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Original Research Article

Experimental study on the effect of microbiological interactions in the scorpion *Tityus serrulatus*

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A B S T R A C T

Keywords

Biological control, entomo-pathogenic fungi, fungal repellency, antibiosis, disharmonious interaction.

The urban sprawl combined with extinction of rural areas and nature reserves in cities favored the development of many pests adapted to sinanthropic ecosystem where the yellow scorpion (Scorpiones: Buthidae, *Tityus serrulatus*, Lutz and Mello, 1922) stood out as urban pests causing serious human accidents of public health importance. The use of fungal pathogens for biological control of pests and the richness of its biodiversity in species and strains of importance in the research for pathogens in invertebrates have developed a great potential for biological control of venomous arthropods. In the period 2006 to 2013, were implemented a mechanical method of catching scorpions in infestation foci of cemetery in Americana, São Paulo, Brazil, using a procedure that consisted in opening tombs to collect scorpions housed internally. During this activity has been found that in many tombs had no scorpions there was a contamination with environmental characteristics of filamentous fungi, compared to other large amounts of them in the absence of fungal growth. Based on these field observations, we performed a bioassay for analysis of possible interactions of microorganisms isolated from these sites of infestation, which would determine repellency or death of specimens of scorpions kept in an experimental system monitored.

Introduction

The Phylum Arthropoda represents more than 80% of the total existing species including the arachnidan (Subphylum Chelicerata - Class Arachnida) where the scorpions are classified (Order Scorpiones). The origin of scorpions is dated from 400 million years ago. The species survived to all the major cataclysms, it was on earth before the dinosaurs. Scientific evidences appoint that scorpions were from aquatic life, as the specie *Praearcturus Gigas*, a great predator with around one meter long.

Nowadays scorpions adapted themselves to all habitats in earth, except on Antarctic Lands, occupying savannas, deserts, cerrado areas, tropical and subtropical forests. About 1,500 species of scorpions are described (distributed into 18 Families) and thirty of then are potentially dangerous to human beings.

The scorpions are a health public problem in several areas, where the incidence and severity of accidents are high. The World Health Organization (WHO) considers it a negligence situation, transformed into a tread to the welfare and life quality of several populations around Earth. There are rare statistical data about death from scorpions, suggesting mortality on 4% of the accidents, mainly children and elderly.

The most poisonous genera are from North Africa and the Middle East (*Androctonus*, *Buthus*, *Hottentotta* and *Leiurus*), South America (*Tityus*), South India (*Mesobuthus*) and Mexico (*Centruroides*), responsible for serious poisoning and death, achieving a population of 2.3 billion people exposed to scorpions (Chippaux and Goyffon, 2008).

In Brazil around 100 scorpions species are known and the major specie for medical importance is the *Tityus serrulatus*, which does not present males and its reproduction happens with parthenogenesis. Lourenço et al (1996) compared the theoretical reproduction rates and population dynamics of two species *Tityus fasciolatus* (sexual reproduction) and *Tityus serrulatus* (parthenogenetic reproduction) concluding that after 5 generations, the sexual specie showed around 2 million new individuals and the parthenogenetic specie 33 million of new individuals. Due to the high toxicity, the highly developed invasive habit and its prolificacy, this parthenogenetic specie is a great health problem classified as an urban plague causing severe accident and death around Brazilian regions.

Scorpions are responsible for the major number of accident notification from poisoning in Brazil and the most infested regions are Minas Gerais, Bahia and São Paulo, respectively. In 2013, at São Paulo State, there were 11,444 accidents and 4 death, with an incidence rate of 27 accidents per 100,000 inhabitants and a more severe epidemiological situation at Americana municipality, with 143 accidents per 100,000 inhabitants, five times the State indice.

Anthropic actions have accelerated the disorientated growth of cities and the extinction of rural areas and natural reserves, favoring the development of several plagues adapted to the sinanthropic environment. Scorpions adapt themselves to big urban centers (62.2% of accidents occur in urban areas) due to the existence of adequate environment for its proliferation as sewage pipes and cemeteries that offer ideal conditions for its maintenance and reproduction. Its highly adaptation skill allow the development of resistance to the major chemical products used for its eradication, generating more exposition and accident risks.

There are eight commercial insecticides registered in Health Ministry for the scorpions control and a huge discussions about technical-scientific use of such tools, lack of laboratory consensual opinion for the evaluation of active molecules efficiency and the application methodology. Some inseticides, specially from piretroid group has the dislodge effect over scorpions in its hiden places, increasing the accident possibilities. Now, the use of micro encapsulated formula contributes for the control process of arachnids and it is the most desirable protocol to reduce the dislodge effect, minimizing the ability of the scorpions to perceive chemical substances due to its quimiorreceptor mechanisms. According to law from Unique Health System (SUS), the cities are responsible for the control of any animal hazardous to human, as well as the technical and scientific responsibility (Souza, 2012).

There are many research appointing to entomopathogen potential on the plague control and fungi can infecte several hosts and it has ability to infect insects (Dictyoptera, Orthoptera, Dermoptera, Culicidae, Muscidae, Simulidae, Tabanidae, Cimicidae, Vespidae, Formicidae, Lepidoptera e Termitidae) e carrapatos (Ixodidae e Argasidae). Fungi species most common for entomopathogenic skills are *Beauveria bassiana* and *Metarhizium anisopliae* with actual application on program control of agriculture and forest finality (İnci et al., 2014).

Entomopathogenic fungi are characterized for causing epizootia, showing high growth rate, ability of survive at wide range of environment, high infectant units, high resistance to physical-chemical barriers from tegument and hemolymph, and several strains with virulence levels, pathogenicity and host reach.

According to Khachatourians and Uribe (2004), fungi diseases in arthropods represent an important opportunity for better comprehension over molecular basis of fungi-insect interactions as well as its application on mycology, biotechnology and entomology by biological control. The cycle fungi-host depends on environmental conditions as temperature, humidity, light, ultraviolet radiation as the nutritional conditions and host susceptibility, presenting phases as: adhesion, germination, appressorium formation, clasp formation, penetration, colonization, reproduction and dissemination.

Gupta et al (1991) isolated by HPLC a toxic secondary metabolite (beauvericine) from mycelium extracts of *Fusarium* (*F. sernitectum* and *F. moniliforme* var. *subglutinans*), with insecticide properties against a bug (*Leptinotarsa decemlineata*), parasite of potato plants.

Souza (2011) verifyed *Metarhizium anisopliae* produced hydrolitic enzymes to infect arthropods and infection structures (appressorium and blastospores) to penetration and dissemination steps on host. Among mechanisms of interaction on tropical environments the facultative mutuality association between yeast and larvae/adult from *Drosophila* species demonstrated an important role for the study of communities and coevolution structure of those two groups involved in microbiological ecology (Morais and Rosa, 2000). Guerra et al (2001) using PDA (potato dextrose agar) added with wide range antibiotic isolated fungi colonies of *Cladosporium* spp., *Beauveria* spp., *Penicillium* spp., *Curvularia* spp., *Aspergillus* spp., *Alternaria* spp., *Nigrospora* spp., *Fusarium* spp., *Metarhizium* spp., *Trichoderma* spp. e *Mycelia sterilia* in *Musca domestica*, *Rhipicephalus sanguineus* and *Cochliomyia macellaria*.

Kanzok and Jacobs-Lorena (2006) demonstrated that contact with conidia of *Beauveria bassiana* can eliminate efficiently the female adults of *Anopheles stephensi* mosquitoes (Malaria vectors) but called attention to the lack of specificity, noting that Metarhizium anisopliae infects approximately 200 arthropod species and its conidia can also kill other important organisms in an ecosystem. Fungal biopesticides were evaluated about potential virulence of entomopathogenic fungi (*Beauveria bassiana* and *Metarhizium anisopliae*) on Malaria transmission, whose control efficacy is threatened by the evolution of resistant mosquitoes to the insecticides (Lynch et al., 2012). The larvicide efficiency of synthetized nanoparticules by *Aspergillus niger* fungus was tested in mosquitoes larvae *Anopheles stephensi*, *Culex quinquefasciatus* and *Aedes aegypti*, with 100% of mortality at every larvae stage, after 48 hours of exposition (Soni and Prakash, 2012). George et al (2013) proved the attractivity tested in bioassay with 95% females from *Anopheles stephensi* for *Beauveria bassiana* spores, evaluating the bioinsecticide potential for malaria vectors control.

Research on biological control using fungi, papers developed with *Metarhizium anisopliae* and *Beauveria bassiana* presents excelent results on ticks *Rhipicephalus sanguineus* (Monteiro et al., 1998), *Rhipicephalus appendiculatus*, *Amblyomma variegatum* and *Boophilus deceloratus* (Kaaya and Hassan, 2000), in fleas *Ctenocephalides felis felis* (Melo, 2006) and in triatomine *Triatoma infestans* (Forlani et al., 2011).

Aspergillus ochraceus was isolated from *Rhipicephalus sanguineus* (naturally infected) causing a disease characterized by the absence of oviposition, evolving to mummification and death (Estrada-Peña et al., 1990). The fungi isolation from ticks was reported by Mwangi et al (1991) isolating *Aspergillus* spp., *Beauveria* spp., *Penicillium* spp., *Torrubiella* spp., *Cephalosporium* spp., *Paecilomyces* spp., *Fusarium* spp. and *Mucor* spp. from naturally infected ticks. Monteiro et al (2003) observed the isolation of fungi in ticks *Rhipicephalus sanguineus* and *Boophilus microplus*, naturally infected by colonies *Alternaria*, *Mucor*, *Fusarium*, *Curvularia*, *Penicillium* and *Aspergillus*. Morais-Urano et al (2012) evaluated the acaricide action of cyclic peptide with biological activity (destruxins) isolated from culture media of *Beauveria felina* in ingurgitated female of *Rhipicephalus* (*Boophilus*) *microplus*, resulting on around 30% of efficiency.

D'Alessandro et al (2012) showed that isolates of *Beauveria bassiana* and *Purpureocillium lilacinum* (=*Paecilomyces lilacinus*) (found in ingurgitated female of *Amblyomma cajennense* harvested from horses) and *P. lilacinum* and *Metarhizium anisopliae* (detected in harvested soil from pastures in Central Brazil) probably act as natural antagonists of *Amblyomma cajennense*, specially during raining season, considering important explore the potential of such pathogens on the development of the main active ingredients of micoacaricidas for control of Spotted Fever vectors and other important disease spread by ticks.

Once there are few references to surveys of biocontrol of scorpions, this study aimed to observe possible antagonistic interactions of microorganisms (harvested and isolated from environmental infestation places) that determined repellency or death of specimens *Tityus serrulatus* maintained in an experimental system monitorated.

Materials and Methods

Sampling and maintenance of microbiological material

Fungi sampling was performed scraping walls from 10 (ten) charnel house. Fungi were cultivated in laboratory culture media

(YEPD - Yeast Extract-Peptone-Dextrose). After colonies development, it was inoculated into two experimental groups of scorpions, to observe repellency or not from microorganisms, as well as evaluate the pathogenicity by death.

Capture and maintenance of scorpions

Tityus serrulatus scorpions were captured during night period, using ultraviolet light (UV) and transported to a vivarium at the Program of Surveillance and Control of Ticks and Scorpions (PVCE) from Health Secretary Americana county, São Paulo State, in conformity with Normative Instruction nº 141, of December 19, 2006 and Normative Instruction nº 154 of March 1, 2007, of the Brazilian Institute of Environment and Renewable Natural Resources - IBAMA and under authorization for scientific finality /SISBIO nº 20562-1.

Specimens selection and definition of amount of scorpions used in research

The amount of 100 (one hundred) selected scorpions were submited to aseptic protocol with 5 seconds immersion on Sodium hypochloride 2%, 5 seconds in distilled water, 5 seconds in alcohol 70% and 5 seconds in distilled water again.

Bioassay for repellency evaluation to fungi environment

In the first experimental group were used five plastic boxes (54 cm lenght x 37 cm wide x 37 cm height, 56.1 liter capacity) connected to each other from a distribution box, by a connexion transparent 15 cm pipe of two inches diameter (Figure 1).

Excepting the distribution box, the other 4 boxes were wrapped in black plastic, avoiding light, mimetizing the scorpion's circadian cycle, which are more active during night time. In two of the four boxes, Petri dishes with fungi colonies were placed in the bottom. Two boxes were used as control. In the first day, 100 scorpions were added to distribution box and readings were performed every 12 hours, counting the number of scorpions on each box, during 45 days. Data from thermo-hygrometry were also collected from inside and outside the experimental environment.

Bioassay for pathogenicity evaluation

In the second experimental group were used ten plastic boxes (39 cm length x 27 cm wide x 14 cm height, 8.6 liters capacity): one control box (without Petri dish) containing 10 (ten) scorpions and nine boxes with 10 scorpions each (submitted to smears of biological material to induce contamination) and a Petri dish with microbiological growth to evaluate pathogenicity (Figure 2).

Statistical analysis of bioassay for fungal repellency

Data collected from first experiment were analyzed considering a factorial assay in a random experimental design with two factors (presence and absence of fungi in the boxes) and periods of observation (7:00 and 19:00 o'clock) during 45 days of observation.

Microbiological analyzes of bioassay for pathogenicity evaluation

For the processing of dead scorpions, there was a degermation of exoskeleton, with passage in iodine alcohol, followed by chlorhexidine 2% and sterile distilled water (to remove the residue of chlorhexidine and iodine). Scorpions were soaked into a sterile saline solution, added of chloramphenicol 200 mg L-1 followed by another passage in sterile distilled water. Each sample was placed into Sabouraud dextrose liquid medium 4%, to prove degermation. The scorpions were segmented into pieces, grinded and seeded in Petri Dishes with solid Sabouraud. From the colony growth, microorganisms were identified. Positive samples for microbiological assay were isolated and the identification of filamentous fungi was performed in Sabouraud dextrose agar in order to reactivate the fungal structures. Microscope slides were prepared using a colony fragment added with blue cotton lactophenol or clarifier (for very dark or black colonies), followed by the technique of cultivation in slide and technique of adhesive tape, that allow to visualize whole structures of fungi, with same dye and clarifier. In slides, we could observe the structures of fungi as mycelium and conidia, hyaline or dark, septate or not, septate conidia with transverse or longitudinal divisions and the shape and size of conidia. Conidia formed from hyphae, from short conidiophores or from complex structures as phialides from vesicles over stipe could be observed also. Presence or absence of sporangia with sporangiospores was observed. When asexual structures were not observed, conidiogenesis were stimulated with specific media. Proves of assimilation of carbonaceous and nitrogenous compounds as well as proves of fermentation were also performed (De Hoog et al., 2000).

Results and Discussion

Differing of bacteria, protozoa and virus, fungi can infect insects not only by the intestine but also by the spiracles and by the tegument surface. Spores (conidia) of entomopathogenic fungi infects host under desirable temperature and humidity, adhere to the cuticle, germinating and penetrating inside, producing hyphae, leading to insect death by tissue destruction or eventually by toxic compounds released by the fungi. Basically, the action mode is germination, apressorius formation, colonization and rarely, fungi can be ingested by the host (Makita et al., 2011).

Despite the existence of several results on fungi activity upon control of invertebrates in Phylum Arthropoda, the majority of such studies are related with species belonging to the Class Insecta (Gupta et al., 1991; Guerra et al., 2001; Melo, 2006; Kanzok and Jacobs-Lorena, 2006; Forlani et al., 2011; Lynch et al., 2012; Soni and Prakash, 2012; George et al., 2013) and to Order Ixodida, inside Class Arachnida (Estrada-Peña et al., 1990; Monteiro et al., 1998; Kaaya and Hassan, 2000; Guerra et al., 2001; Monteiro et al., 2003; Morais-Urano et al., 2012; D'Alessandro et al., 2012).

In the Class Arachnida (Order Araneae), Beys-da-Silva et al (2013) proved the efficiency of control of brown spider (*Loxosceles* sp.) with a mortality rate of 100% (for young and adults) from 9 to 12 days after application by aspersion of a conidia suspension of *Metarhizium anisopliae*, suggesting the development of strategies specific to public health problems, in order to reduce the loxoscelism in Brazil and other countries which holds thousands cases per year.

Nowadays, reports on scorpions experiments with entomopathogenic fungi is null and a single report from Santana-Neto et al (2010) show the natural occurency of *Fusarium solani* in *Tityus stigmurus*, by observation of infected species where the fungus can be isolated from quelicera and intersegment areas, decreasing the locomotion and feeding, leading to host death.

Table.1 Average values from counting adult females of *Tityus serrulatus* per treatment and period of observation.

Treatment	Morning (7 am)	Afternoon (7 pm)
Control	50.47 A a*	42.62 B a
Fungal Colonies	38.53 A b	33.89 B b

* Average of treatments with different capital letters in the lines or different lowercase letters in the columns differ significantly ($P<0.05$) by Tukey's test.

Table.2 Average values from counting adult females with offspring of *Tityus serrulatus* per treatment and period of observation.

Treatment	Morning (7 am)	Afternoon (7 pm)
Control	6.67 A a*	6.11 A a
Fungal Colonies	2.16 A b	2.22 A b

* Average of treatments with different capital letters in the lines or different lowercase letters in the columns differ significantly ($P<0.05$) by Tukey's test.

Table.3 Amount of fungal strains isolated in bioassay for repellency

Fungi	Number of strains
Penicillium spp.	09
Mucor spp.	08
Fusarium spp.	06
Aspergillus spp.	06
Scopulariopsis spp.	04
Aspergillus fumigatus	03
Syncephalastrum spp.	02
Trichosporon spp.	02
Absidia spp.	02
Rhizopus spp.	01
Neospora spp.	01
Micelia sterilia	01
Cladosporium spp.	01
Cunninghamella spp.	01

Table.4 Percentage of fungal isolates in bioassay for pathogenicity

Fungi isolated	Presence in the samples
Fusarium spp.	70%
Cladosporium spp.	20%
Paecilomyces spp.	10%
Phialophora spp.	10%
Acremonium spp.	10%
Micelia sterilia	10%

Figure.1 Scheme of experimental design, with the distribution box in the center, connected to others by polypropylene pipe.

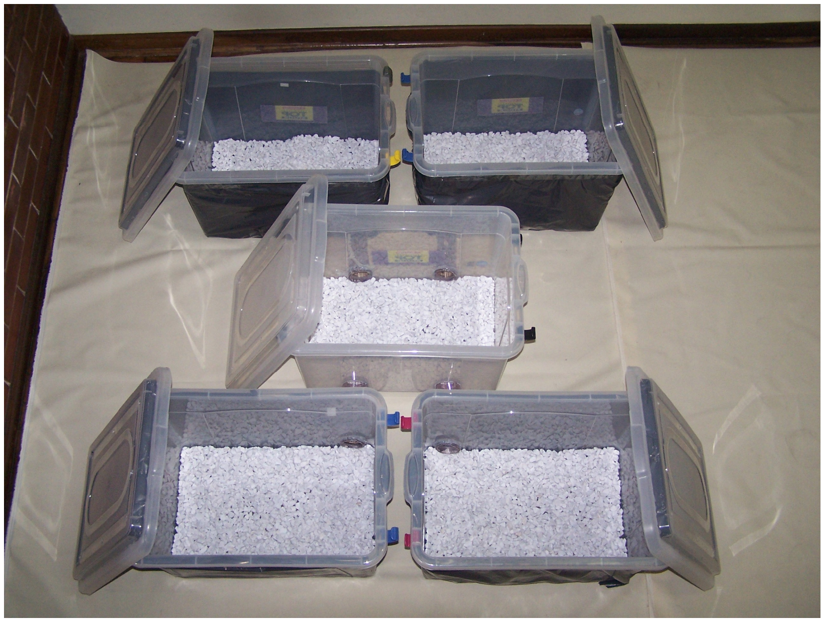

Figure.2 Detail of the box containing scorpions and a Petri dish with microbial growth for evaluation of pathogenicity

From 2006 to 2013, a mechanical method for scorpions capture has been implanted by the Health Department Americana county, São Paulo state, focusing infested areas such as cemeteries (Brites-Neto and Brasil, 2012). During the procedure of opening graves, it was observed that graves with no scorpions presented some hyphae fungi. A hypothesis was raised about a biological barrier harmful to scorpions in comparison to captures done at graves infested with scorpions, with no fungi contamination. Based on these observations were evaluated the possible mechanisms involved in this interaction.

For the bioassay, the evaluation of repellency of fungal environment was researched to *Tityus serrulatus* and an analysis of variance (ANOVA) was performed with the counting number of scorpions (adult females and females with offspring) in the two boxes from each treatment (control and exposure to the fungus), during two periods of observation (7 am and 7 pm), previously transformed into square root, minimizing the experimental error. The averages of counts of adult females of *Tityus serrulatus* and its comparison by the Tukey's test (α=0.05) are presented on Table 1.

It was verified an increase on the number of adult females during morning observations ($P<0.05$), in the control group and the treatment. A higher number of adult females were found in the control boxes, independent on the period observed, proving that environment with fungal colonies significantly reduced ($P<0.05$) the staying of adult females.

The averages of counts of adult females with offspring of *Tityus serrulatus* and its comparison by the Tukey's test (α=0.05) are presented on Table 2.

It was found that the averages of counts of adult females with offspring did not differ significantly ($P>0.05$) according to the period observed (morning or afternoon), for control and treatment groups. A higher number of adult females with offspring were found in the control boxes, independent on the period observed, proving that environment with fungal colonies significantly reduced ($P<0.05$) the staying of adult females with offspring.

In the bioassay for repellency observation, twelve genera and two species of fungi were isolated and identified, from microbial growth into Petri dishes and from dead scorpions processing (Table 3). In the bioassay for pathogenicity evaluation, five genera and one species could be isolated and identified by microbial growth of collected fungi and from 16 dead scorpions processed and seeded into Petri Dishes (Table 4).

The present study concludes, the great potential of pathogenic fungi and the richness of its diversity highlights the research importance on searching new compounds as bioinsecticides or in the biological control of venomous arthropods. In this bioassay was proved that there is an antagonism between scorpions and fungal strains of *Fusarium* spp., *Cladosporium* spp., *Paecilomyces* spp., *Phialophora* spp., *Acremonium* spp., *Aspergillus* spp., *Scopulariopsis* spp., *Cunninghamella* spp., *Penicillium* spp., *Neospora* spp., *Syncephalastrum* spp., *Trichosporon* spp., *Absidia* spp., *Rhizopu*s spp., *Mucor* spp., *Aspergillus fumigatus* and *Micelia sterilia*, by observation of repellency for scorpions Tityus serrulatus in contaminated environments experimentally by these fungal varieties. Based upon these studies and observations, new research on biocontrol of scorpions can reduce accidents with this arthropod, minimizing epidemiological risks and its relevance for Public Health.

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