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Original Research Article

Susceptibility of different life stages of *Blattella germanica* (Blattodea: Blattellidae) and *Periplaneta fuliginosa* (Blattodea: Blattidae) to entomopathogenic fungi

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Keywords

Entomo-pathogenic fungi, cockroaches, bait, direct contact, lethal time

A B S T R A C T

The susceptibility of nymphs and adults of the German cockroach, *Blattella germanica* Linnaeus (Blattodea: Blattellidae) and the smokybrown cockroach, *Periplaneta fuliginosa* Serville (Blattodea: Blattidae) to Argentinian isolates of the entomopathogenic fungi *Metarhizium anisopliae* (Metschn.) Sorokin (CEP 085) and *Beauveria bassiana* (Bals.-Criv.) Vuill. (CEP 077) was evaluated. Fungi were tested by using two different methods: bait and direct contact. Mortality was monitored daily for twenty days to obtain LT_{50}. *M. anisopliae* produced 60 and 93% mortality in nymphs and adults of *B. germanica*, respectively, when conidia were applied by direct contact. The LT_{50} for adults was 3.8 days, and 8.6 days for nymphs. Direct contact of *B. bassiana* produced 80% mortality on adults of *B. germanica* with a LT_{50} of 4.9 days, and for nymphs 40 % mortality in 10 days. When *B. germanica* was exposed to bait, the level of mortality was significant in adults. Nymphs of *P. fuliginosa* were treated with bait with *M. anisopliae* and *B. bassiana* and they caused 50% mortality with a LT_{50} of 22 days, and LT_{50} of 27 days respectively. Nymphs and adults of *P. fuliginosa* treated by direct contact and adults treated with bait showed that mortality level was not significantly different as compared to the control. Results showed differences in susceptibility between the two species of cockroaches and between nymphs and adults of the same species. In addition, different responses to the fungal species with the two methods that were used in the bioassays have been demonstrated. This is the first report of susceptibility of *P. fuliginosa* to entomopathogenic fungi. This study demonstrates the potential of fungi as biocontrol agents against this pest.

Introduction

Cockroaches can carry pathogens in their digestive tracts, teguments and excrements. Contamination with pathogens can occur in regurgitation of food, by direct contact or by deposition of feces in places inhabited by humans (Perez, 1989). In human residences

they can contribute to increase allergic processes since they are agents of induction and exacerbation of asthma disease (Rosário *et al.*, 1999; Nowak-Wegrzyn *et al.*, 2009; Mindykowski *et al.*, 2010). In different types of places of manufacture or food manipulation, they can act as mechanical vectors, carrying pathogenic agents, such as fungi, *Aspergillus* spp., *Penicillium* spp., and *Candida* spp, *Geotrichum* spp. (Lemos *et al*., 2006), and bacteria such as *Escherichia coli*, *Serratia marcescens*, *Staphylococcus aureus* (Pai *et al*., 2005).

The most common cockroach household in Argentina, as in the rest of the world, is *Blattella germanica* (Linnaeus) (Blattodea: Blattellidae), the German cockroach.

Periplaneta fuliginosa (Serville) (Blattodea: Blattidae), the smokybrown cockroach, is closely related to the American cockroach and has become a major pest in many parts of the world (Suiter & Koehler, 2003); it has also been reported from Asia, Europe, Australia, North and South America. In Argentina, it has been recorded in Buenos Aires and Tucumán provinces (Crespo & Valverde, 2008). It thrives in warm and humid climates; smoky brown cockroaches periodically invade homes, (Appel & Smith, 2002).

Cockroaches have been mostly controlled by using chemical insecticides. However, many factors such as insecticide resistance, contamination of stored food, and residual effects have caused the interest on searching alternative control methods (Wickham, 1995; Cochran, 1999; Taiarol *et al*., 2001). Entomopathogenic fungi act mostly by contact, penetrating the insect host cuticle through mechanical forces and enzymatic action in order to colonize the insect hemocele (Gupta *et al*., 1991; St. Leger *et al*., 1991). Previous studies focused on the effects of the entomopathogenic fungi *Beauveria bassiana* (Bals.- Criv.) Vuill., (Sordariomycetes: Hypocreales: Cordycipitaceae) and *Metarhizium anisopliae* (Metschn.) Sorokin (Sordariomycetes: Hypocreales: Clavicipitaceae) against *B. germanica* and *P. americana*, evaluated its virulence and host range (Murali Mohan *et al.*, 1999; Quesada Moraga *et al*., 2004; Hubner-Campos *et al*., 2013). Other authors reported studies on the evaluation of *B. bassiana* effects on *B. germanica* (Pachamuthu *et al*., 1999; Zurek *et al*., 2002) and others reports were about virulence, transmission, use of baits and differential susceptibility of nymphs and adults of *B. germanica* to *M. anisopliae* (Lopes & Alves, 2011).

The overall objective of the present study was to evaluate differential susceptibility of nymphs and adults of *B. germanica* and *P. fuliginosa* to native isolates of the entomopathogenic fungi *M. anisopliae* and *B. bassiana* comparing between direct contact and bait treatment methods.

Material and Methods

Insects: Nymphs and adults of *B. germanica* and *P. fuliginosa* were collected from households and at the zoological park in La Plata, Buenos Aires, Argentina, between July 2010 and September 2011. Cockroaches were confined in plastic boxes with plastic lids (30 x 25 x 20 cm) and provided with cardboard shelters. They were fed with commercial dog-food (Purina Dog Chow®, Nestlé Argentina S.A., Buenos Aires) and tap water *ad libitum*. The cockroaches were maintained in an incubator at 26 ± 2°C, 56 ± 10% RH, and a photoperiod of 12:12 light: dark.

Fungal cultures: The strain CEP 085 of *Metarhizium anisopliae sensu lato*

(Metschn.) Sorokin and strain CEP 077 of *B. bassiana* (Bals.- Criv.) Vuill. were isolated in 2004 from Los Hornos, Buenos Aires, Argentina, from unidentified Hemiptera Cercopidae and from *Balacha melanocephala* Cercopidae (Hemiptera), respectively. These fungi were selected based on the results of a preliminary experiment. Two strains of *M. anisopliae*, *B. bassiana*, *Nomuraea rileyi* and *Isaria fumosorosea* were tested against cockroaches. From all those strains we selected the most pathogenic: *M. anisopliae* 085 and *B. bassiana* 077.

These isolates were cultured on Sabouraud dextrose agar + 1% yeast extract (SDAY 1%) medium, incubated at 25 ± 1°C and 12 h photophase. Conidia were harvested from 15 day old cultures. They were scraped with a sterile looper and collected into sterile plastic tubes (45 cm^3) containing 5 ml of 0.01% (v/v) Tween 80 (Merck, México). A suspension of the conidia was vortexed for 5 min; the concentration of propagules was quantified by using a haemocytometer (Neubauer chamber) and the suspension was adjusted to 1 x 10^9 conidia/ml, it concentration was based on the results of a preliminary experiment. Conidial germination percentage was calculated according to Lane et al., (1988).

Bioassays: Aggressivity of the fungal strains of *M. anisopliae* and *B. bassiana*, towards nymphs and adults of *B. germanica* and *P. fuliginosa,* was evaluated by direct contact and bait. Groups of ten adults and ten third instars nymphs cockroaches were exposed to 1 ml of a suspension containing $1x10^9$ conidia per milliliter of *M. anisopliae* or *B. bassiana* on a dry filter paper disc (9 mm diam.). Each filter paper was placed into the bottom of a sterilized Petri dish (100 mm diam.). Controls were treated with discs of filter paper, with Tween 80, 0.01% (v/v). After 24 h, cockroaches were transferred to plastic cups (250 cm^3). Five nymphs of cockroaches were placed per container and adults were placed individually in plastic cup, and incubated at 25 ± 1°C, > 70% RH, and 12 h photophase. Food and water were placed inside the containers and they were replaced and changed every two days. Mortality was monitored daily up to twenty days post treatment.

In another experimental series, the conidial suspension was applied on bait. Bait was prepared with dog food (Purina Dog Chow®, Nestlé Argentina S.A., Buenos Aires) mixed with 1% medium agar water. One ml of the conidial suspension was added to 4 ml of bait, and 4 ml of this mixture was applied on the bottom of 35 mm sterilized Petri dishes. Groups of ten adults and ten third instars nymphs cockroaches were exposed to these baits for 72 h. Then, cockroaches were transferred to plastic cups (250 cm^3) and bait assays were maintained under the conditions mentioned for the direct contact assays, as mentioned above.

Each treatment was replicated three times with 10 adults and 10 nymphs of cockroaches per treatment.

In both experimental series, dead cockroaches were removed daily and placed on slides inside Petri dishes (100 mm diam.) that were containing filter paper moistened with sterile distilled water. Insects were superficially sterilized with 70% ethanol followed by a second bath in sterile distilled water and then in antibiotic (40.000 units/ml chloramphenicol, (Parafarm®, Argentina); and 80.000 units/ml streptomycin and the last bath was done using sterile distilled water. Each bath had duration of ten seconds. The cadavers were placed on wet filter paper disks into sterile 100 mm Petri

dishes sealed with parafilm® and maintained in an incubator chamber at 25 ± 1ºC and 70 ± 5 % RH. Emergence of mycelia was monitored for a total of eight days post challenged. Infected insects with evidence of external fungal growth were examined under a stereomicroscope. Fungal structures were stained with 0.01 % (w/v) lactophenol/cotton blue 1 % (w/v). Fungal slide preparations were observed under Olympus microscope to verify the fungal species.

Statistical analysis: An ANOVA test was performed to determine significant difference among the mortality of cockroaches among treatments. An angular transformation of the data was done, using the arc cosine of the square root of the mortality percentage before the analysis, to stabilize the variance error (Zar, 1996). Tukey´s test ($P < 0.05$) was performed when significant differences appeared among the treatments. Statistical calculations were performed using Statgraphics Centurion 15.2 program (StatPoint, Inc., 2007). Median Lethal time (LT_{50}) values were calculated with their respective 95% confidence intervals (IC) by using the statistical software for correlated data developed by Throne *et al*., (1995) and Mathematica software 5.2 (Wolfram). Differences between values were considered significant ($P < 0.05$) if the respective 95% IC did not overlap.

Results and Discussion

The first dead cockroaches were observed after 5 days post treatment. Mortality levels of nymphs of *B. germanica* during the 20 days of treatment by direct contact with *M. anisopliae* was 60 % after 15 days; while with *B. bassiana* was 40 % after 10 days and using bait with *M. anisopliae* was 10 % after 15 days post treatment (Table 1). The mortality in the control was 0%. Mortality due to direct contact treatment *M. anisopliae* or *B. bassiana* was highly significant on nymphs compared with the control and bait ($F= 33.9$; $df= 3$; $P= 0.0000$) (Table 1). The LT_{50} obtained from nymphs treated with direct contact with *M. anisopliae* was 8.63 days, with limits between 3.9 and 27.8 (1.29±0.4). LT_{50} for rest of the treatment was not calculated, since mortality percentage was lower than 50%.

Mortality levels of adults of *B. germanica* during the 20 days of treatment by direct contact with *M. anisopliae* or *B. bassiana*, and bait with *M. anisopliae* reached values up to 93.3% after 10 days; 80% after 10 days; 40 % after 20 days post treatment, respectively (Table 1). Mortality due to direct contact treatment *M. anisopliae* or *B. bassiana* was highly significant on adults compared with the control, and mortality due to bait with *M. anisopliae* was significant compared with the control (Table 1) ($F= 31,29$; $df= 3$; $P= 0.0000$). Finally, the LT_{50} obtained from adults treated with direct contact with *M. anisopliae* was 3.8 days, with limits between 2.5 and 5.4 (2.7±0.6) and LT_{50} obtained from adults treated with direct contact with *B. bassiana* was 4.9 days, with limits between 3 and 7.7 (2.3±0.5). The percentage of mortality in adults of *B. germanica* was higher than nymphs with a lower LT_{50}.

Mortality levels of nymphs of *P. fuliginosa* during the 20 days of treatment by direct contact with *M. anisopliae* or *B. bassiana* reaches values 13.3 % after 10 days post treatment. Mortality levels of nymphs treated with bait reach values 50 % after 20 days with both fungi (Table 2). Mortality during the control was 10%. Mortality of nymphs of *P. fuliginosa* due to bait treatment *M. anisopliae* or *B. bassiana* was highly significant compared with the control

and direct contact with both fungi (F= 12,47; df= 4; P= 0.0000). LT_{50} obtained from nymphs treated with bait with *M. anisopliae* was 22 days, with limits between 12 and 75 (1.65 ± 0.5) and LT_{50} obtained from nymphs treated with bait with *B. bassiana* was 27 days, with limits between 11 and 100 (1,1 ± 0.3).

The mortality rate in adults of *P. fuliginosa* with *M. anisopliae* and *B. bassiana* were from 20% to 33.3 % at 15 days with direct contact and treatment with bait mortality was less than 20% with both fungi (Table 2). However, adults of *P. fuliginosa* treated with bait and direct contact did not show significant differences with the control (F=2.04; df=4; P=2.04). Nymphs and adults of *P. fuliginosa* were more resistant to infection with these isolates of *B. bassiana* and *M. anisopliae* with direct contact treatment than *B. germanica*.

Adults of *B. germanica* were more susceptible to *M. anisopliae* and *B. bassiana* infection than nymphs when direct contact was used; this behaviour was repeated under bait treatment method with *M. anisopliae*. Direct contact treatment when compared to bait treatment was more effective. Our results of treatment with bait agreed with results recorded by (Lopes & Alves, 2011), they reported that survival of *B. germanica* nymphs treated with *M. anisopliae* using bait, were not affected, therefore they recorded mortality rates of adults of 28% after 15 days post-application. This differential susceptibility at various life stages can be attributed to interaction between the insect integument being penetrated by the fungus and ecdysis of nymphs stages. Ecdysis has been reported to be an important factor in insect resistance to fungal infection, particularly when the time interval between successive molting is short (Lopes & Alves, 2011; Ekesi & Maniania, 2000).

Our results showed that *B. germanica* adults treated with direct contact with *B. bassiana* generated a higher level of mortality which was not significantly different from those insects exposed to *M. anisopliae* with direct contact.

The bait treatment was less efficient than direct contact. In nymphs of *B. germanica* mortality only reached 10% and 40% in adults. In the direct contact treatment contact cockroaches could get higher percentage of conidia.

In the present study mortality of nymphs and adults of *P. fuliginosa* were not significantly different treated by direct contact with *M. anisopliae* and *B. bassiana* and also there was not statistical differences in adults treated with bait. However, adults shown higher mortality than the nymphs by direct contact. Nonetheless, mortality of nymphs treated with bait was significantly different from that obtained in the control. Nymphs under bait treatment showed higher level of mortality when compared to adults. We must consider that infection may also occur via natural openings, such as the mouth, or *per os* penetration, however, it only was effective in nymphs of *P. fuliginosa*.

In conclusion, *P. fuliginosa* susceptibility to the tested entomopathogenic fungi was significantly lower than in *B. germanica*. These differences between treatments and fungal species may be due to the surface structure and the chemical composition of the host cuticle. The waxy coat of *P. fuliginosa* may have affected the adhesion of conidia Otherwise, it has been reported elsewere that cuticular lipids have a profound effect on fungal spore germination and differentiation: they can be toxic, fungistatic, or occasionally, for some pathogenic species, stimulatory Gołębiowski et al. (2011).

Table.1 Cumulative mortality (%) of nymphs and adults of Blattella germanica exposed to direct contact and baits, with M. anisopliae (CEP 085) and B. bassiana (CEP 077)

Treatment	Nymphs Days after inoculation					Adults Days after inoculation				
Contact direct	5 day	10 day	15 day	20 day	**Sig**	5 day	10 day	15 day	20 day	**Sig**
Control	0 ± 0	0 ± 0	0 ± 0	0 ± 0	a	3.3 ± 0.6	10 ± 0	10 ± 0	10 ± 0	a
M. anisopliae	40 ± 1	53.3 ± 0.6	60 ± 1	60 ± 1	b	73.3 ± 2.1	93.3 ± 0.6	93.3 ± 0.6	93.3 ± 0.6	c
B. bassiana	16.7 ± 1.2	40 ± 1.7	40 ± 1.7	40 ± 1.7	b	50 ± 1	80 ± 1	80 ± 1	80 ± 1	bc
Baits	5 day	10 day	15 day	20 day	**Sig**	5 day	10 day	15 day	20 day	**Sig**
Control	0 ± 0	0 ± 0	0 ± 0	0 ± 0	a	0±0	3.3 ± 0.6	10 ± 1	13.3 ± 0.6	a
M. anisopliae	0 ± 0	0 ± 0	10 ± 1	10 ± 1	a	0±0	23.3 ± 2.1	33.3 ± 3	40 ± 2.6	b
B. bassiana	ND					ND				

For each formulation mean (± standar error); Sig: significance. Different letters on the same column indicate significant differences according to Tukey´s test (P< 0.05). ND: not determined.

Table.2 Cumulative mortality (%) of nymphs and adults of *Periplaneta fuliginosa* exposed to direct contact and baits, with *M. anisopliae* (CEP 085) and *B. bassiana* (CEP 077)

Treatment	Nymphs Days after inoculation					Adults Days after inoculation				
Contact direct	5 day	10 day	15 day	20 day	**Sig**	5 day	10 day	15 day	20 day	**Sig**
Control	0 ± 0	10 ± 1	13.3 ± 1	13.3 ± 1	a	10 ± 1	13.3 ± 0.6	13.3 ± 0.6	16.7 ± 0.6	a
M. anisopliae	3.3 ± 0.6	13.3 ± 0.6	13.3 ± 0.6	13.3 ± 0.6	a	6.7 ± 0.6	16.7 ± 0.6	20 ± 1.7	20 ± 1.7	a
B. bassiana	0 ± 0	13.3 ± 0.6	13.3 ± 0.6	13.3 ± 0.6	a	10 ± 0	16.7 ± 0.6	30 ± 2.6	33.3 ± 2.5	a
Baits	5 day	10 day	15 day	20 day	**Sig**	5 day	10 day	15 day	20 day	**Sig**
Control	0±0	0±0	6.7 ± 0.6	10 ± 0	a	3.3 ± 0.6	3.3 ± 0.6	3.3 ± 0.6	6.7 ± 0.6	a
M. anisopliae	10±1	26.7±1.5	40 ± 1	50 ± 1	b	3.3 ± 0.6	6.7 ± 0.6	13.3 ± 0.6	16.7 ± 0.6	a
B. bassiana	23.3±0.6	36.7±1.5	43.3 ± 1.2	50 ± 1	b	3.3 ± 0.6	13.3 ± 1.2	20 ± 2	20 ± 2	a

For each formulation mean (± standar error); Sig: significance, different letters on the same column indicate significant differences according to Tukey´s test (P< 0.05).

However, this is the first report of susceptibility of *P. fuliginosa* to entomopathogenic fungi *M. anisopliae* and *B. bassiana* throughout the use of bait and direct contact methods.

These studies showed differences in mortality between different species of cockroaches and between nymphs and adults in the same species. We can conclude that *M. anisopliae* CEP 085 and *B. bassiana* CEP 077 strains showed potential as a biological control agent of nymphs and adults *B germanica* by direct contact treatment.

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