Journal of Invertebrate Pathology 153 (2018) 30–34

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Management of the American cockroach's oothecae: The potential of entomopathogenic fungi control

Mariah Valente Baggio-Deibler[a,*], Marcelo da Costa Ferreira[a], Antonio Carlos Monteiro[b], Andressa de Souza-Pollo[c], Manoel Victor Franco Lemos[d]

[a] Department of Plant Protection, UNESP, Jaboticabal, SP, Brazil
[b] Department of Plant Production, UNESP, Jaboticabal, SP, Brazil
[c] Department of Preventive Veterinary Medicine and Animal Reproduction, UNESP, Jaboticabal, SP, Brazil
[d] Department of Applied Biology, UNESP, Jaboticabal, SP, Brazil

ARTICLE INFO

Keywords:
Biocontrol
Periplaneta americana
Ovicide
Ootheca
Extrusion

ABSTRACT

The *Periplaneta americana* species is an annoyance to man, causing allergies and damage to clothes and documents. It has the ability to spread pathogens and requires control measures. Control with natural enemies is less aggressive and can currently be applied with less risk than other techniques, such as chemical control, which is the main method used worldwide to control its post-embryonic stages. The potential microbial control of nymphs and adults of this pest has been shown, but little is known about its oothecae. There are isolates of fungal species that can be used to achieve this aim, but they may have innate differences in their virulence and ability to spread. This study aimed to identify fungal isolates JAB 68 and IBCB 35 through genetic sequencing of the ITS1-5.8S-ITS2 region, analyze their ability to synthesize chitinase, and investigate and compare their aggressiveness against *P. americana* oothecae and their influence on nymph eclosion. Fungal suspensions were inoculated into minimal medium containing glucose (control) as the sole carbon source and 1% colloidal chitin to determine the chitinolytic activity on the 4th, 7th and 10th days and sporulation on the 10th day. To obtain mortality, extrusion and the compiled number of hatched nymphs, oothecae were sprayed with suspensions of the isolates as follows: T1 – no application; T2 – aqueous solution of Tween 80® 0.1% (vehicle suspension for treatments T3 to T8); T3 – 2×10^9 conidia/mL of the JAB 68 isolate; T4 – 2×10^8 con./mL of the JAB 68 isolate; T5 – 2×10^7 con./mL of the JAB 68 isolate; T6 – 2×10^9 con./mL of the IBCB 35 isolate; T7 – 2×10^8 con./mL of the IBCB 35 isolate; T8 – 2×10^7 con./mL of the IBCB 35 isolate. The JAB 68 and IBCB 35 isolates were identified as belonging to the species *Metarhizium anisopliae* and *Beauveria bassiana*, respectively. Chitinolytic activity and extrusion were good parameters for evaluating the fungi's action on oothecal control. The most aggressive entomopathogen was *M. anisopliae* isolate JAB 68, with shorter time for fungus extrusion at a concentration of 2×10^7 con./mL. *B. bassiana* reduced the number of hatched nymphs at a concentration of 2×10^8 con./mL. Both fungi are capable of infecting and killing *P. americana*'s oothecae and reducing the number of nymphs hatched.

1. Introduction

Periplaneta americana (Linneaus, 1785) (Blattodea: Blattidae) are cosmopolitan cockroaches that occur frequently in urban sewage galleries and transit in anthropic environments, spreading pathogens; they are considered hazardous organisms to humans causing serious health problems such as allergies, asthma, and others (Thyssen et al., 2004; Pinto et al., 2007; Kassiri and Kazemi, 2012). These insects have oviparous reproduction with sets of 7–16 eggs inside of ovate capsules named oothecae. Females can lay approximately 27 oothecae per year, and 32 days is the average incubation period for eggs at 30 °C (Vianna et al., 2001), which clearly shows the great multiplication ability of this insect.

P. americana control has been primarily accomplished by the application of chemical insecticides, either in liquid or powder formulations, targeting its post-embryonic stages because of the difficulty of these products have penetrating through ootheca to reach the eggs. *P. americana* oothecae are composed of proteins (87%), oxalate (8%), diphenol (4%), lipids (1%) and calcium (6.5%) (Hackman and Goldberg, 1960; Kramer et al., 1991). The oxalate and calcium are responsible for

* Corresponding author.
E-mail address: mariahbdeibler@gmail.com (M.V. Baggio-Deibler).

https://doi.org/10.1016/j.jip.2018.02.008
Received 15 April 2017; Received in revised form 22 November 2017; Accepted 9 February 2018
Available online 10 February 2018

hardening the structure, making the passage of chemicals more difficult. Nonetheless, the high protein content can facilitate penetration of entomopathogenic fungi, considering the proteolytic activity of species such as *Metarhizium anisopliae* (Metschnikoff, 1879) and *Beauveria bassiana* (Balsamo) Vuillemin (1912) (Petlamul and Prasertsan, 2012) that are used in biological control programs. Therefore, the discovery of a new strategy to control the eggs of this pest, such as microbial control with fungus, could reduce the number of nymphs and adults of this organism in the environment, preventing risks of disease dissemination or of chemical contamination by the pesticides used to control them in the internal and external environments.

Intra- and inter-specific differences between fungi in pathogenicity and virulence to insects may occur depending on the species and the isolate used (Hubner-Campos et al., 2013; Islam et al., 2014). That is the reason why species identification is needed. One acceptable tool used for this purpose is genetic sequencing of the ITS1-5.8S-ITS2 region (Souza et al., 2013).

The choice of entomopathogens as an insect control agent is performed using mortality and lethal time parameters. However, fungi may have other important differences, such as sporulation on the host's body (Alves and Lecuona, 1998) and secretion of enzymes such as chitinases related to deposition and degradation of chitin, hatching of eggs and insect exoskeleton degradation (Xiuli et al., 1988), which can be correlated with the isolates' virulence and pathogenicity (Pelizza et al., 2012). Despite the lack of chitin in the composition of oothecae, fungal chitinolytic activity study can be an interesting tool to determine differences in fungal aggressiveness toward the host prior to conducting experiments aiming at controlling the pest in field conditions.

Only a few studies in the literature have reported fungal pathogenicity to cockroach oothecae. Isolates of *Metarhizium* spp. and *B. bassiana* have been considered the most promising for the control of *P. americana* oothecae (Mohan et al., 1999; Hubner-Campos et al., 2013). Penetration of *Aspergillus westerdijkiae* (Frisvad and Samson, 2004) through the integument of *P. americana*'s oothecae was shown by Baggio et al. (2016).

The aim of this study was to identify fungal isolates JAB 68 and IBCB 35, analyze their ability to synthesize chitinase, and investigate and compare their aggressiveness against *P. americana* oothecae and their influence on nymph eclosion.

2. Material and methods

The oothecae used were obtained from the mass rearing of the Laboratory of Nucleus Studies and Application Technology Development (NEDTA) Department of Plant Protection, Faculdade de Ciências Agrárias e Veterinárias (FCAV) from the Universidade Estadual Paulista (UNESP), Jaboticabal – São Paulo State, Brazil.

The isolates JAB 68 and IBCB 35 were provided by the Applied Microbiology Laboratory of the Department of Plant Production, FCAV, UNESP, Jaboticabal.

2.1. Identification of fungal isolates by genetic sequencing of the ITS1-5.8S-ITS2 region

Fungi were cultured for 7 days in 40 mL of potato dextrose medium at 27 °C in the dark. The mycelium of each isolate was filtered and placed to dry at 60 °C for 12 h. They were macerated with liquid nitrogen and DNA extraction was performed according to the Kuramae-Izioka Protocol (1997) with DNA precipitation in absolute ethanol.

Primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCC TCCGCTTATTGATAT GC-3′) (White et al., 1990) were used to amplify the fragment of ITS1-5.8S-ITS2. For PCR reactions, a 1× buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 1.0 U of Taq DNA polymerase (Invitrogen), 4 pmol primer, 60 ng of genomic DNA and ultrapure water were used to obtain a final volume of 20 μL. The amplification reactions were performed in a Nexus thermocycler (Eppendorf) using 1 cycle at 95 °C for 3 min, 36 cycles at 94 °C for 40 s, 60 °C for 40 s and 72 °C for one minute and 1 cycle at 72 °C for 10 min.

The amplified DNA fragment was subjected to sequencing PCR using BigDye Terminator v3.1 (Applied Biosystems) according to the manufacturer's instructions. The primers used were the same ones used for the amplification step. DNA sequencing was performed on the ABI 3100 sequencer (Applied Biosystems). The sequences obtained were evaluated using PHRED/PHRAP/CONSED (Ewing and Green, 1998; Green, 1996; Gordon et al., 1998). Sections of contiguous sequences of bases with quality equal or superior to 20 were accepted and used for analysis.

The obtained nucleotide sequences were compared to GenBank database sequences accessed through the National Center for Biotechnology Information (NCBI).

For phylogenetic relationships analysis, ITS sequences from this study and others from GenBank were aligned by MUSCLE (Edgar, 2004) with the software MEGA 6.06 (Tamura et al., 2013). The same program was used to determine the most suitable evolutionary model to be applied to sequences in Bayesian analysis using the Akaike Information Criterion (AIC) (Posada and Buckley, 2004). The phylogenetic tree was generated by MrBayes 3.2.3 software (Ronquist and Huelsenbeck, 2003) set for the Kimura-2 substitution model (Kimura, 1980) and Gamma distribution. The Markov Chain Monte Carlo (MCMC) algorithm performed analysis with four chains simultaneously, three hot and one cold. Four separate runs were performed with 1,000,000 generations, and the chains were sampled every 200 generations. At the analysis end, 25% of the trees were discarded as burn-in, considering a desirable standard deviation corresponding to less than 0.01. The tree generated by MrBayes was edited graphically by TreeGraph 2.3.0 software (Stöver and Müller, 2010).

2.2. Chitinolytic activity of fungal isolates

Fungi were inoculated into minimal medium (Pontecorvo et al., 1953) with the only carbon sources being glucose (control) and 1% colloidal chitin. Commercial chitin (Sigma-Aldrich) was hydrolyzed with fuming hydrochloric acid (Merck) with sequential washes using deionized water to obtain colloidal chitin (Hankin and Anagnostakis, 1975). The Petri dishes were stored at 27 ± 0.5 °C in the dark.

The diameter of fungal colonies and halos indicative of chitinolytic activity were measured with a millimetric ruler at 4, 7 and 10 days of incubation. With these data, the enzymatic index of chitin degradation was calculated, which corresponds to the ratio between the diameter of the colony and the degradation halo diameter (Rosato et al., 1981), and the lower the value obtained, the greater the chitinolytic activity. On the tenth day of cultivation, the conidia produced by colonies of fungi were collected and counted using a Neubauer chamber.

The experimental design was completely randomized with four treatments and five replications. Analysis of variance was performed by F test and comparison of means by Tukey test ($p \leq 0.05$).

2.3. Infection of Periplaneta americana's oothecae

Oothecae 1–3 days of age were randomly selected, cleaned with damp paper and submitted to the following treatments: T1 – no application (control); T2 – aqueous solution of Tween 80® 0.1% (vehicle suspensions); T3 – suspension containing 2×10^9 conidia/mL of the JAB 68 isolate; T4 – suspension with 2×10^8 con./mL of the JAB 68 isolate; T5 – suspension with 2×10^7 con./mL of the JAB 68 isolate; T6 – suspension with 2×10^9 con./mL of the IBCB 35 isolate; T7 – suspension with 2×10^8 con./mL of the IBCB 35 isolate; T8 – suspension with 2×10^7 con./mL of the IBCB 35 isolate.

Groups of 10 oothecae were sprayed with 1 mL of conidial suspension with the aid of a manual sprayer. They were placed in Petri dishes containing moistened cotton at a temperature of 27 ± 0.5 °C, RH > 80% and the absence of light. Mortality was assessed daily for

62 days.

Oothecae without fungus extrusion and without hatching nymphs were disinfected with sodium hypochlorite solution (3%), washed with deionized water, dissected and pieces of their internal content deposited on potato dextrose agar (PDA) medium to determine entomopathogen growth and confirm the causal agent of insect death of this stage.

Oothecae with no hatching nymphs were considered for total ootheca mortality analysis. The average time (in days) of fungus extrusion and the total number of hatched nymphs from all ten oothecae of each treatment were also evaluated.

Data were compiled and the results submitted to statistical analysis by Sisvar program – V. 5.3. The experimental design was completely randomized with 8 treatments, 5 repetitions per treatment and 10 oothecae per repetition. The analysis of variance was conducted by F test in a factorial design, with eight treatments × 2 fungi for ootheca mortality and number of hatched nymphs per treatment, and 6 treatments × 2 fungi for the average time of fungal extrusion, with the comparison of means by Tukey test ($p \leq 0.05$).

3. Results and discussion

3.1. Identification of fungal isolates by genetic sequencing of the ITS1-5.8S-ITS2 region

The technique of sequencing the ITS1-5.8S-ITS2 region is known as a precise and laborious method for cataloging molecular differences among fungi (Xu, 2006), and it proved to be an efficient tool for identifying the fungal species of this work.

The nucleotide sequences of isolates JAB 68 and IBCB 35 aligned with 100% similarity to the GenBank sequences of *M. anisopliae* and *B. bassiana*, respectively. The phylogram presented three main groups, one group composed of sequences of *Metarhizium* spp., the second composed of *Beauveria* spp. and the third was the external grouping (Fig. 1). The sequences were deposited in GenBank with the following encodings: KF958306 *Metarhizium anisopliae* JAB 68 and KF958305 *Beauveria bassiana* IBCB 35.

In the phylogram, *M. anisopliae* JAB 68 grouped with other *Metarhizium* species from GenBank but presented genetic distance from them (Fig. 1). The same was observed for isolate IBCB 35, which grouped with other isolates of *Beauveria* from GenBank, also showing genetic distance from them. This result indicates that the JAB 68 and IBCB 35 isolates from Brazil could be identified by genetic sequencing of the ITS1-5.8S-ITS2 region (Fig. 1).

Other studies also used the sequencing of the ITS1-5.8S-ITS2 region to identify isolates of *B. bassiana* and *M. anisopliae* with potential use in controlling insects such as *Ostrinia nubilalis* (Lepidoptera: Pyralidae) (Demir et al., 2012), *Galleria mellonella* larvae (Lepidoptera: Pyralidae) and *Tenebrio molitor* (Coleoptera: Tenebrionidae) (Mora et al., 2016).

3.2. Chitinolytic activity of fungal isolates

The analyzed fungal isolates showed differences in enzyme production and sporulation. The chitinolytic activity of *B. bassiana* isolate IBCB 35 was significantly higher (0.64) on the fourth day when compared with the JAB 68 isolate of *M. anisopliae* (0.84), but on the seventh and tenth days of culture, no significant difference of enzyme synthesis capacity was shown between fungi. The amount of conidia produced by isolate IBCB 35 in medium containing chitin was smaller than that produced by the isolate JAB 68 grown in the same medium, suggesting that JAB 68 has a greater capacity to channel the nutritional benefits of chitin lysis for conidiogenesis.

Chitinase secretion differs depending on the fungal isolate, and it decreases after 96 h of incubation (Petlamul and Prasertsan, 2012) as shown in Table 1. This occurs because the structure of the cell wall of these microorganisms is mainly composed of chitin (Seidl, 2008) and when in excess in their cells, chitinases can be harmful to secreting fungi.

3.3. Infection of Periplaneta americana oothecae

Both fungi were able to infect the oothecae of *P. americana* and showed similar results for total mortality ($F = 23.6055$, $df = 7$, $p < 0.0001$) (Fig. 2).

The entomopathogens sprayed on *P. americana* oothecae caused great mortality, differing from the control with no fungus application and the negative control (Tween 80® 0.1%). There was no significant difference in insect mortality considering the evaluated fungal concentrations and species of fungi. The results were similar to a study of the horizontal transmission of *M. anisopliae* in *Blattella germanica* (Linnaeus, 1767) in which ootheca viability was reduced 48–85% (Quesada-Moraga et al., 2004). Hubner-Campos et al. (2013) found a lower percentage of *P. americana* oothecae killed by fungi than that presented in this work, with approximately 31% mortality for isolate IP

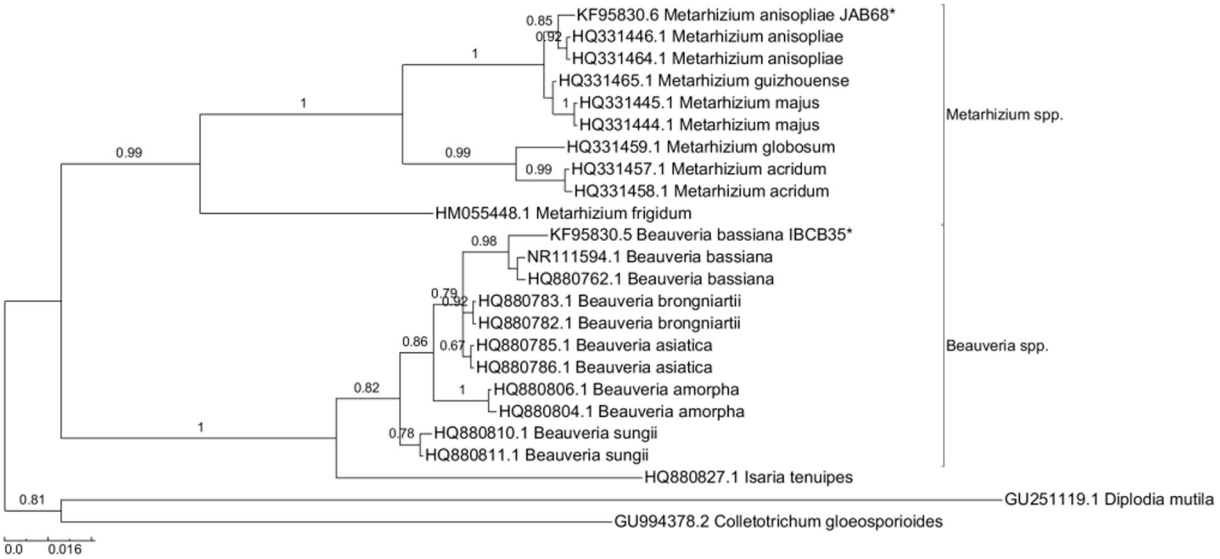

Fig. 1. Bayesian analysis of ITS sequences of isolates JAB 68 and IBCB 35 and species-type sequences from the GenBank database showing clustering of isolates according to their genetic similarity. Numbers between branches refer to Bayesian support values.

Table 1
Enzymatic activity index and sporulation of isolates IBCB 35 of *Beauveria bassiana* and JAB 68 of *Metarhizium anisopliae* in minimal medium containing chitin as a specific substrate for detecting chitinolytic activity.

Treatments	Enzymatic activity index			Sporulation[1] ($\times 10^8$ conidia/mm^2 colony)
	4 days	7 days	10 days	
No chitin + *M. anisopliae*	–	–	–	2.08 ± 0.35b
Chitin + *M. anisopliae*	0.84 ± 0.02b	0.90 ± 0.00a	0.90 ± 0.01a	1.25 ± 0.15b
No chitin + *B. bassiana*	–	–	–	1.71 ± 0.24b
Chitin + *B. bassiana*	0.64 ± 0.08a	0.94 ± 0.01a	0.90 ± 0.01a	0.22 ± 0.05a
F test	13.86	38.71	93.81	14.74

Original mean (± standard error of the mean), but statistical analysis was performed by the F test with [1]data transformed into $\sqrt{x+1}$. Means followed by the same letter in the column do not differ by Tukey test ($p \leq 0.05$). For all variables $p < 0.0001$ and df = 3.

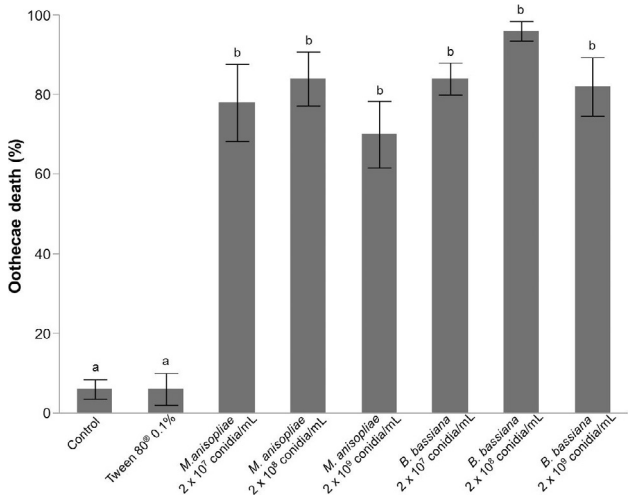

Fig. 2. Ootheca mortality of *Periplaneta americana* sprayed with suspensions of different concentrations of isolates JAB 68 of *Metarhizium anisopliae* and IBCB 35 of *Beauveria bassiana* under laboratory conditions. Original mean (± standard error of the mean); those followed by the same letter do not differ significantly by Tukey test ($p \leq 0.05$). The statistical analysis was conducted by F test with data transformed into arcsine $\sqrt{x/100}$.

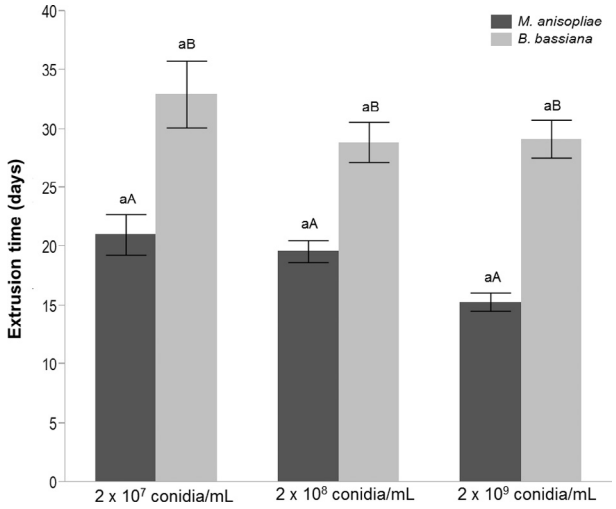

Fig. 3. Average time for extrusion of isolates JAB 68 of *Metarhizium anisopliae* and IBCB 35 of *Beauveria bassiana* on oothecae of *Periplaneta americana* treated with different concentrations of fungal suspensions in laboratory conditions. Original mean (± standard error of the mean), but statistical analysis was performed by the F test with data transformed into $\sqrt{(x+1)}$. Similar capital letters between treatments (fungi) and similar lowercase letters between concentrations of conidia tested do not differ statistically by Tukey test ($p \leq 0.05$).

46 of *M. anisopliae* and 23% mortality for IP 34 of *M. robertsii* at the concentration 5×10^5 con./mL.

Promising results have been verified for a fungal isolate of *Aspergillus westerdijkiae* species, confirming its infection, colonization and extrusion on oothecae of *P. americana* with an ootheca mortality rate of 60% caused by the fungus at a concentration of 3×10^8 con./mL (Baggio et al., 2016).

There was no relationship between the concentration of fungal conidia and extrusion time (Treatment F = 3.821, df = 2, p = 0.0363, Fungus: F = 68.762, df = 1, $p < 0.0001$. Treatment vs Fungi: F = 0.878; df = 2; p = 0.4287), but it was observed that *M. anisopliae* was more effective, taking from 15 to 21 days for its extrusion on the ootheca surface, while for *B. bassiana* it occurred 29–32 days after inoculation (Fig. 3). The same interspecific difference in performance of fungi was found in infections of three species of white grubs (Dhoj-GC et al., 2008), ticks of the species *Boophilus annulatus* (Say, 1821) (Pirali-Kheirabadi et al., 2007), and the large traces of wax from *Galleria mellonella* (Linnaeus, 1758) and *Tenebrio molitor* (Linnaeus, 1758) (Oreste et al., 2012).

The total number of hatched nymphs was significantly affected by the fungal species applied (F = 15.8670, df = 7, $p < 0.0001$), with little difference between the conidia concentrations used. The suspension of 2×10^8 con./mL can be considered the most effective because it provided approximately 75% and 95% reduction in the number of hatched nymphs from the application of *M. anisopliae* and *B. bassiana*, respectively, when compared to control (Fig. 4). This event shows the potential for reduction of offspring by the microbial agents used. A

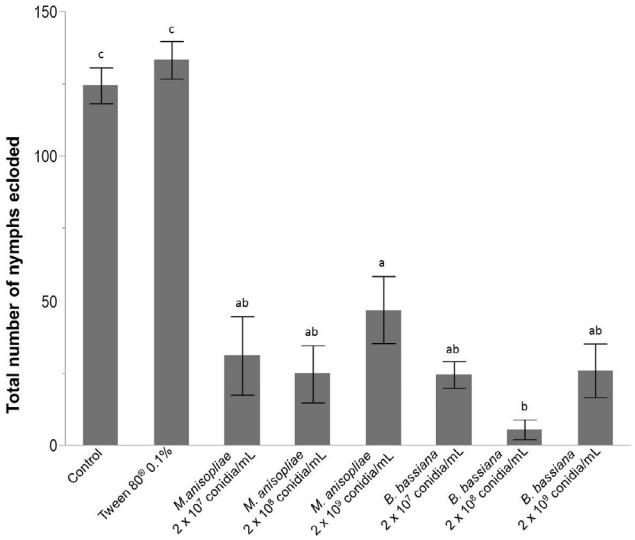

Fig. 4. Total number of hatched nymphs from *Periplaneta americana* oothecae sprayed with fungal suspensions of different concentrations in laboratory conditions. Original mean (± standard error of the mean), but statistical analysis was performed by the F test with data transformed into $\sqrt{(x+1)}$. Treatments with the same lowercase letter do not differ by Tukey test ($p \leq 0.05$).

similar result was observed for hatched larvae of *Choristoneura rosaceana* (Harris, 1841), decreasing by 90% following the treatment of eggs with suspensions of *B. bassiana* at a concentration of 1×10^8 con./mL (Cossentine, 2014).

Cockroach control is a worldwide problem, especially in the embryonic phase, due to a lack of chemical products available to kill this specific stage. Therefore, it requires new combat strategies involving new research. The promising results of this study showed that *M. anisopliae* and *B. bassiana* caused high mortality to *P. americana* oothecae in all evaluated conidia concentrations, suggesting that they can be successfully used in the control of this insect's embryonic stage as an alternative to chemical control. However, control of this stage of the insect's life cycle is still a challenge, especially in field conditions, because the environments these insects inhabit probably exert a great influence on the pathogenic action of fungi, suggesting the need for further specific studies for field application.

4. Conclusion

The fungal isolates JAB 68 and IBCB 35, identified as *Metarhizium anisopliae* and *Beauveria bassiana* species, respectively, are pathogenic to the eggs of *Periplaneta americana*. Their chitinolytic activity and extrusion were good parameters in evaluating the action of the fungi on ootheca control. Both fungi are capable of infecting and killing oothecae and reducing the number of nymphs hatched. There is high potential for using conidial suspensions of these isolates to control the insect, leading to a possible reduction of chemical applications to control its post-embryonic stages. The concentration of the conidia suspensions used exerted little influence on the fungal action. The most aggressive isolate was JAB 68 of *M. anisopliae* at a concentration of 2×10^7 con./mL, presenting shorter extrusion time on oothecae. Isolate IBCB 35 of *B. bassiana* significantly reduced the number of hatched nymphs at a concentration of 2×10^8 con./mL.

Acknowledgements

To Ana Carolina Peterossi, Dinalva Alves Mochi and all the students from NEDTA-FCAV/UNESP for helping conduct the experiments.

To Jake Deibler from Rent-a-Hero Studios LLC for providing the graphic design of the graphical abstract.

Funding: This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) (Ph.D. Degree scholarship) and by Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) (Process no. 2012/14503-1).

References

Alves, S.B., Lecuona, R.E., 1998. Epizootia aplicada ao controle microbiano de insetos. In: Alves, S. (Ed.), Controle microbiano de insetos, second ed. FEALQ, Piracicaba, pp. 97–170.

Baggio, M.V., Ferreira, M.C., Monteiro, A.C., Maldonado Junior, W., Lemos, M.V.F., 2016. Pathogenicity of *Aspergillus westerdijkiae* to females and oothecae of *Periplaneta americana*. Ciênc Rural. 46, 20–25.

Cossentine, J.E., 2014. Laboratory and field evaluations of the susceptibility of immature *Choristoneura rosaceana* (Lepidoptera: Tortricidae) to *Beauveria bassiana* (Hypocreales: Cordycipitaceae). Biocontrol Sci. Technol. 23, 396–408.

Demir, I., Seçil, E.S., Demirbağ, Z., Sevim, A., 2012. Molecular characterization and virulence of the entomopathogenic fungus *Beauveria bassiana* from *Ostrinia nubilalis* (Hubner) (Lepidoptera: Pyralidae). Türk Entomol. Bült. 2, 23–30.

Dhoj-GC, Y., Keller, S., Nagel, P., Kafle, L., 2008. Virulence of *Metarhizium anisopliae* and *Beauveria bassiana* against common white grubs in Nepal. Formos Entomol. 28, 11–20.

Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32, 1792–1797.

Ewing, B., Green, P., 1998. Basecalling of automated sequencer traces using PHRED. II. Error probabilities. Genome Res. 8, 186–194.

Gordon, D., Abajian, C., Green, P., 1998. CONSED: a graphical tool for sequence finishing. Genome Res. 8, 195–202.

Green, P., 1996. PHRAP Documentation. < http://bozeman.mbt.washington.edu/phrap.docs/phrap.html > (accessed on 23 March 2017).

Hankin, L., Anagnostakis, S.L., 1975. The use of solid media for detection of enzyme production by fungi. Mycologia 67, 597–607.

Hackman, R.H., Goldberg, M., 1960. Composition of the oothecae of three Orthoptera. J. Insect Physiol. 5, 73–78.

Hubner-Campos, R.F., Leles, R.N., Rodrigues, J., Luz, C., 2013. Efficacy of entomopathogenic hypocrealean fungi against *Periplaneta americana*. Parasitol. Int. 62, 517–521.

Islam, M.T., Omar, D., Shabanimofrad, M., 2014. Molecular identification and virulence of six isolates of *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes) to *Bemisia tabaci* Q biotype. J. Asia Pac. Entomol. 17, 237–241.

Kassiri, H., Kazemi, S., 2012. Cockroaches (*Periplaneta americana* (L.), Dictyoptera: Blattidae) as carriers of bacterial pathogens, Khorramshahr County, Iran. Jundishapur J. Microbiol. 5, 320–322.

Kimura, M., 1980. A simple method for estimating evolutionary rate of base substitution through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–120.

Kramer, K.J., Christensen, A.M., Morgan, T.D., Schaefer, J., Czapla, T.H., Hopkins, T.L., 1991. Analysis of cockroach oothecae and exuviae by solid-state 13C-NMR spectroscopy. Insect Biochem. Mol. Biol. 21, 149–156.

Kuramae-Izioka, E.E., 1997. A rapid, easy and high yield protocol for total genomic DNA isolation of *Colletotrichum gloesporioides* and *Fusarium oxysporum*. Rev. UNIMAR 19, 683–689.

Mohan, C.H.M., Lakshmi, A., Devi, U., 1999. Laboratory evaluation of the pathogenicity of three isolates of the entomopathogenic fungus *Beauveria bassiana* (Bals.) Vuillemin on the American cockroach (*Periplaneta americana*). Biocontrol Sci. Technol. 9, 29–33.

Mora, M.A.E., Chacón-Orozco, J.G., Harakava, R., Rouws, J.R.C., Fraga, M.E., 2016. Molecular characterization and virulence of *Beauveria bassiana* and *Metarhizium anisopliae* against *Galleria mellonella* (Lepidoptera: Pyralidae) and *Tenebrio molitor* (Coleoptera: Tenebrionidae) larvae. Afr. J. Microbiol. Res. 10, 662–668.

Oreste, M., Bubici, G., Poliseno, M., Triggiani, O., Tarasco, E., 2012. Pathogenicity of *Beauveria bassiana* (Bals.-Criv.) Vuill. and *Metarhizium anisopliae* (Metschn.) Sorokin against *Galleria mellonella* L. and *Tenebrio molitor* L. in laboratory assays. REDIA 95, 43–48.

Pelizza, S.A., Elíades, L.A., Saparrat, M.C.N., Cabello, M.N., Scorsetti, A.C., Lange, C.E., 2012. Screening of Argentine native fungal strains for biocontrol of the grasshopper *Tropidacris collaris*: relationship between fungal pathogenicity and chitinolytic enzyme activity. World J. Microb. Biot. 28, 1359–1366.

Petlamul, W., Prasertsan, P., 2012. Evaluation of strains of *Metarhizium anisopliae* and *Beauveria bassiana* against *Spodoptera litura* on the basis of their virulence, germination rate, conidia production, radial growth and enzyme activity. Mycobiology 40, 111–116.

Pinto, A.S., Rossi, M.M., Salmeron, E., Pinto, C.S., 2007. O meio urbano e as pragas urbanas. In: Pinto, A.S., Rossi, M.M., Salmeron, E. Manejo de pragas urbanas, Piracicaba, CP2, pp. 13.

Pirali-Kheirabadi, K.H., Haddadzadeh, H.R., Razzaghi-Abyaneh, M., Zare, R., Ranjbar-Bahadori, S.H., Rahbari, S., Nabian, S., Rezaeian, M., 2007. Preliminary study on virulence of some isolates of entomopathogenic fungi in different developmental stages of *Boophilus annulatus* in Iran. Online J. Vet. Res. 62, 113–118.

Pontecorvo, G., Roper, J.A., Hemmons, L.M., Macdonald, K.D., Bufton, A.W., 1953. The genetic of *Aspergillus nidulans*. Adv. Genet. 5, 141–238.

Posada, D., Buckley, T.R., 2004. Model selection and model averaging in phylogenetics: advantages of Akaike information criterion and bayesian approaches over likelihood ratio tests. Syst. Biol. 53, 793–808.

Quesada-Moraga, E., Santos-Quiro's, R., Valverde-García, P., Santiago-Álvarez, C., 2004. Virulence, horizontal transmission, and sublethal reproductive effects of *Metarhizium anisopliae* (Anamorphic fungi) on the German cockroach (Blattodea: Blattellidae). J. Invertebr. Pathol. 87, 51–58.

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.

Rosato, Y.B., Messias, C.L., Azevedo, J.L., 1981. Production of extracellular enzymes in isolates of *Metarhizium anisopliae*. J. Invertebr. Pathol. 38, 1–3.

Seidl, V., 2008. Chitinases of filamentous fungi: a large group of diverse proteins with multiple physiological functions. Fungal Biol. Rev. 22, 36–42.

Souza, A., Delphino Carboni, R.C., Wickert, E., Macedo Lemos, E.G., Goes, A., 2013. Lack of host specificity of *Colletotrichum* spp. isolates associated with anthracnose symptoms on mango in Brazil. Plant Pathol. 62, 1038–1047.

Stöver, B.C., Müller, K.F., 2010. TreeGraph 2: combining and visualizing evidence from different phylogenetic analyses. BMC Bioinf. 11, 7.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.

Thyssen, P.J., Moretti, T.C., Ueta, M.T., Ribeiro, O.B., 2004. O papel de insetos (Blattodea, Diptera e Hymenoptera) como possíveis vetores mecânicos de helmintos em ambiente domiciliar e peridomiciliar. Cad. Saúde Pública. 20, 1096–1102.

Vianna, E.E.S., Berne, M.E.A., Ribeiro, P.B., 2001. Desenvolvimento e longevidade de *Periplaneta americana* Linneu, 1758 (Blattodea: Blattidae). Rev. Bras. Agrocienc. 7, 111–115.

White, T.J., Bruns, T., Lee, S., Taylor, J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelgard, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego, USA, pp. 315–322.

Xiuli, H., Danme, A., Qian, R., 1988. Study of chitinase in cultures of *Beauveria bassiana*. In: Proceedings from the 4th Congress on Chitin and Chitosan. Elsevier Science Publishers LTD, Trondheim, pp. 309–317.

Xu, J., 2006. Fundamentals of fungal molecular population genetic analyses. Curr. Iss. Mol. Biol. 8, 75–90.