BIOLOGICAL CONTROL

Grooming Capacity Inhibition in *Cornitermes cumulans* (Kollar) (Isoptera: Termitidae) Inoculated With Entomopathogenic Fungi and Treated With Imidacloprid

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Inibição da Capacidade de Limpeza em *Cornitermes cumulans* (Kollar) (Isoptera: Termitidae) Inoculados com Fungos Entomopatogênicos e Tratados com Imidaclopride

RESUMO – Este estudo foi realizado para comprovar a inibição do comportamento de limpeza de *C. cumulans* quando tratado com conídios de fungos entomopatogênicos e sub-doses do inseticida imidaclopride. As observações foram realizadas através de microscópio eletrônico de varredura. Os operários mostraram um eficiente mecanismo de limpeza o que possibilitou a remoção, nas primeiras horas após a aplicação, de praticamente todos os conídios da cutícula dos soldados e dos outros operários. O inseticida imidaclopride, quando utilizado em doses sub-letais, inibiu o comportamento de limpeza, permitindo que os conídios germinassem e penetrassem a cutícula do inseto provocando a infeção.

PALAVRAS CHAVE: Insecta, controle biológico, *Beauveria bassiana, Metarhizium anisopliae,* cupim de montículo.

ABRSTRACT – This study was carried out to attest inhibition in *C. cumulans* grooming behavior when treated with entomopathogenic fungi and sub-doses of imidacloprid insecticide. Observations were made under the scanning electronic microscope. The termite workers showed an efficient grooming behavior which allowed the removal of all conidia from the cuticle of soldiers and other workers, in the first hours after fungal application. The insecticide imidacloprid, when used in sublethal concentrations, inhibited grooming behavior and allowed conidia to germinate and penetrate the insect cuticle, causing infection.

KEY WORDS: Insecta, biological control, *Beauveria bassiana, Metarhizium anisopliae,* mount termite.

Grooming behavior among social insects is important as a defense mechanism against pathogens and parasites. This behavior prevents contamination and attack on colonies and is essential for the establishment and maintenance of nests in natural highly contaminated environments such as the soil. For entomopathogenic fungi, which act mainly through contact, exposure to the conidia allows their removal from the host body surface, reducing the chances of infection and, consequently, control efficiency. Because of this behavior, large quantities of conidia are necessary to cause colony death, making control by these fungi uneffective and economically unfeasible.

The insecticide imidacloprid has induced the spontaneous occurrence of pathogens especially in insects with subterranean habits, such as termites. Thus, when imidacloprid was applied to the soil and baits, there was a synergism with the entomopathogens, causing *Reticulitermes flavipes* (Kollar) workers death probably acting as a stressor on the termites by affecting their defense mechanisms (W. M. Zeck 1992, unpublished data). Boucias *et al*. (1996) studied the effects of imidacloprid and its interaction with *Beauveria bassiana* (Bals.) on *R. flavipes* and concluded that the defense mechanism is basically carried out by the grooming behavior among individuals. Trophalaxis and gallery construction in the soil also act as protection against pathogens. They further observed that imidacloprid in non-lethal doses did not cause alterations either in the insect cuticle or in the immune system. Synergism has already been found between imidacloprid and *Metarhyzium anisopliae* (Metsch.) in the control of two *Blattella germanica* (L.) strains, one resistant and the other susceptible to imidacloprid (Kaakeh *et al*. 1997). It was also observed, when non-lethal doses of imidacloprid were combined with *M. anisopliae* and *B. bassiana,* a significant increase in larval mortality at the first instar of *Diaprepes abbreviatus* (L.) (Quintela & McCoy 1997).

There was also a synergetic effect, in the mortality of the scarab species *Cyclocephala hirta* LeConte and against a more nematode-resistant scarab species *Cyclocephala pasadenae* Casey when the entomopathogenic nematode *Heterorhabditis bacteriophora* (Poinar) was associated with imidacloprid (Koppenhöfer & Kaya 1998).

When *M. anisopliae* and sub-doses of imidacloprid were used for the control of *C. cumulans* in pastures, big nests of this termite were controlled using significantly lower concentrations of the fungus (Neves & Alves 1999).

Thus, this research was carried out to study the inhibition of *C. cumulans* grooming behavior when treated with entomopathogenic fungi and sub-doses of imidacloprid insecticide.

Material and Methods

C. cumulans termites were collected from field colonies in Piracicaba county, state of São Paulo, Brazil. *M. anisopliae* (1037) and *B. bassiana* (447) isolates were used from the collection of entomopathogens kept in the Insect Pathology Laboratory at ESALQ/USP. All the scanning electronic microscope observations were made on a Zeiss DMS 940 A.

Determination of the Sublethal Imidacloprid Concentration. Imidacloprid concentrations used were 0; 0.1; 1; 10; 100 and 1000 ppm of active ingredient in water. Circular pieces of filter paper were deepened in the different suspensions and allowed to dry overnight. After drying they were placed in plastic petri dishes (60 mm diameter 20 mm high). Moistened filter paper were placed in the lid of the dishes which were then left in a B.O.D. incubator at 25±0.5°C in the dark. The mortality was assessed and movements/activity scores (0; 2; 5; 8 and 10) were established (0 - no movements – activity; 2 - little movements only by stimulation; 5 - rapid movements when stimulated; 8 - movements without stimulation and 10 - movements like control) 6, 12, 24, 48 and 72 hours after placing

the insects in contact with imidacloprid. The experimental design was complete randomized, with five replications (dishes) with 15 insects per dish (two soldiers and 13 workers). Mortality data were submitted to Probit analyses. The corrected mortality was calculated by the difference between total death in the treatment and the control. The data were submitted to ANOVA and means compared by the Tukey's test ($P \leq 0.05$).

Synergism Between *M. anisopliae, B. bassiana* and Imidacloprid. Inhibition in grooming behavior was tested by spraying the termites with either 0.5 ml of *M. anisopliae* conidia suspension (isolate 1037) or *B. bassiana* (isolate 447) at concentrations of 7.3 X 10^8 and 1.6 X 10^9 conidia/ml, respectively. They were then placed on filter paper, in Petri dishes, treated or not with imidacloprid, as previously described. The following treatments were assessed: in treatment one (control) and treatment three (100 ppm imidacloprid) *B. bassiana* and *M. anisopliae* were applied separately; in treatment two (0 ppm of imidacloprid) and four (100 ppm imidacloprid) soldiers only (25) were used to observe their grooming capacity without workers, in these treatments *M. anisopliae* was applied and, in treatment five (10 ppm of imidacloprid) it was only used *M. anisopliae* and 100 workers and 15 soldiers. After treatment, the insects were kept in growth chamber at 25±0.5°C in the dark. Four insects were removed from each treatment (two soldiers and two workers, or four soldiers from treatments 2 and 4) immediately after the application and after 6, 12 , 24, 48 and 72 hours. These insects were killed in ether vapor, fixed in OsO_4 vapor for 48 hours and dehydrated in a diseccator for 72 hours. The fixed insects were coated with gold using a sputter coater and observed under scanning electronic microscope.

Results and Discussion

Determination of Imidacloprid Sublethal Doses. The accumulated percent mortality was low (0 – 2.7%), for all imidacloprid concentrations, up to 24 hours after treatment except for the highest concentration (1000 ppm), which had a 54.7% mortality rate (24 hours). After 48 hours, mortality was high to 100 ppm (58.7%) and 1000 ppm (66.7%) treatments, relatively low to 10 ppm (18.7%) and to 1 ppm (13.4%) treatments and very low in the 0.1 ppm treatment (2.7%) (Table 1). Seventy-two hours after application mortality was high (36% – 79%) in all the treatments but low (12%) in the 0.1 ppm concentration (Table 1). Boucias *et al*. (1996) found mortality close to 95%, 60%, 40%, 15% and lower than 5% for *R. flavipes*, respectively at the 1000, 100, 10, 1 and 0.1 ppm imidacloprid concentrations on the fourth day after inoculation (96 hours), which are close to the death levels observed for *C. cumulans* at three days (72 hours).

Mortality data do not fit the Probit's model since, in the majority of the treatments, a highly significant χ^2 and a high heterogeneity were observed.

Activity/movements was slightly altered (score 8) at the lowest concentration (0.1 ppm) up to 72 hours after inoculation, medium (score 5) for 1.0 ppm concentration up to 48 hours, low for the 10 ppm and 100 ppm treatments after 12 hours (score 2 and 1.6 respectively) and nil (score 0) for the highest concentration (1000 ppm) at the first assessment.

In the associated termite control strategy, using imidacloprid in sub-doses and *B. bassiana* or *M. anisopliae*, the grooming activity should be inhibited or reduced soon after the first hours of application and last as long as possible without insect death. Also the establishment of the pathogen should take place up to a level where it could compete and beat the antagonists present in the host. Thus, the 100 and 10 ppm concentrations were chosen once they provide a sharp reduction in activity soon after contamination and a lower death rate up to 24 hours.

***C. cumulans* Grooming Capacity Inhibition.** The high grooming capacity in termites may be observed by comparing the quantity

Table 1. Percent mortality of *C. cumulans* for the treatments with different imidacloprid concentrations and means of the activity scores.

Treat. (ppm)	Hours after treatment									
	6		12		24		48		72	
	Mort.[1] n=5	Activ.[2] n=5	Mort. n=5	Activ. n=5	Mort. n=5	Activ. n=5	Mort. n=5	Activ. n=5	Mort. n=5	Activ. n=5
Control	-	10.0±0.00 a	-	10.0±0.00 a	-	10.0±0.00 a	-	10.0±0.00 a	-	10.0±0.00 a
0.1	0	10.0±0.00 a	0	9.6±0.64 a	0	9.2±0.96 a	2.7	8.4±0.64 b	12.0	8.0±0.00 b
1	2.7	8.0±0.00 b	2.7	5.0±0.00 b	2.7	5.0±0.00 b	13.4	5.0±0.00 c	36.0	2.0±0.00 c
10	0	5.0±0.00 c	0	2.0 ± 0.00 c	2.7	2.0±0.00 c	18.7	2.0±0.00 d	52.0	0.0±0.00 d
100	1.3	2.0±0.00 d	1.3	0.0 ±0.00 d	2.7	1.6±0.64 c	58.7	0.0±0.00 e	68.0	0.0±0.00 d
1000	36.0	0.0±0.0 0 e	48.0	0.0 ±0.00 d	54.7	0.0±0.00 d	66.7	0.0±0.00 e	78.7	0.0±0.00 d

[1]Corrected accumulated mortality percentage.

[2]Movements/Activity scores: 0 (no movements - activity), 2 (little movements only by stimulation), 5 (rapid movements when stimulated), 8 (movements without stimulation) and 10 (movements like control).

Means followed by different letters within each column are significantly different (P≤0.05) from control treatment.

of *B. bassiana* and *M. anisopliae* conidia deposited on the worker femur soon after application (Fig. 1 A) and six hours after application (Fig. 1B).

Grooming is carried out by the workers, among themselves and on the soldiers, probably using structures similar to brushes found at the end of the mandibles (Fig. 1 C and D). However, conidia were neither observed on these structures nor deposited on other regions of the termite body. Thus two hypotheses can be considered about the places were the conidia, removed from the body surface, are eliminated or deposited. The first is that they are ingested and deactivated in the worker digestive tracts. Several workers were dissected and their digestive tracts observed under an optic microscope. No conidia were found, either germinated or not. Some entomopathogenic fungi invaded their host through the digestive tract (Alves 1998). In many cases, physical and chemical factors prevent this infection via the alimentary canal such as in *Leptinotarsa decemlineata* (Say), where the rapid passage of the food prevents infection by *B. bassiana* (Allee *et al*. 1990). Other insects have a microbiota with fungistatic and/or fungicide properties which deactivate ingested conidia. In *Schistocerca gregaria* (Forska), besides the quick passage of the food, bacteria in the digestive tract produce phenolic substances with anti-fungus action (Dillon & Charnley 1986). The occurrence of infection via the digestive tract in *R. flavipes* has not been confirmed (Boucias *et al*. 1996). These authors further observed that the microorganism communities isolated from the termite digestive tract showed high fungistatic activity for *B. bassiana* conidia and inhibited their germination. There is probably the same defense pattern in *C. cumulans*, as the conidia removal mechanism is identical to that reported for *R. flavipes* and also because these insects develop their colonies in potentially extremely contaminated places such as the soil. On the other hand, Bao and Yendol (1971) found that the majority of *B. bassiana* conidia lost their integrity in *R. flavipes* intestine, but some were observed germinating and penetrating the epithelium.

Another hypothesis for conidia deactivation is that they are deposited in some substrate. The conidia in *R. flavipes* were transferred or deposited in the gallery and tunnel complex and deactivated by the anti-fungi activity of the soil and the microbiota in the insect intestine (Boucias *et al*. 1996). This deactivation may also happen in *C. cumulans*, because this insect builds its nest and isolates dead insects using saliva and fecal contents, as observed in bioassays and in the field.

In spite of the grooming made by the insects in the intersegmentary regions of the antenna, palp and mainly the tarsomeres and claws, not all the conidia were always removed from the insects not exposed to imidacloprid (Fig. 1 E and F). It is presumed that, under natural conditions, where the number of conidia in contact with the insects is probably smaller than in the direct spraying in the bioassys, few conidia remain on the insect after grooming.

In the bioassays the soldiers, when left without workers, were not able to groom because probably this activity is carried out only by the workers, among each other and on the soldiers.

Insect activity is considerably reduced with the use of a stressor even at the lowest concentrations. Thus, for *M. anisopliae*, conidia removal was not observed when the insects came into contact with imidacloprid (stressor), both at the higher (100 ppm) (Fig. 2 A) and with the lower (10 ppm) (Fig. 2 B) concentrations. In contrast, the conidia were removed in the first six hours after application in the control insects (Fig. 1 B). The same results were observed for the *B. bassiana* fungus. The control insects had a large quantity of conidia on the body shortly after application (Fig. 2 C). However, six hours later, practically all the conidia had been removed (Fig. 2D). On the other hand, when under the effect of the stressor, the grooming capacity was drastically inhibited after application as shown in Fig. 2 E (six hours) and F (24 hours).

The cuticle of imidacloprid treated and untreated insects did not show morphologi-

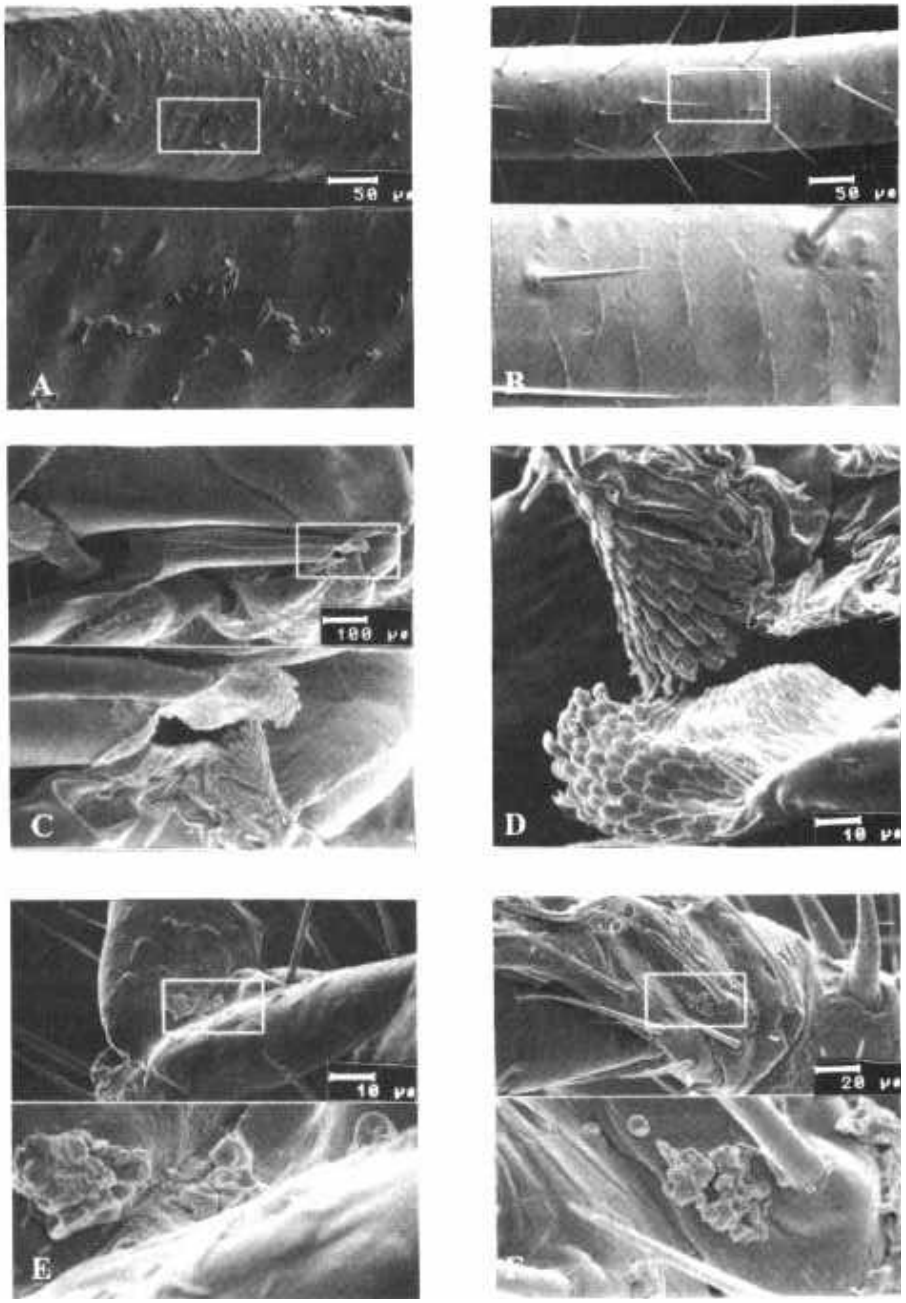

Figure 1. Photomicrographs (S.E.M.) of *C. cumulans* treated with entomopatogenic fungi; **A** – Worker femur after application – 0 h (220 x 4); **B** - Worker femur after grooming- 6 h (200 x 4); **C** - Worker head showing a possible grooming structure (maxillae extremity) (100 x 4); **D** - Possible grooming structure in detail (1000 x); **E** - *B. bassiana* retained conidia between a worker claws - 6 h (1000 x 4); **F** - *B. bassiana* retained conidia between a worker tarsomere - 6 h (500 x 4).

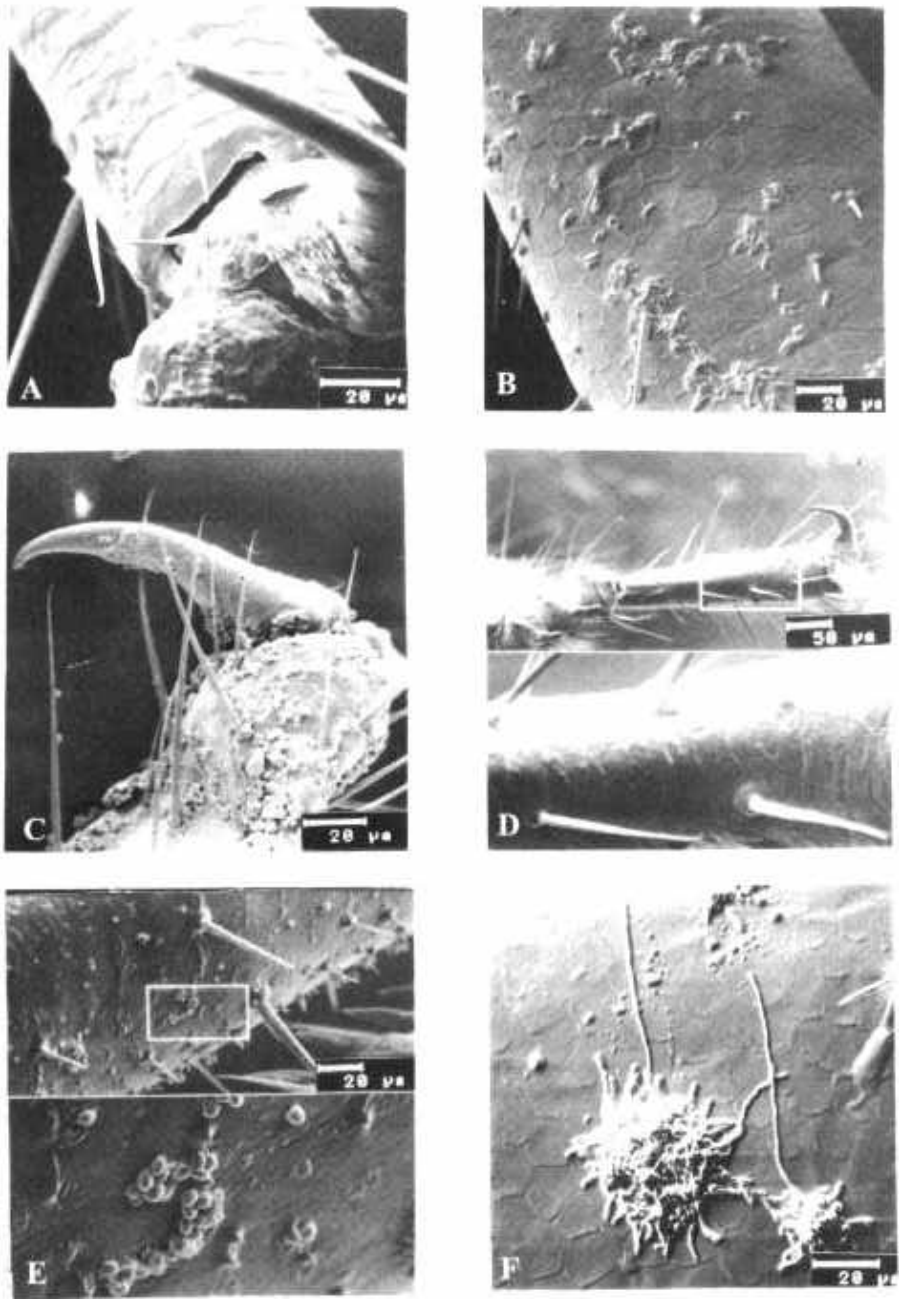

Figure 2. Photomicrographs (S.E.M.) of *C. cumulans* treated with entomopatogenic fungi ; **A** - *M. anisopliae* on a soldier claw (with stressor) - 6 h (875 x); **B** - *M. anisopliae* on a soldier tibia (with stressor) - 6 h (500 x); **C** - *B. bassiana* on worker tarsus and claw - 0 h (700 x); **D** – Worker tarsus and claw - 6 h (200 x 4); **E** - *B. bassiana* on soldier femur (with stressor) - 6 h (500 x 4); **F** - *B. bassiana* on soldier head– (with stressor) - 24 h (725 x).

cal differences. Neither did hemolinfa samples show alterations in the phagocyte response to the imidacloprid treated and untreated termites (Boucias *et al*. 1996).

Imidacloprid synergism with *B. bassiana* and *M. anisopliae*, which increased the mortality of the *D. abbreviatus* larva, occurred because the insects had become less mobile, causing a decrease in the conidia removal by contact with the substrate (Quintela & McCoy 1997). This hypothesis is suported by Quintela & McCoy (1998) who observed that larva death and mycosis in *D. abbreviatus* were highly correlated with soil mobility in all the bioassays. The larvae treated with imidacloprid stayed longer in the treated soil zones and thus a greater number of conidia probably adhered to the cuticle. Similarly, Boucias *et al*. (1996) observed that sublethal imidacloprid doses affected gallery construction behavior (tunnelling) and grooming in *R. flavipes*, which are important resistance factors to infection by mycopathogens.

Thus our results show that imidacloprid affects *C. cumulans* workers activity and the grooming capacity allowing the permanence and later germination of the entomopathogenic fungi on the insect cuticle, improving the efficiency of these pathogens. Probably this explain why a smaller fungus concentration was necessary for control of field big nests of *C. cumulans* when the fungus is applied with imidacloprid sub-dosages.

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