as substrate for the fungus [1]. 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 [4, 5].

Acromyrmex lundi and *Acromyrmex heyeri* are some of the species of leaf-cutting ants found

in Uruguay [6]. Nests of *A. lundi*, "black ant", are located under ground at a depth of one or two meters with only one chamber where mushroom is cultivated (garden). These nests are of substantial size, more than a half meter in diameter. *A. heyeri*, "red ant", build their nests with plant materials and mud arranged in layers. The 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 [7]. Damages caused by these ants result in important production losses in agriculture and forestry [8]. As *Metarhizium anisopliae* is a well known entomopathogen, the main aim of this study was to evaluate the ability of this fungus for reducing ant populations under field conditions.

The pathogenic mechanism of this fungus starts with the adhesion of the conidia in the insect cuticle, formation of an appressorium and penetration by mechanical pressure and enzymatic activity. The hemocele is invaded by the mycelium leading to death of the insect. Finally hyphae emerge and grow saprophytically on the ant body, sporulating and infecting other ants. During the process of infection of ants, the fungus secretes chitinase to digest the cuticle and the hemocele is invaded by the mycelium. When the insect is dead the fungus grows saprophytically on the ant body, sporulating on the insect and forming abundant conidia [9, 10, 11].

Chemical pesticides are commonly used for ant control. As these pesticides will probably be eliminated in the medium term, a transient solution could be the use of pesticides and fungi as combined control.

The aim of this work was to evaluate the median lethal dose of *M. anisopliae* strain MVHC 1878, the viability during medium term storage and the efficiency of different conidia formulations for the biological control of *Acromyrmex* spp. under field conditions.

MATERIALS AND METHODS

M. anisopliae MVHC 1878 was isolated from a prairie soil located at the southeast of Uruguay. The identification was done based on the micro-morphological characteristics and confirmed by sequencing of ITS 1, 5.8S, ITS 2 rDNA region and comparing with DNA sequences from GenBank. This isolate was selected after preliminary pathogenicity tests against *A. heyeri* and *A. lundi* under laboratory conditions [12].

M. anisopliae was grown on Potato Dextrose Agar (PDA) (Baker) plates at 25 ºC for 14 days. Conidia were harvested by scraping each plate with a glass rod, suspended in sterile distilled water with 0.05% Tween 80 and vortexed for 3 min to produce a homogenous suspension. The concentration of the stock suspension was checked with a haemocytometer (Hausser Scientific) and adjusted to 10^{10} conidia ml^{-1} using sterile distilled water with 0.05% Tween 80. Each insect was inoculated with 0.5 µl of 10^8, 10^7, 10^6, and 10^4 conidia ml^{-1} in water with Tween 80 (0.05%). Six insects per plate were inoculated and 10 replicates were made. Ants were placed in Petri dishes with two drinking-troughs containing 300 µl of sugar water (50% w/v) and cotton saturated in water to maintain a constant relative humidity. Insects without fungal infection, under the conditions described above were used as control. Mortality was evaluated daily. Dead ants were transferred to a humid chamber, allowing mycelial growth and sporulation of the fungus, in order to confirm if death was due to infection by the inoculated fungus. The median lethal dose was calculated using the Probit program. The time considered to calculate median lethal dose was 10 days since at this moment the percentage of mortality in control group was lower than 20%.

Conidia production was carried out in bags containing 200 g of rice with 50 ml of distilled water autoclaved at 121 ºC for 20 minutes. They were inoculated with 40 ml of conidia suspension, placed on shelves and incubated for 15 days at 25 °C under 12 h light : 12 h dark cycle to allow sporulation. Conidia were separated from the substrate with a spores collector (MycoHarvester®) and conidia production was determined using Neubahuer chamber after serial dilution.

From a stock of powder with 4 x 10^8 conidia/g without rice residues obtained with the MycoHarvester®, 1.5 x 10^4 conidia were suspended in 10 ml of each conidial carriers. Mineral oil in water 10% (MO), emulsifiable oil in water 10% (EO), sunflower oil in water 10% (SO), tween 80 0.05% in water and distilled water (W) were the carriers used.

As conidia survival is dependent of temperature and water potential, one ml of each liquid formulation was poured on three Petri dishes of 90 mm containing malt-agar with glycerol adjusted to the following conditions: Ψ -0.69 MPa, -1.38 MPa, -2.78 MPa, -4.19 MPa and temperatures: 15 ºC, 20 ºC, 25 ºC, 30 ºC and 37 °C. After 24 and 48 hours of incubation the percentage of conidia germination was evaluated.

Water potentials were obtained by adding glycerol to culture medium as described in [13]:

glycerol g/l	0	51.6	102.2	152.96
Ψ	-0.69 MPa	-1.38 MPa	-2.78 MPa	-4.19 MPa

Three solid formulations, containing 10 g of conidia without the addition of carrier, 1 g of conidia with 9 g of maltodextrin and 1 g of conidia with 9 g of rice flour were assessed. The percentage of germination of conidia in these formulations was evaluated. Conidia were previously dried in a desiccator cabinet for four days to reduce the moisture content to around 5%, because an optimal moisture content for long term dried conidia storage was found to be 4-5% [14].

The activity of *M. anisopliae* as biological control agent (BCA) under field conditions was evaluated in several sites. Ten plots of 0.5-1 ha were delimited in grasslands, sorghum and *Eucalyptus* plantations, and a total of 100 nests, 5 in each plot were treated with the BCA and 5 with only the carrier as control. Table 1 shows the treatments performed and the distribution of *A. heyeri* nests (60) and *A. lundi* nests (40) in different sites and plantations at the end of the spring and during the summer when ants were more active. Nests of *A. lundi* located in *Eucalyptus* plantations were treated with 10 g of powder containing 10^8 conidia g^{-1} and nests located in grasslands with 30 g of powder containing 10^{10} conidia g^{-1}, insufflated through the nest hole. Three applications were realized, one each week and the activity of nests was recorded weekly during 4 weeks.

Nests of *A. heyeri* were sprayed with different volumes of liquid formulations according to the nest size (Table 1). In the biggest nests, with several holes, the conidia suspension used was higher than in those with few holes. An application once a week for 3 weeks was performed. Nests

Table 1. Treatments of ant nests with *M. anisopliae* conidia formulations.

Ant species	Nº T	Nº C	Formulation	Doses	Nº A	Cultures	Sites	Date
A. heyeri/G	5	5	W+T 0.05%	10^7 con/ml; 1L (T1)	3	sorghum	San José	D
A. heyeri/G	5	5	W+T 0.05%	10^7 con/ml; 1L (T2)	3	sorghum	San José	D
A. heyeri/G	5	5	W+T 0.05%	10^8 con/ml; 1L (T3)	3	grassland	Canelones	N
A. heyeri/G	5	5	W+T 0.05%	10^{10} con/ml; 2L (T4)	3	grassland	Canelones	N
A. heyeri/G	5	5	W+T 0.05%	10^7 con/ml; 2L (T5)	3	*Eucalyptus*	Soriano	M
A. heyeri/G	5	5	W+T 0.05%	10^7 con/ml; 2L (T6)	3	*Eucalyptus*	Soriano	M
A. lundi/S	5	5	rice flour	10^{10} con/g; 30g (T7)	3	grassland	Lavalleja	F
A. lundi/S	5	5	rice flour	10^{10} con/g; 30g (T8)	3	grassland	Lavalleja	F
A. lundi/S	5	5	rice flour	10^8 con/g; 10g (T9)	3	*Eucalyptus*	Soriano	J
A. lundi/S	5	5	rice flour	10^8 con/g; 10g (T10)	3	*Eucalyptus*	Soriano	J

Nº T : number of Treated nests; Nº C : number of Control nests; Nº A: number of applications; G : over-ground nests; S: under-ground nest. Mean temperature at the hours of applications was 25-30 ºC.
T1-T10 : Treatments. D : December, N : November, J : January, F : February, M : March.

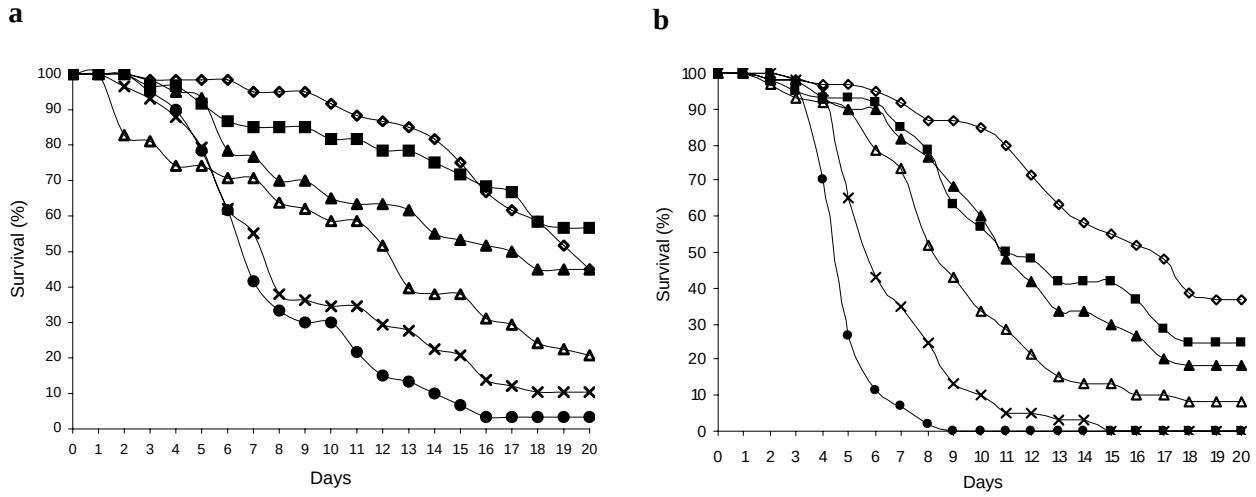


Figure 1 (a-b). Survival of infected ants with different doses of conidia. **a.** *A. heyeri* and **b.** *A. lundi*. Symbols indicate, ◊ : Control; ■ : 10^4; ▲ : 10^5; Δ : 10^6; x : 10^7; ● : 10^8.

were mechanically disorganized during applications. Control nests were similarly treated with carrier. The reduction of activity was evaluated weekly during 30 days.

Fisher test for multiple comparisons was performed on the number of inactive nests in relation to the controls. This test allows to compare the means of the *t* levels of a factor (concentration of conidia applied to ant hills), after having rejected the null hypothesis of equality of means using ANOVA.

The number of conidia of *M. anisopliae* corresponding to the median lethal dose for *A. heyeri* was 1961 and mortality did not reach 100%, 20 days after being infected (Figure 1 a) and the number of conidia for *A. lundi* was 94000 and the mortality reached 100% after 9 days (Figure 1 b). Dead ants when transferred to humid chamber evidenced the presence of mycelia of *M. anisopliae* growing and sporulating on them.

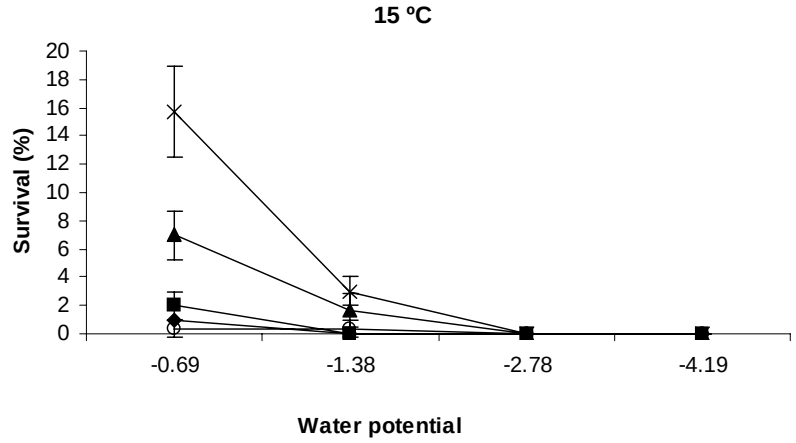
Figure 2 (a-e) shows the percentages of germination at 15 ºC, 20 ºC, 25 ºC, 30 ºC and 37 ºC at different water potential, after 48 hours of incubation. At 15 °C all formulations were ineffective. At 20 °C and -0.69 MPa, 80-100% of conidia germinated when they were suspended in EO, T and MO. At -1.38 MPa, 60-70% of conidia germinated when suspended in the same products. At 25 °C, 75-85% of conidia suspended in MO germinated at all water potential assayed except at -4.19 MPa. At 30 °C and -0.69 MPa, conidia suspended in W had the highest germination (70%) and at -1.38 MPa the germination was reduced to 25%. Finally, at 37 °C and -0.69 MPa, 40% of the conidia suspended in MO and 30% of those suspended in T germinated. In general, for field conditions during warmer time, T would be advisable to protect the conidia from dessication.

As nearly 75% of conidia mixed with rice flour germinated when stocked at 5 ºC after one month, conidia mixed with rice flour was used for applications of the BCA. As for nests of *A. lundi* located in a grassland after three applications with 30 g of 10^{10}/conidia/g with rice flour (RF) to each nest, 100% become inactive after four weeks. Whereas, 40% and 60% of nests located in *Eucalyptus* plantations after three applications with 10 g containing 10^8/conidia/g became inactive, at the 4th week (Table 2).

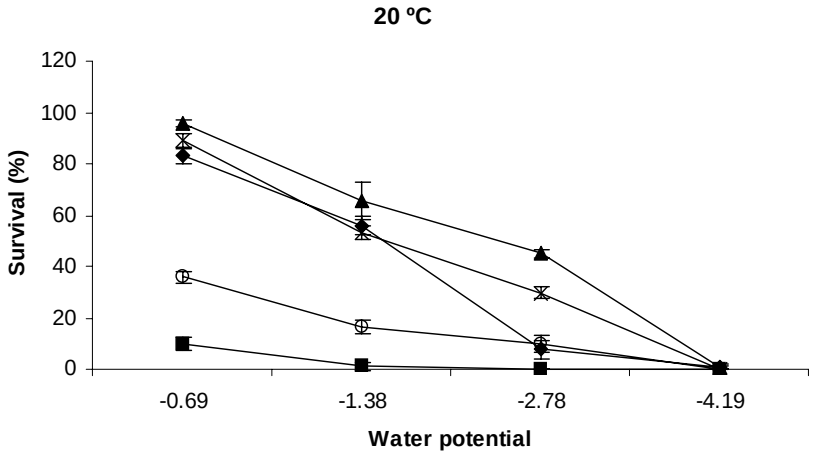
The application of 2 L of 10^7 conidia ml^{-1} on nests of *A. heyeri*, located in *Eucalyptus* plantations were completely controlled at the 4th week. Similar results were obtained when nests located in grassland were treated with 2 L of 10^{10} conidia ml^{-1}. The applications of 1 L with 10^8 conidia ml^{-1} to each nest reduced 60% of nest activity in similar grassland. The application of 1 L with 10^7 conidia ml^{-1} controlled 60% of nests in two sorghum plantations at week 4 (Table 2).

According with the Fisher test, significant differences ($p < 0.05$) among control and the

a


15 ºC
Survival (%)
20
18
16
14
12
10
8
6
4
2
0
-0.69
-1.38
-2.78
-4.19
Water potential

b


20 ºC
Survival (%)
120
100
80
60
40
20
0
-0.69
-1.38
-2.78
-4.19
Water potential

c

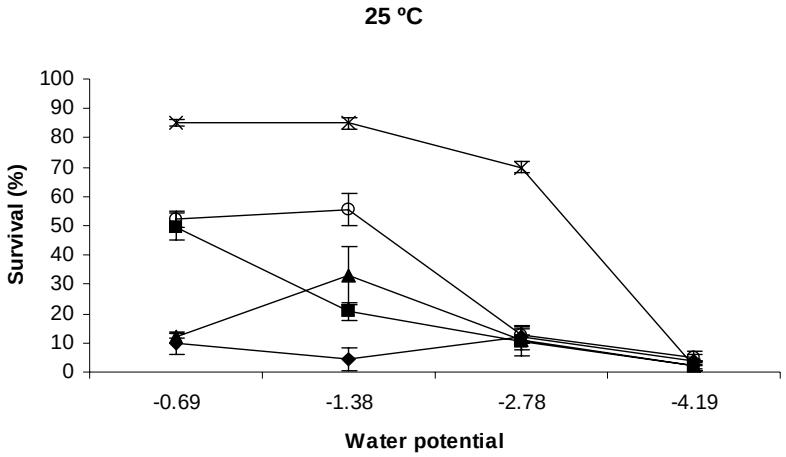
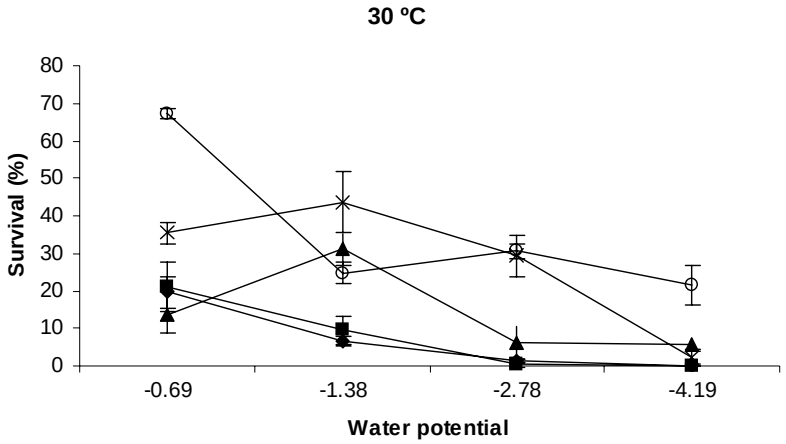

25 ºC
Survival (%)
100
90
80
70
60
50
40
30
20
10
0
-0.69
-1.38
-2.78
-4.19
Water potential

Figure 2

Figure 2 continued..

d



e

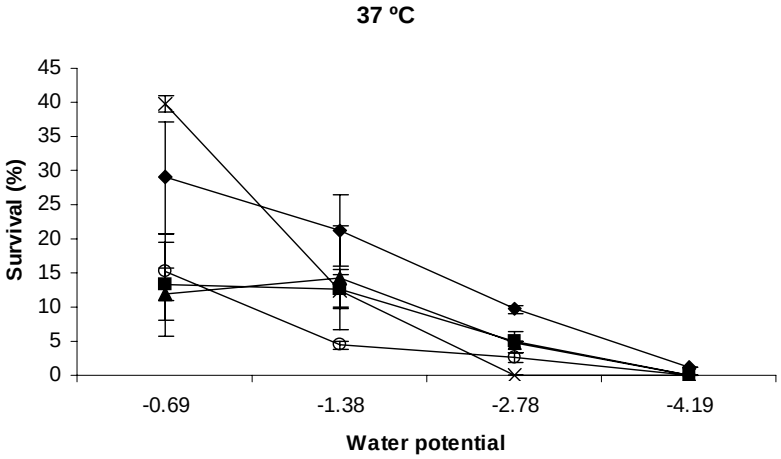


Figure 2 (a-e). Percentage and standard deviation of *Metarhizium* conidia germination at different water potential and temperature. Symbols indicate, ♦ : tween (T); ■ : sunflower oil (SO); ▲ : emulsifying oil (EO); X : mineral oil (MO); ○ : water (W).

treatments T4, T5 and T6 on *A. heyeri* nests and treatments T7 and T8 of *A. lundi* nests existed (Table 3). Then it was assumed that the two variables, conidia concentration and percentage of inactive nests were associated.

DISCUSSION

The strategies of fungal applications differed according to the architecture and size of nests. In agreement with this fact the garden emplacement over or under ground determines the most efficient formulation for reaching and infecting as many ants as possible. Nests of *A. heyeri* were completely controlled when 2 L of 10^7 conidia ml^{-1} was used showing that higher volumes at the same concentration were more efficient in controlling ants. Nests of *A. lundi* exhibiting galleries and chambers under-ground were inactivated with dry conidia formulations. It could be expected that the gallery system allows a higher viability and permanence of the conidia inside the nest, favoring individual infection.

Table 2. Inactive nests after treatment with *M. anisopliae*.

Ant species	Treatment	% of T Inactive nests	% of C Inactive nests	Cultures
A. heyeri/G	T1	60	20	sorghum
A. heyeri/G	T2	60	0	sorghum
A. heyeri/G	T3	60	0	grassland
A. heyeri/G	T4	100	20	grassland
A. heyeri/G	T5	100	20	*Eucalyptus*
A. heyeri/G	T6	100	0	*Eucalyptus*
A. lundi/S	T7	100	0	grassland
A. lundi/S	T8	100	0	grassland
A. lundi/S	T9	60	0	*Eucalyptus*
A. lundi/S	T10	40	20	*Eucalyptus*

T : Treated nests; C : Control nests; G : over-ground nests; S : under-ground nests. T1-T10 : number of conidia introduced in nests. T1 : 1 x 10^{10}, T2 : 1 x 10^{10}, T3 : 1 x 10^{11}, T4 : 2 x 10^{13}, T5 : 2 x 10^{10}, T6 : 2 x 10^{10}, T7 : 3 x 10^{11}, T8 : 3 x 10^{11}, T9 : 1 x 10^{9}, T10 : 1 x 10^{9}.

Table 3. Fisher test for multiple comparisons among diferent treatments and control.

Acromyrmex heyeri							
Treatment	Control	T1	T2	T3	T4	T5	T6
Control	-	0.083	0.083	0.083	**0.004**	**0.004**	**0.004**
T1	-	-	0.476	0.476	0.222	0.222	0.222
T2	-	-	-	0.476	0.222	0.222	0.222
T3	-	-	-	-	0.222	0.222	0.222
T4	-	-	-	-	-	1	1
T5	-	-	-	-	-	-	1
T6	-	-	-	-	-	-	-

Acromyrmex lundi					
Treatment	Control	T7	T8	T9	T10
Control	-	**0.004**	**0.004**	0.083	0.222
T7	-	-	1	0.222	0.083
T8	-	-	-	0.222	0.083
T9	-	-	-	-	0.5
T10	-	-	-	-	-

Number of conidia, T1 : 1 x 10^{10}, T2 : 1 x 10^{10}, T3 : 1 x 10^{11}, T4 : 2 x 10^{13}, T5 : 2 x 10^{10}, T6 : 2 x 10^{10}, T7 : 3 x 10^{11}, T8 : 3 x 10^{11}, T9 : 1 x 10^{9}, T10 : 1 x 10^{9}. The numerical characters in bold indicate significant differences ($p < 0.05$) among control and the treatments.

M. anisopliae could constitute a viable alternative for pesticide replacement considering the low cost of production. Highly virulent isolates may not be ideal candidates for biological control programs, especially in the case of social insects, since disease symptoms produced by *Metarhizium* can be detected and ants can leave the nest avoiding the fungus, but to date it is not reported.

From an environmental point of view, the use of *M. anisopliae* is recognized worldwide as safe for both human and animal health [15]. They don't accumulate in the environment since ant populations are naturally regulated in the soil and products used in formulation are biodegradable. Moreover, it must be taken into account that chemical pesticides for ants will be probably eliminated in the medium term due to their high toxicity [16]. An environment-friendly solution could be the use of biological agents with low doses of chemical products, compatible with conidia germination [17, 18].

CONCLUSION

Leaf-cutting ants (*Acromyrmex* spp.) were efficiently controlled with *M. anisopliae* conidia formulations under field conditions. The strategy of fungal applications differed according to the ant species since they have different nest architecture and size. The effectiveness of the strain of *M. anisopliae* used for the mortality of ants and to reduce the activity of ant nests could constitute a viable alternative for reducing the concentration of pesticide or its replacement.

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CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

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