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ORIGINAL RESEARCH

Contribution of the horizontal transmission of the entomopathogenic fungus *Beauveria bassiana* to the overall performance of a fungal powder formulation against *Triatoma infestans*

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Abstract: Control of domiciliated *Triatoma infestans*, the major Chagas disease vector in southern South America, is currently achieved by indoor residual spraying of infested houses with chemical insecticides. However, in recent years this strategy has been threatened by the emergence of pyrethroid-resistant bug populations. As an alternative approach, we have previously demonstrated the efficacy of the entomopathogenic fungus *Beauveria bassiana* to control *T. infestans* bugs regardless of their pyrethroid susceptibility. In this work, we tested the virulence and residual activity of a powdered fungal formulation, and studied the significance of the horizontal transmission process (autodissemination) to fungal infection of bugs. The *B. bassiana*-based formulation was highly virulent against all *T. infestans* stages, and maintained its insecticidal capability for at least 5 months under natural ambient conditions. We showed that horizontal transmission of conidia is associated to bug density, and contributes significantly to the overall population infection event.

Keywords: Chagas disease vectors, triatomines, biocontrol, autodissemination

Introduction

Chagas disease is the most important parasitic disease in Latin America; its incidence is almost three times the combined incidence of other parasitic diseases.[1] The number of people infected by *Tripanosoma cruzi*, the causative agent of Chagas disease, was estimated at 16–18 million; with a further 40 million considered at risk.[2] The major vector of *T. cruzi* in the Southern Cone area of South America is the "kissing bug" *Triatoma infestans*, usually infesting poorly constructed dwellings, and hiding in thatched roofs and cracks in the walls. Domiciliated *T. infestans* populations have been successfully controlled for more than 30 years by indoor residual spraying of pyrethroid insecticides. However, recent control failures due to pyrethroid resistance were detected in Bolivia and in some neighboring areas of Argentina.[3,4] Currently, there is a very strong need to develop new alternatives to control pyrethroid-resistant *T. infestans*.

We have already attempted to modify the chemical control paradigm by incorporating entomopathogenic fungi as a new biological control tool against this vector. Its efficacy was proven after showing that pyrethroid-susceptible and pyrethroid-resistant *T. infestans* are both killed by the entomopathogenic fungus *Beauveria bassiana*.[5] The major mode of entry for most entomopathogenic fungi is by penetration through the insect host cuticle. Fungal infection occurs after adhesion to the cuticle, germination, and finally penetration to the hemocoel. Surface structure and the chemical composition

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of the host cuticle are both assumed to affect the attachment and adhesion of fungal appressoria to the cuticle.[6]

Fungal horizontal transmission, or autodissemination, is a passive transference of conidia from either contaminated insects or sporulating mycosed cadavers to healthy insects;[7] therefore, it is essential for this event that a direct contact between potential insect hosts should take place. The characteristic *T. infestans* aggregation behavior in small nests – induced, among others, by contact aggregation pheromones[8] – facilitates insect contact, and consequently could help *B. bassiana* transmission by autodissemination, already shown to happen in laboratory assays.[5]

An attraction–infection trap based on *B. bassiana* conidia formulation combined with a chemical attractant[9] has already been tested for indoor *T. infestans* control.[5,10] Due to the successful results obtained, this fungal formulation has been incorporated in the control program of pyrethroid-resistant triatomines in the Argentinian province of Salta; national validation of the trap method is still pending. As part of a larger research project addressed to understand the complex interactions between entomopathogenic fungi and triatomines, the objectives of present study were: 1) to evaluate the efficacy of the *B. bassiana* powder formulation against all *T. infestans* stages, 2) to assess the residual activity of the fungal formulation for extended time periods in natural ambient conditions, 3) to evaluate the fungal horizontal transmission capacity of individual bugs, and 4) to determine the effect of bug density on horizontal transmission. These results, combined with other ongoing studies, will contribute to modeling the population dynamics of indoor *T. infestans* exposed to *B. bassiana* infection.

Materials and methods

Fungal strain and formulation

Commercial powder of *Beauveria bassiana* strain GHA (Laverlam International, Butte, MT) (1.27×10^{11} conidia/g, 98% viable) was formulated with diatomaceous earth (DE) (Perma-Guard Inc, Albuquerque, NM) obtaining a conidia: DE powder (2:1 w:w).[5]

Insects

The *T. infestans* insects came from a colony regularly maintained and reared at 30°C, 50%–60% relative humidity, under a 12-hour dark/12-hour light photo cycle, and fed on chickens, at the INIBIOLP, Facultad de Ciencias Médicas, La Plata, Argentina. The colony is renewed yearly by incorporating first generation insects, usually from Formosa province, provided by the Servicio Nacional de Chagas, Cordoba, Argentina. For all the bioassays, 2 week-old insects were used, 1 week after a blood meal.

Susceptibility of eggs, nymphs, and adults of *T. infestans* to the *B. bassiana* formulation

Bugs were contaminated with the fungus after contact with the powder formulation. Separate assays were carried out with eggs, nymphs (first to fifth instar), and adults. Nymphs and adults were allowed to be in contact with the powder for 5 minutes in a 15 cm Petri dish (2.6×10^8 conidia/cm^2); groups of ten eggs were deposited in a 20 mL vial with the same amount of fungal formulation, and mixed for 5 minutes with gentle agitation. Both eggs and insects were placed on separate 250 mL plastic containers covered with a muslin cloth, and maintained afterwards at 26°C, 50% relative humidity; insects were not fed again during the assay period. Insects or eggs, similarly treated, but without the fungal formulation, were used as controls. Four to twelve replicates of each treatment (with ten insects or eggs per replicate) were performed. Egg hatch and insect mortality were checked daily. Cadavers were washed in 70% ethanol for 30 seconds and then rinsed in sterile distilled water for 2 minutes to eliminate any residual nongerminated spores on the insect surface. Bodies were allowed to dry, and then placed in individual humid chambers – generated by a wet cotton plug – within sealed petri dishes, and maintained at 25°C to confirm fungal infection. After 4 days, cadavers were observed under a binocular microscope; the presence of white mycelium confirmed death by fungal infection. Only insects that showed conspicuous fungal growth were scored as infected by *B. bassiana*. Median lethal time (MLT) was estimated as Σ (days$_n$ $\times$ dead nymphs$_n$)/total dead nymphs.[5]

Residual activity of fungal formulation

The fungal formulation was tested for residual activity up to 5 months after preparation and storing in a 15 cm Petri dish, at natural ambient conditions in an experimental house (see next page). In the time period assayed, from July to December 2010, temperatures varied from 10°C ± 4°C to 22°C ± 5°C, and relative humidity from 34% to 100%. At monthly intervals, aliquots of the fungal formulation were used to contaminate first and third instar nymphs as described above. Mortality was checked daily, dead insects were placed in individual humid chambers to confirm death by fungal infection. For each nymph instar and time period, five replicates with 10 to 16 insects per replicate were performed.

Fungal horizontal transmission at different time periods

To find out for how long one bug could contaminate any other bug by autodissemination, several third instar nymphs were contaminated with the fungal formulation as described on previous page, and maintained under rearing conditions. One of the treated insects was randomly selected and placed in a 20 mL vial, then one healthy nymph was added to the vial; both insects remained together in the vial for 5 minutes. Then, the formerly healthy insect was separated and maintained under rearing conditions. This procedure was repeated three more times using different healthy bugs. The whole experiment was repeated at additional time periods, ie, 2, 4, and 7 days after initial contamination. Mortality of the formerly healthy bugs was checked daily, cadavers were placed in humid chambers to confirm fungal infection, and MLT was estimated as described.[5] There were four to eight replicates for each time period assayed.

Effect of insect density on fungal horizontal transmission

The effect of insect density on fungal autodissemination was studied in experimental houses. The houses (1 × 1 × 1 m) were built with stacked bricks (unplastered walls), and medium density fiberboard (MDF) was used in floors and roofs. To prevent insect run-away, an aluminum structure with mosquito net sides completely surrounded each experimental house. In all experiments, ten fourth instar nymphs, previously contaminated with the fungal powder formulation as described above, were released inside each house. After 30 minutes (time enough for insects to fully hide in the adobe bricks) healthy fourth instar nymphs were released into each house; the number of insects was either 25 (low density), 60 (middle density), or 130 (high density). Bugs were maintained in the houses for 1 month at ambient conditions without feeding. After that period, the houses were totally dismantled, and dead and live insects were collected. Mortality was checked weekly for 1 more month. At this time, total cadavers were evaluated for fungal infection as described above. Mortality was calculated as N × 100/Nt, where: N = number of dead nymphs by autodissemination, Nt = total nymphs in each house. The contaminated insects were not considered for mortality estimation. There were three replicates for each bug density condition. Replicates were performed in January, February and March 2011, with mean temperatures of 24°C ± 1°C, 22°C ± 1°C, and 20°C ± 1°C, respectively. The corresponding mean relative humidity (and minimum–maximum humidity) values were 74.1% (32%–99%), 67.4% (25%–91%), and 66.2% (19%–93%), respectively.

Results

Bug susceptibility to fungal formulation

Both nymphs and adults were highly susceptible to fungal powder formulation (Table 1). For nymphs, mortalities ranged from 81.7% ± 6.2% (fourth instar) to 100% (first instar), and MLT from 5.1 ± 0.4 days (first instar) to 9.1 ± 1.5 days (fourth instar), at the dose tested. For adults, mortality was 87.5% ± 14.0% and MLT was 10.9 ± 2.4 days. After one-way analysis of variance (ANOVA), we found no significant differences ($F = 1.3950$; df = 5,28; $P = 0.2564$) in mortality percentage between all insect stages. For MLT, ANOVA parameters were $F = 5.6499$; df = 5,28; $P = 0.0010$. After Tukey's post-hoc test, significant differences ($P < 0.05$) were only detected between the first instar and all other stages. No mortality was observed in the controls. Hatching was not significantly different ($P > 0.05$, Student's *t*-test) between fungus-treated (73.8% ± 6.2%) and control (77.5% ± 7.2%) eggs.

Residual activity of fungal formulation

Residual activity of fungal formulation showed no significant differences ($P > 0.05$, Student's *t*-test) either in the mortality percentage or the MLT, between fresh and 5-month-old formulations, tested both in first and third instar nymphs. For first instar nymphs, mortalities were 100% and 95.2% ± 2.1% with fresh and 5-month-old formulations, respectively. Median lethal times recorded were 5.1 ± 0.3 days (fresh formulation) and 5.8 ± 0.1 days (5-month-old formulation) (Figure 1). Third instar mortality values were 93.0% ± 2.4% and 69.6% ± 7.9% for the fresh and 5-month-old formulations, respectively. The corresponding TLM values were 8.7 ± 0.5 days and 10.4 ± 0.6 days (Figure 1).

Table 1 Virulence of *B. bassiana* powder formulation (conidia: DE, 2:1w:w) against different stages of *T. infestans*

Insect stage	Mortality (%)	MLT (days)
Nymph I	100	5.1 ± 0.4
Nymph II	87.0 ± 12.0	8.2 ± 2.0
Nymph III	92.7 ± 4.8	8.7 ± 0.9
Nymph IV	81.7 ± 6.2	9.1 ± 1.5
Nymph V	89.5 ± 13.9	8.3 ± 1.6
Adult	87.5 ± 14.0	10.9 ± 2.4

Notes: Insects were treated as described in Materials and methods. Mortality was checked daily and dead insects were placed in individual humid chambers to confirm fungal infection. Values are means ± SD. Four to twelve replicates (ten bugs per replicate) were performed.
Abbreviations: MLT, median lethal time; DE, diatomaceous earth.

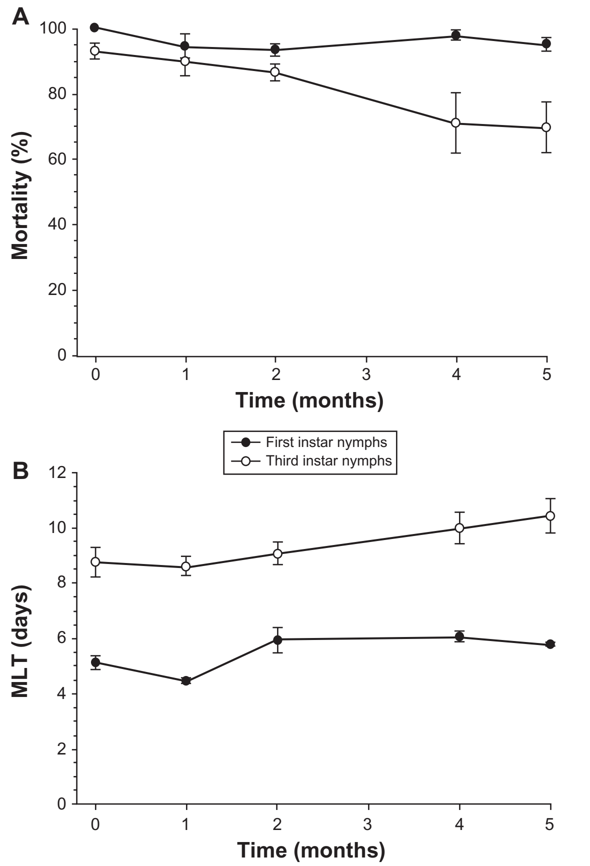

Figure 1 Residual activity of fungal formulation after exposition at natural ambient conditions. (**A**) Mortality percentage of insects killed by fungi; (**B**) Median lethal time. Values are means ± SD. Formulation was prepared and stored at ambient temperature, humidity, and natural dark-light photo cycle. At monthly intervals, an aliquot was used for mortality assays as described in Materials and methods.

Horizontal transmission of *B. bassiana* conidia

Laboratory assay

The autodissemination capability of *B. bassiana* in *T. infestans* remained unchanged up to the longer time period tested, as is shown in Table 2. Mortality caused by horizontal transmission was 56.2% ± 12.0% when contact between healthy and contaminated insects occurred immediately after the latter was exposed to the fungus. A mortality of 46.9% ± 12.0% was estimated when contact took place 1 week after insect contamination. The corresponding MLT values were 14.4 ± 3.0 days and 18.5 ± 1.4 days. No significant differences were detected for both parameters after ANOVA analysis (Table 2).

Artificial house assays

Table 3 shows the effect of insect density on autodissemination mortality. At a fixed number of fungus-contaminated bugs (ten per house), mortality was significantly different after repeated measures ANOVA ($F = 10.732$; df $= 2,4$; $P = 0.0247$). Tukey's post-hoc test showed a significant difference ($P < 0.05$) in bug mortality by horizontal transmission between "low density" and "high density" houses, with a healthy/contaminated bug ratio of 2.5 and 13, respectively. Mortality numbers were 11.9% ± 3.6% for "low density" houses and 29.5% ± 6.2% for "high density" houses. At "middle" insect density (healthy/contaminated bug ratio = 6), an intermediate mortality (20.5% ± 7.1%) was estimated, without significant differences from the former numbers ($P > 0.05$).

Discussion

Triatomine susceptibility to fungal infection was shown in *Rhodnius prolixus*.[11,12] Since then, several strains of *B. bassiana* and *Metarhizium anisopliae* highly virulent against *T. infestans* have been reported.[13–15] However, little progress has been made in fungal formulation for practical applications. To this end, we have previously reported that a powder formulation containing fungal conidia and diatomaceous earth, contained in an attraction–infection trap, was successfully tested in indoor control of *T. infestans* in two field sites next to the Argentina–Bolivia border.[5] In this work, we demonstrated that all nymph instars and adults are highly susceptible to *B. bassiana* powder formulation, with mortalities ranging from 82% to 100%, and the time to kill between 5 and 10 days, at the dose tested. Egg hatching was not affected by fungi. There is no previous information about the ovicidal effects of entomopathogenic fungi against triatomines; although for other bloodsucking insects the activity depends on fungal species and strains.[16]

Table 2 Horizontal transmission capability of *B. bassiana* after *T. infestans* fungus-inoculation, at different time periods: mortality and MLT of formerly healthy nymphs

Parameter	Time period after fungal inoculation				ANOVA (*F*; df; *P*)
	0 days	2 days	4 days	7 days	
Mortality (%)	56.2 ± 12.0	43.7 ± 6.2	43.7 ± 18.7	46.9 ± 12.0	0.1557; 3,16; 0.9245
MLT (days)	14.4 ± 3.0	14.1 ± 3.5	15.4 ± 1.5	18.5 ± 1.4	0.9588; 3,34; 0.4233

Notes: Fungus-treated third instar nymphs were placed in contact with healthy third instar nymphs at different time periods from fungus inoculation, as described in Materials and methods. Mortality and MLT numbers for initially healthy bugs are means ± SEM. There were four to eight replicates (with 16 to 32 originally healthy insects) for each time period assayed.
Abbreviation: MLT, median lethal time.

Table 3 Effect of *T. infestans* density on *B. bassiana* horizontal transmission in experimental houses

Density (number of insects per house)	Mortality by autodissemination (%)[a]
35	11.9 ± 3.6*
70	20.5 ± 7.1
140	29.5 ± 6.2*

Notes: For each density condition, ten fungus-inoculated fourth instar nymphs were released in experimental houses containing 25, 60, and 130 healthy fourth instar nymphs. The total number of insects per house is that shown in the first column. Fungus-inoculated insects were obtained as described in Materials and methods. Numbers are means ± SEM of three replicates. [a]Calculated as N × 100/Nt, where: N: number of dead nymphs by autodissemination, Nt: total nymphs in each house. *Differences are significant ($P < 0.05$) after Tukey's post-hoc test. (ANOVA: $F = 10.732$; df = 2,4; $P = 0.0247$).

M. anisopliae, but not *B. bassiana,* was shown to fully inhibit eclosion of *Anopheles gambiae* eggs.[17] Although *B. bassiana* conidia showed no ovicidal effect in *T. infestans*, all the emerging 1st instar nymphs died by fungal infection when conidia were in the surrounding area (data not shown). After setting attraction–infection traps in rural houses, a considerable number of eggs were laid inside the traps (data not shown). This behavior can be helped by adding aggregation and/or sex pheromones to the trap.[8,18]

The fungal formulation was used as a powder, avoiding water suspensions that might increase the odds of spore germination (and consequently increasing loss of viability) before being in contact with the insect surface. Preliminary data showed that this formulation was able to retain high germination rates up to 3 months under natural ambient conditions.[5] Here, we demonstrated that virulence against first and third instars did not change significantly up to 5 months in the same conditions. Using the fungal formulation inside the attraction–infection trap[5,9] can help protect conidia from UV light; thus, residual activity of the bioinsecticide could last even longer. The fungus-containing trap has an additional advantage compared to traditional water suspension spraying: insects walking through the trap will exit with a much higher load of conidia than those walking the same distance on a fungus-sprayed wall, and hence might improve the chances of spreading the fungus via horizontal transmission.

Autodissemination has been shown to occur during copulation in other bloodsucking insects, such as tsetse flies and mosquitoes.[19,20] In *T. infestans*, we hypothesized that horizontal transfer of conidia would take place not only during the brief mating events; typical bug aggregation behavior (remaining in close contact in reduced size nests for most of the day) should also favor fungal transmission. In the experimental design, we took advantage of the characteristic starvation resistance of triatomines. In particular, *T. infestans* fourth stage insects were reported to survive for about 3 months.[21] Furthermore, no difference between *B. bassiana* infection of fed and starved bugs were reported.[22] Thus, bugs remained unfed throughout the entire assay. Here we show that horizontal fungal transmission (by previously infected insects) is possible over a wide timeframe. In the conditions tested, this time period lasted for 1 week after contamination; then, most fungus-infected insects are expected to die (Table 1). Additionally, fungal autodissemination was shown to increase with increasing bug density. Although we do not know whether this trend would be maintained at higher density levels, our results suggest that horizontal transmission might be relevant in highly *T. infestans*-infested houses.

Fungal biopesticides are already registered for agricultural use throughout the world. The use of entomopathogenic fungi has important advantages over the use of chemical insecticides: in addition to the much lower potential to resistance development in the insect target, they are environmentally friendly, and not harmful to birds, fish, or mammals. The US Environmental Protection Agency expects no risk to humans when using products containing *B. bassiana.*[23]

The results of this study may help to improve the use of a biological control approach based in attraction–infection traps to control Chagas disease vectors. This low cost, low tech, and ecologically friendly methodology was shown to be useful to control pyrethroid-resistant domiciliated bugs.[5] Next, these data will be incorporated into a mathematical model to better understand the population dynamics of fungus-infected bugs, and optimize this methodology for its use in national vector control programs.

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Disclosure

The authors report no conflicts of interest in this work.

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