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Pathogenicity of *Aspergillus westerdijkiae* to females and oothecae of *Periplaneta americana*

Patogenicidade de isolado de *Aspergillus westerdijkiae* a fêmeas e ootecas de *Periplaneta americana*

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ABSTRACT

Cockroach control is performed by the application of chemical insecticides which exert high selective pressure on populations and introduces synthetic substances in the environment, motivating the search for other methods of control such as entomopathogenic fungi. The objectives of this study were to investigate the pathogenicity of the JAB 42 *Aspergillus westerdijkiae* to females and oothecae of *Periplaneta americana* and to demonstrate its mechanism of action on oothecae. Suspensions containing 10^6 to 10^8 conidia/ml were used to infect females and oothecae. Mortality and other variables such as scanning electron microscopy were used to demonstrate the mechanism of action of the fungus. The isolated JAB 42 *A. westerdijkiae* is pathogenic to oothecae of *P. americana*, with low capacity to kill females. Adhesion, germination, penetration and extrusion of the fungus on the cockroach oothecae were observed.

Key words: cockroach, biological control, ovicide.

RESUMO

O controle de baratas realizado através da aplicação de inseticidas químicos exerce alta pressão seletiva sobre as populações e introduz substâncias sintéticas no ambiente, motivando a procura por outros métodos de controle, como os fungos entomopatogênicos. Os objetivos deste trabalho foram investigar a patogenicidade do isolado JAB 42 de *Aspergillus westerdijkiae* a fêmeas e ootecas de *Periplaneta americana* e demonstrar o mecanismo de ação sobre ootecas. Suspensões contendo 10^6 a 10^8 conídios/mL do isolado foram usadas para infectar fêmeas e ootecas. A ação do fungo foi analisada pela mortalidade e outras variáveis, e por microscopia eletrônica de varredura. O isolado JAB 42 de *A. westerdijkiae* é patogênico a ootecas de *P. americana*, tendo baixa capacidade de matar

fêmeas. Foi observada a adesão, germinação, penetração e extrusão do fungo sobre ootecas da barata.

Palavras-chave: barata, controle biológico, ovicida.

INTRODUCTION

Treatment against household pests is an area of growing interest in the world and aims to control urban pests such as cockroaches (KASSIRI & KAZEMI, 2012; MARICONI, 1999). The management of this insect is commonly performed only with the application of synthetic chemical insecticides. Less aggressive methods to the environment with low selective pressure on populations for tolerance or resistance have shown promising results, such as the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* (HERNANEZ-RAMIREZ et al., 2008; HUBNER-CAMPOS et al., 2013).

There are reports that *Aspergillus flavus* is pathogenic to *Blattella germanica* (KULSHRESTHA & PATHAK, 1997). *A. westerdijkiae* was found in the parasitoid *Prorops nasuta* cuticle (VEGA et al., 2006). The present research isolated this fungus from *P. americana* corpse, but there is no information on the pathogenic action of this microorganism to

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insects. *A. westerdijkiae* produces ochratoxins, but it is not a common cause of food spoilage, and it is not considered a good indicator of mycotoxin production (WORLD HEALTH ORGANIZATION, 2008). Therefore, it would be interesting to evaluate the potential of this fungus in *P. americana* control.

Most of the studies target the post-embryonic periods (HERNANEZ-RAMIREZ et al., 2008) of insects, but they should be essentially about spreading stages of the organism, such as females and eggs. *P. americana* can lay 51 oothecae in 25 months, generating about 816 descendants within two years (GALLO et al., 2002).

Little is known about the infection of oothecae by fungi, especially those of the genus *Aspergillus*, but the possibility of finding new pathogenic isolates to *P. americana* oothecae can bring new perspectives of control, since no chemical insecticides are effective so far when applied topically on this phase of the pest. This study aimed to investigate the pathogenicity of the isolate JAB 42 of *A. westerdijkiae* to females and oothecae of *P. americana* and to demonstrate its mechanism of action on oothecae, for use in biological control programs.

MATERIAL AND METHODS

Infection of *P. americana* females and oothecae

It was used the isolate JAB 42 of *A. westerdijkiae*, identified by Dr. Stephen W. Peterson (Agricultural Research Service - US Department of Agriculture), obtained from a *P. americana* adult, kept in the collection of Microbiology Laboratory of FCAV/UNESP. The oothecae and females of *P. americana* were obtained from mass rearing of the laboratory NEDTA/FCAV/UNESP.

Treatments were: T1 – no application (control); T2 – solution of Tween 80® (polysorbate 80) 0.1% (vehicle of suspensions); T3 - suspension of 3×10^8 conidia/mL; T4 - suspension of 3×10^7 con./mL and T5 – suspension of 3×10^6 con./mL.

Females were anesthetized with CO₂ and 100mL of suspensions were applied on their abdomens, using hand sprayer. Specimens were stored in plastic containers (10cm x 10cm) with perforated lids for aeration at $27 \pm 0.5^\circ\text{C}$ in the dark, RH $\geq 80\%$, and fed with water and dog food in abundance. Data were recorded for 20 days. Oothecae were cleaned with paper towel with deionized water and subjected to the same treatment and conditions as females. They were kept in Petri dishes containing moistened cotton and mortality was checked daily for 62 days.

Dead females and oothecae that did not show fungus extrusion were washed with sodium hypochlorite, dissected and its internal content introduced on PDA (potato dextrose agar) to confirm death caused by the fungus. The variables analyzed were: total mortality (TM), death confirmed by the fungus (DF), extrusion (EX) and mean death time caused by the fungus (TDF). The average time of fungal extrusion (TFE), total number of hatched nymphs per treatment (NN) and the average number of hatched nymphs per ootheca (NNO) were also evaluated.

The experimental design was completely randomized for females and in randomized blocks for oothecae, with a total of 250 individuals per experiment divided into five treatments and five replications. The analysis of variance was performed by F test and means were compared with Tukey test ($P \leq 0.05$). TM, EX and DF variables were transformed into arcsine $\sqrt{x/100}$ and TDF/TFE was submitted to the Log-rank test, by Kaplan-Meier method using JMP® Pro 11.1.1 program. All variables satisfy the assumptions of homogeneity of variances and normality of the treatments.

Electron microscopy scanning of pathogenic action of isolate JAB 42 *A. westerdijkiae* in *P. americana* oothecae

Forty five *P. americana* oothecae aged between 7 to 15 days were immersed for three minutes in suspension containing 6×10^8 con./mL of the isolate JAB 42 of *A. westerdijkiae* according to the protocol described by GARCIA et al. (2005). It was used the methodology of SANTOS & MAIA (1997), described below, to conduct the electron micrographs.

The oothecae were chemically fixed with 3% glutaraldehyde in sodium phosphate buffer 0.1M, pH 7.2 to 7.4, and placed in a refrigerator for 72h. The fixing process was performed during periods of 0h, 1h, 14h, 18h, 24h, 48h, 72h, 96h and 144h after application of the fungal suspension. Five oothecae were used for each treatment.

The oothecae were washed six times with pure buffer solution every 15 minutes to be post-fixed with osmium tetroxide 2% for at least 1h. Then, they were again washed with pure buffer and the samples were dehydrated with graded series of ethanol and subjected to drying with CO₂ until the critical point. Finally, oothecae were covered with gold particles of 35nm and electronically micrographed in the scanning electron microscope JEOL JSM 5410, operating at 15kV.

RESULTS AND DISCUSSION

Infection of *P. americana* females and oothecae

Total mortality of *P. americana* females sprayed with suspensions of *A. westerdijkiae* isolate ranged between 14 and 32% and did not differ significantly from the control (4%) and treatment with Tween 80® solution (20%) (Table 1). Although considered to be essentially a non-toxic adjuvant, Tween can cause the death of insects with differences in susceptibility of individuals of different species or within a population (OLIVEIRA et al., 2011).

In treatment 3 (3×10^7 con./mL) there was an average of 12% more dead females than in treatment T2 (Tween 80®) (Table 1), suggesting that the fungus is able to infect and kill female *P. americana*, regardless of the effect of Tween 80®. The fungus took 8.7 days to cause the death of females, the average between the three treatment suspensions, and in 12% of adults of treatments 3, 4 and 5 it was possible to verify its extrusion (Table 1).

It was observed that oothecae mortality risen due to the increase in the concentration of fungal suspension (Table 2). MNYONE et al. (2009) report a similar biological phenomenon with adults of *Anopheles gambiae*, in which mortality increased at interval concentrations of 10^7 - 10^{10} conidia/mL of *M. anisopliae* and *B. bassiana*. These concentrations can be easily achieved in laboratory for further use in the control of insects on field trials.

The highest mortality caused by the fungus in this experiment was 58% (T3) and differed from those in the controls (T1 and T2) and from a less concentrated suspension (T5) (Table 2). Mortality was found to be greater than 31% and 23% for oothecae treated with 2,5µL of 5×10^5 conidia of *M.*

anisopliae IP 46 and *M. robertsii* IP 34, respectively (HUBNER-CAMPOS et al., 2013).

In the mean time of 20.5 days it was possible to observe fungal extrusion on oothecae, particularly in the treatment concentration of 3×10^8 con./mL (Table 2). Oothecae with extruded fungus may be new pathogen inoculum sources.

In 14%, 12% and 2% of oothecae with fungus extrusion, which received treatments T3, T4 and T5, respectively, there was emergence of nymphs. This shows that the fungus is able to penetrate and infect the eggs but possibly without causing suffocation of the oothecae, as mentioned by HUBNER-CAMPOS et al. (2013).

There was no significant difference in the average number of nymphs per oothecae, but the application of *A. westerdijkiae* at a concentration of 3×10^8 con./mL reduced by 48.20% and 35.15% the total number of nymphs when compared with the total number obtained in the T1 and T2 controls, respectively (Table 2). The reduction in the number of nymphs hatched has been observed for females of *B. germanica* treated with suspensions of *M. anisopliae* (QUESADA-MORAGA et al., 2004).

The difference in susceptibility of females and oothecae to *A. westerdijkiae* can be attributed to the life stage of the insect. Nymphs of *B. germanica* and *P. americana* oothecae were also reported to be less susceptible to the fungus *M. anisopliae* when compared to adults (LOPES & ALVES, 2011; HUBNER-CAMPOS et al., 2013).

The infecting ability of *Aspergillus* can depend on intrinsic factors of the fungus, such as adhesion of conidia to the host (SHAHID et al., 2012) and the release of toxins (SHANKAR, 2013). There are also factors not related to the fungus that can influence infection process, such as the self-cleaning behavior of *P. americana*

Table 1 - Total mortality (TM), death confirmed by the fungus (DF), extrusion (EX) and mean death time by the fungus (DTF) of *P. americana* female sprayed with suspensions of different concentrations of the isolate JAB 42 of *A. westerdijkiae*.

Treatments	TM (%) ¹	DF (%) ¹	EX (%) ¹	DTF (days) ²
T1- Control	4.0 ± 2.2 a	0.0 ± 0.0 a	0.0 ± 0.0 a	-
T2- Tween 80® at 0.1%	20.0 ± 6.3 a	0.0 ± 0.0 a	0.0 ± 0.0 a	-
T3- 3×10^8 con./mL	14.0 ± 6.1 a	12.0 ± 5.2 ab	12.0 ± 5.2 a	9.0 ± 3.7 a
T4- 3×10^7 con./mL	32.0 ± 10.0 a	28.0 ± 8.7 b	12.0 ± 3.3 a	10.9 ± 1.6 a
T5- 3×10^6 con./mL	20.0 ± 5.7 a	14.0 ± 3.6 ab	12.0 ± 3.3 a	6.3 ± 2.0 a
	F = 2.124 ^{ns}	F = 7.692*	F = 4.772*	X ² = 2.2212 ^{ns}
CV (%)	68.43	77.60	88.86	

Means ± standard error. Statistical analysis was conducted with data transformed into arcsine for TM, DF and EX. Means followed by the same letter in the column do not differ by ¹Tukey tests (P = 0.05) and ²log-Rank by comparing pairs of isolates by analysis of survival by Kaplan-Meier method. F - F test, X² - Chi-square, *F test significant at 5% of probability. ^{ns}not significant. CV: coefficient of variation.

Table 2 - Total mortality (TM) death confirmed by the fungus (DF), extrusion (EX), average time of fungal extrusion (TFE), total number of hatched nymphs (NN) and average number of nymphs hatched per ootheca (NNO) of *P. americana* oothecae sprayed with suspensions of different concentrations of the isolate JAB 42 of *A. westerdijkiae*.

Treatments	TM (%) ¹	DF (%) ¹	EX (%) ¹	TFE ²	NN ¹	NNO ¹
T1- Control	6.0 ± 2.2 c	0.0 ± 0.0 c	0.0 ± 0.0 c	-	137.4 ± 3.7 a	14.6 ± 0.1 a
T2- Tween 80® a 0.1%	20.0 ± 4.9 bc	0.0 ± 0.0 c	0.0 ± 0.0 c	-	109.8 ± 6.1 ab	13.8 ± 0.2 a
T3- 3 x 10 ⁸ con./mL	60.0 ± 8.0 a	58.0 ± 9.5 a	36.0 ± 6.1 a	18.0 ± 2.5 a	71.2 ± 10.8 b	13.5 ± 0.6 a
T4- 3 x 10 ⁷ con./mL	46.0 ± 4.6 ab	46.0 ± 4.6 ab	26.0 ± 4.6 ab	21.5 ± 2.3 a	87.0 ± 8.4 b	13.6 ± 0.2 a
T5- 3 x 10 ⁶ con./mL	34.0 ± 6.7 ab	28.0 ± 7.7 b	16.0 ± 6.1 b	22.0 ± 6.3 a	95.2 ± 11.0 b	13.9 ± 0.2 a
	F = 12.84**	F = 34.15**	F = 20.48**	X ² = 1.5728 ^{ns}	7.02**	1.61 ^{ns}
LSD	18.26	17.02	15.83		40.05	1.41
CV (%)	29.08	36.41	47.92		21.14	5.34

Means ± standard error. Statistical analysis was conducted with data transformed into arcsine $\sqrt{x/100}$ for TM, DF and EX. Means followed by the same letter in the column do not differ by ¹Tukey tests (P = 0.05) and ²log-Rank by comparing pairs of isolates by analysis of survival by Kaplan-Meier method. F - F test, X² - Chi-square, *F test significant at 5% of probability. ^{ns}not significant. LSD: Least Significant Difference. CV: coefficient of variation.

females (SMITH & VALENTINE, 1985), which can remove much of the conidia applied on their abdomens. This shows that oothecae can facilitate the pathogenic action of the fungus.

Few studies have been conducted aiming the control of oothecae (MOHAN, 1999; HUBNER-

CAMPOS et al., 2013). However, the use of fungi shows to be efficient against this phase of pest cycle. Given the result of this research it is important to consider the use of the isolate JAB 42 of *A. westerdijkiae* in management of *P. americana* oothecae.

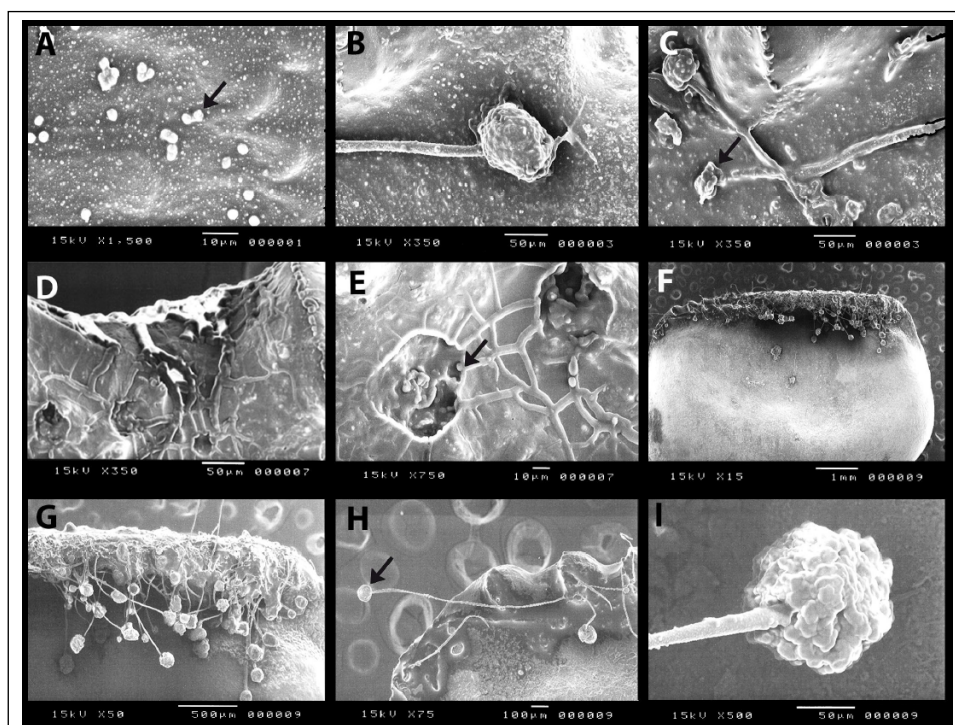


Figure 1 - Scanning electron micrography of *P. americana* oothecae at different times after immersion in a conidial suspension of the isolate JAB 42 of *A. westerdijkiae*. A - conidia present in the ootheca (arrow) 1h after infection; B - germ tube formation 14h post infection; C - appressoria formation (arrow) 14h post infection; D and E - hyphae formed on the ootheca tegument partially digested with apparent penetration (arrow) three days after infection; F and G - pathogen extrusion and formation of the fruiting body on the outer surface of the ootheca six days after infection; H and I - conidiophore on ootheca six days after infection.

Electron microscopy scanning of pathogenic action of isolate JAB 42 *A. westerdijkiae* in *P. americana* oothecae

The electron micrographs show germination, penetration and extrusion of *A. westerdijkiae* in *P. americana* oothecae (Figure 1). A similar result was found in a study on the infection of eggs of *Rhipicephalus sanguineus* by *M. anisopliae* (GARCIA et al., 2005). This shows that both fungi present the same process of infection.

The presence of conidia on the surface of ootheca was observed 1h after immersion in the fungal suspension (Figure 1A). Thereafter it was observed the formation of the germ tube (Figure 1B) and appressoria fixing to the integument of ootheca (Figure 1C). After 14h of immersion of oothecae, the penetration of the fungus process was initiated in the host.

The formation of hyphae and penetration into the ootheca occurred on the third day (Figures 1D and 1E) through holes present in the tegument of the ootheca. Such perforations may result from the action of enzymes secreted by the fungus, especially proteases, as ootheca is composed of 87% proteins (KRAMER et al., 1991). The fungus extruded on the sixth day (Figures 1F and 1G) with the presence of conidiophores (Figures 1H and 1I). Extrusion of *A. westerdijkiae* occurred through the teeth in the upper side of the ootheca and the superior openings existing for air exchange (MAYA et al., 2000).

CONCLUSION

The isolate JAB 42 of *A. westerdijkiae* is pathogenic to oothecae of *P. americana*, with low capacity to kill females. The penetration occurs through the cuticle of the ootheca and the pathogenic action culminates with the fungus extrusion.

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