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Pathogenicity and virulence of four isolates of *Metarhizium anisopliae* on selected natural enemies: *Cryptolaemus montrouzieri, Anagyrus kamali, Lysiphlebus testaceipes* and *Bracon thurberiphagae*

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ABSTRACT

Integrated Pest Management (IPM) involves the use of cultural, chemical and biological agents and more recently genetic modification to limit pest incidence in crop production. The effectiveness of the biological agents is dependent on the compatibility of the living organisms with the cultural and chemical components as well as other living organisms used in the programme. In this paper the pathogenicity of four isolates (ARSEF 932, ARSEF 954, IMI 152222 and IMI 299982) of the entomopathogenic fungus, *Metarhizium anisopliae* was tested against selected natural enemies *viz., Cryptolaemus montrouzieri, Anagyrus kamali*, *Lysiphlebus testaceipes* and *Bracon thurberiphagae*. Bioassays and probit analyses were used to determine the LC_{50} and LT_{50} of each fungal isolates against the natural enemies. The results of this study will be used to assist in the determination of the compatibility of *M. anisopliae* with the selected natural enemies when developing IPM programs in which these natural enemies are used as biological agents.

Keywords: Entomopathogenic fungus, natural enemies, compatibility, Integrated Pest Management (IPM)

INTRODUCTION

Biological pest control in the Caribbean was almost completely neglected with the advent of chemical pesticides in the early 1930s (Cruz and Segarra, 1992). However, the birth of the Integrated Pest Management (IPM) concept has given new life to biological control in insect pest management in the Caribbean. Pest management is an ecological affair, which results in management of the pest and its natural enemies in an agroecosystem. The size of a pest population and the damage it inflicts is, largely, a reflection of the design and management of a particular agroecosystem (Cruz and Segarra, 1992). One important management strategy is to develop and maintain the species diversity of the associated plant and animal community.

The success of a biological control agent, including entomopathogens is based on maintaining the balance of the ecosystem in which the organism which exerts control is introduced. Creating a balanced ecosystem means that the interactions between the various organisms that share the ecosystem / niche must be carefully studied. The entomopathogens used in biological control include bacteria, fungi, viruses, nematodes and protozoa. Entomopathogens are considered as an alternative to chemical insecticides primarily because they are more environmentally friendly. More than 750 species of entomopathogenic fungi with potential as biopesticides from the Deuteromycetes have been recognized including, the genera *Beauveria*, *Metarhizium*, *Verticillium*, *Nomuraea* and *Hirsutella* (Dent, 2000). Individual species of fungi such as *Metarhizium anisopliae* (Metsch) Sorokin (Deuteromycetes: Hyphomycete) have wide host ranges including species of Coleoptera, Lepidoptera, Orthoptera, Hemiptera and Diptera (Hall and Papierok, 1982; Toledo *et al*., 2008).

The objective of this study is to determine the pathogenicity of one of the most widely used entomopathogens, *M. anisopliae*, on the natural enemies of the pests of some of the more important crops of the Caribbean namely, *Cryptolaemus montrouzieri* Mulsant (Coleoptera:Coccinellidae) a predator of the Pink Hibiscus Mealybug, *Maconelli coccus hirsutus* Green (Hemiptera: Pseudococcidae) that attacks Malvaceous and Leguminous plants; *Anagyrus kamali* Moursi (Hymenoptera: Encyrtidae) a parasitoid also of the Pink Hibiscus Mealybug; *Lysiphlebus testaceipes* Cresson (Hymenoptera: Aphidiidae), a parasitoid of *Toxoptera citricida* (Kirkaldy) (Homoptera: Aphididae) that attacks citrus and *Bracon thurberiphagae* Muesebeck (Hymenoptera: Braconidae), a parasitoid that attacks

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Ancylostomia stercorea Zeller (Lepidoptera: Pyralidae), a pod borer of pigeon pea, *Cajanus cajan* L Millsp.

MATERIAL AND METHODS

Metarhizium anisopliae

Pure cultures of *M. anisopliae* isolates ARSEF 932 and ARSEF 954 were obtained from USDA-ARS Collection of Entomopathogenic Fungal Cultures, Cornell, USA while IMI 152222 and IMI 299982 were obtained from CAB International Mycological Institute, United Kingdom. IMI 299982 was originally isolated from the sugarcane froghopper, *Aenolamia varia saccharina* (Distant) (Homoptera:Cercopidae) in Trinidad and is thus a local strain. Twenty 9cm Petri dishes of each isolate were cultured on Potato Dextrose Agar (PDA) and kept at room temperature (24^0C). Spores were harvested from the Petri dishes containing the pure isolates four weeks post inoculation. Spores were harvested by adding a mixture of distilled water and Tween 80® to each Petri dish and gently scraping the PDA surface with a sterilized spatula. This spore suspension was poured into a 100 ml beaker and placed on a magnetic stirrer (Fisher Thermix Stirring Hot Plate Model 310T) without heat for fifteen minutes. Each isolate suspension was kept at room temperature and used to formulate four spore concentrations (10^9,10^8, 10^7 and 10^6 spores/ml) based on the initial concentration which was determined using a Neubauer Haemacyto meter®.

INSECTS

Cryptolaemus montrouzieri

C. montrouzieri grubs were collected from Macoya and Aranguez, Trinidad at weekly intervals. This beetle was found predominately on wild okra (*Malachra alceifolia*) which grows on the roadsides and on abandoned or uncultivated agricultural plots and which supports a high population of *M. hirsutus* that is preyed upon by the grubs and adults of *C. montrouzieri*. Grubs were fed on *M. hirsutus* on *Malachra alceifolia* and kept in large plastic containers (40cm x 40cm x 40cm) covered with organza mesh cloth (150 mesh / cm^2) until pupation and adult eclosion.

Anagyrus kamali

Same age *A. kamali* adults were obtained from the Ministry of Agriculture, Lands and Marine Resources Multiplication Centre, Point Fortin, Trinidad. The wasps were transported to the Entomology Laboratory, University of the West Indies, St. Augustine, Trinidad in batches of 150 in 250ml plastic bottles, released into mesh cages (30cm x 30cm x 45cm) and fed on diluted honey. Adults were utilized in bioassays within two days of arrival.

Lysiphlebus testaceipes

Young citrus shoots with mummified Brown Citrus Aphids, *Toxoptera citricida* were collected from the citrus orchard at the University of the West Indies Field Station (UFS), Trinidad and taken to the Entomology Laboratory at the University of the West Indies, St. Augustine, Trinidad. The shoots were placed in 3 L glass jars and covered with organza mesh cloth (150 mesh / cm^2) held in place by a rubber band. Eclosing wasps of the same age were used in bioassays.

Bracon thurberiphagae

Cajanus cajan pods infested with the pigeonpea podborer, *Ancylostomia stercorea* were collected from the UFS and placed in a nylon mesh cage (1.25m x 0.6m x 1.2m) at the Entomology Laboratory, University of the West Indies, St. Augustine, Trinidad. Adult *B. thurberiphagae* emerged daily and were collected using an aspirator. Same age wasps were used in bioassays.

BIOASSAY AND DATA ANALYSIS

Leaf dip bioassay for each insect species was conducted in a similar manner. Leaves of the appropriate plant species (*Malachra alceifolia* for *C. montrouzieri* and *A. kamali, Citrus sinensis* for *L. testaceipes* and *Cajanus cajan* for *Bracon thurberiphagae*) were sterilized using 0.5% Clorox® solution, washed with distilled water then air-dried. Four different spore concentrations (1 x 10^9, 1 x 10^8, 1 x 10^7 and 1 x 10^6 spores/ml) from each of four isolates (ARSEF 932, ARSEF 954, IMI 152222 and IMI 299982) of *M. anisopliae* were used to treat the leaves. Water and Tween 80® solution was applied to the sterilized leaves for the control treatment. The leaves were then air-dried and placed in a Petri dish with mesh (150 mesh / cm^2) covered top. There were five replicates for each concentration of each isolate with 10 test insects per replicate. All insects were provided with honey as a food source during the bioassays. Mortality was recorded daily until death of the last adult in each bioassay. Adult cadavers were incubated individually in clean Petri dishes on moist cotton wool to encourage fungal sporulation and confirm mortality was due to *M. anisopliae.* Mortality data was analyzed using EPA Probit Analysis (Ver.1.3) to determine LC_{50} and LT_{50} values.

RESULTS

The results of the pathogenicity and virulence of four isolates of *M. anisopliae* to four natural enemies are presented in Tables 1 and 2. ARSEF 932 was the most pathogenic isolate tested (LC_{50} = 1.87 x 10^7 spores/ml) against *C. montrouzieri*, although this was not significantly different ($p>0.05$) from IMI 152222 (Table 1).

Table 1. Pathogenicity (LC_{50}) for different isolates of *Metarhizium anisopliae* on four natural enemies.

Natural enemy / *Metarhizium anisopliae* isolate	LC_{50} (spores/ml) (95% C.I.)*	χ^2	SE of LC_{50}	Probit Equation
Cryptolaemus montrouzieri				
ARSEF 932	$1.87x10^7$ ($9.92x10^6$ – $3.15x10^7$)a	1.955	1.38	Y=0.834x - 1.063
ARSEF 954I	$6.53x10^8$ ($2.64x10^8$ – $1.62x10^9$)b	3.188	1.59	Y=0.682x – 1.015
MI 152222I	$3.17x10^7$ ($1.09x10^7$ - $9.23x10^7$)a	2.275	1.72	Y=0.498x + 1.264
MI 299982	$9.81x10^{10}$ ($3.18x10^9$ – $3.03x10^{12}$)c	0.139	1.75	Y=0.167x + 3.163
Anagyrus kamali				
ARSEF 932	7.37 x 10^9 ($1.58x10^9$ - $3.44x10^{10}$)a	1.434	2.19	Y=0.413x + 0.927
ARSEF 954	4.10 x 10^9 ($1.05x10^9$ - $1.60x10^{10}$)a	0.339	2.00	Y=0.456x + 0.615
IMI 152222	4.17 x 10^8 ($1.57x10^8$ - $1.10x10^9$)bc	1.967	1.64	Y=0.606x – 0.226
IMI 299982	1.36 x 10^8 ($7.12x10^7$ – $2.60x10^8$)c	2.969	1.39	Y=1.029x – 3.366
Lysiphlebus testaceipes				
ARSEF 932	$2.82x10^5$ ($9.38x10^4$ – $8.49x10^5$)a	2.197	1.75	Y=0.660x + 1.401
ARSEF 954	$1.56x10^5$ ($4.83x10^4$ – $5.03x10^6$)ab	3.236	0.17	Y=0.156x + 5.813
IMI 152222	$1.36x10^6$ ($6.53x10^5$ – $2.85x10^6$)a	1.261	1.46	Y=0.952x – 0.843
IMI 299982	$1.48x10^6$ ($9.01x10^5$ – $2.44x10^6$)b	0.026	1.29	Y=1.595x – 4.842
Bracon thurberiphagae				
ARSEF 932	$7.59x10^6$ ($2.16x10^6$ – $2.66x10^7$)a	1.036	1.90	Y=0.373x + 2.433
ARSEF 954	$7.00x10^5$ ($1.90x10^5$ - $2.58x10^6$)a1.	0.946	194	Y=0.374x + 2.814
IMI 152222	$72x10^7$ ($7.19x10^6$ – $4.12x10^7$)b	3.379	1.56	Y=0.487x + 1.475
IMI 299982	$7.00x10^7$ ($2.65x10^7$ – $1.85x10^8$)ab	0.181	1.64	Y=0.555x + 0.648

* Values followed by the same letter for a species are not significantly different (p = 0.05)

This isolate also gave the lowest LT_{50} of 4.07 days against *C. montrouzieri*. Adults of this predator survived the longest when exposed to ARSEF 954 (LT_{50} = 12.43 days) (Table 2).

A. kamali adults were most susceptible to IMI 299982 (LC_{50} =1.36 x 10^8 spores/ml) but this was not significantly different (p>0.05) isolate IMI 152222 (Table 1). Despite being the most pathogenic among isolates tested against *A. kamali* adults, IMI 299982 was the least virulent with a LT_{50} of 13.01 days (Table 2).

The most pathogenic isolate tested against both *L. testaceipes* and *B. thurberiphagae* was ARSEF 954 with LC_{50} values of 1.56 x 10^5 and 7.00 x 10^5 spores/ml respectively (Table 1) with corresponding LT_{50} values of 2.29 and 5.32 days, respectively (Table 2).

DISCUSSION

Goettel and Johnson (1992) note that entomopathogenic fungi constitute an autochthonous element of the natural microflora and that side effects of mycopesticides on non-target arthropods are considered small compared to synthetic chemicals. There is no doubt however, that using microorganisms for management of pest arthropods poses potential risks to non-target organisms, but these risks can be avoided or minimized through careful choice of agents. Compared with chemical insecticides,

Table 2. Virulence (LT_{50}) for different isolates of *Metarhizium anisopliae* on four natural enemies.

Natural enemy / *Metarhizium anisopliae* Isolate	LT_{50} (days) (95% C.I.)*	χ^2	SE of LT_{50}
Cryptolaemus montrouzieri			
ARSEF 932	4.07 (2.98 – 5.55)a	4.499	1.17
ARSEF 954I	12.43 (9.31 – 16.59)b	1.110	1.16
MI 152222I	8.45 (6.32 – 11.30) b	5.917	1.16
MI 299982	5.95 (4.67 – 7.57)a	7.438	1.13
Anagyrus kamali			
ARSEF 932	5.54 (4.82 – 6.37)a	1.068	1.07
ARSEF 954	5.11 (3.74 – 6.99)a	6.337	1.17
IMI 152222	3.66 (2.99 – 4.86)a	0.294	1.11
IMI 299982	13.01 (11.73 – 14.43)b	6.016	1.05
Lysiphlebus testaceipes			
ARSEF 932	1.25 (1.04 – 1.49)a	0.736	1.10
ARSEF 954	2.29 (2.01 – 2.62)b	2.361	1.07
IMI 152222	1.86 (1.59 – 2.18)b	2.951	1.08
IMI 299982	1.68 (1.44 – 1.96)a	1.662	1.09
Bracon thurberiphagae			
ARSEF 932	8.01 (7.11 – 9.03)a	2.866	1.06
ARSEF 954	5.32 (4.44 – 6.38)b	4.977	1.10
IMI 152222	3.32 (2.27 – 4.88)b	1.288	1.22
IMI 299982	1.15 (0.68 – 1.93)c	0.446	1.30

* Values followed by the same letter for a species are not significantly different (p = 0.05)

entomopathogenic organisms offer a better and much safer alternative for use in Integrated Pest Management systems (Goettel and Hajek, 2001). With this in mind, a series of bioassays were conducted using four isolates of *M. anisopliae* against four commonly used natural enemies – *C. montrouzieri, A. kamali, L. testaceipes* and *B. thurberiphagae.* Two parameters were used to assess the suitability of isolates for incorporation into IPM programmes – pathogenicity and virulence. The LC_{50} of an entomopathogenic fungal isolate on population provides an indication of the pathogenicity of the isolate on the population. A higher LC_{50} value is indicative of a less lethal isolate and *vice versa*. The LT_{50} of an entomopathogenic fungal isolate on population provides an indication of the virulence of the isolate on the population and is of considerable importance, as the longer the isolate takes to achieve 50% mortality of the insect population the greater the likelihood that adults will have to reproduce.

With this attribute in mind, ARSEF 954 was determined to be the safest isolate against C. *montrouzieri* since it had the highest LT_{50} of 12.43 ± 1.16 days. Apart from causing death of the insects, fungal spores may have adverse effects on other parameters of the insects' activities that were not tested in this experiment including: number of offspring produced, its ability to hunt for prey and reproductive capacity. Some adult *C. montrouzieri* were observed surviving for more than 30 days post infection at all concentrations of every isolates (personal observations). The highest LT_{50} was achieved by ARSEF 932 in 4.07 ± 1.17 days.

IMI 299982 was the most pathogenic isolate against *A. kamali* with a LC_{50} = 1.36 x 10^8 spores/ml; however the LT_{50} of IMI 299982 was 13.01 ± 1.05 days. At this length of time, isolate IMI 299982 took the longest time of all the isolates to achieve 50% mortality. From this perspective, IMI 299982 is the safest isolate against *A. kamali*. Despite the fact that it recorded the highest percentage mortality and the lowest LC_{50} it took a relatively long time to do so. This gives the insects the opportunity to continue reproduction over a longer period of time and hence the possibility of replenishing the population. Indeed, fungal spores survive longer on the protected and more humid abxial leaf surface than on the adxial surface and consequently puts insects dwelling on the undersides of leaves at greater risk of infection than those on the upper leaf surface (Hajek and Butler, 2000).

The results indicate that the isolates of *M. anisopliae* tested against *L. testaceipes* are very pathogenic to this species. This is supported by the low LT_{50} and LC_{50} values at the lowest spore concentration of the isolates tested. The mean generation time of this species is 6.77 days and has a net reproductive rate (R_0) of 444 offspring during this time but with younger females contributing more to the population than older females (Aziz and Khan, 2008). This is particularly important as the low LT_{50} values indicate that death is rapid and may cause high mortality in a short living species. However, *L. testaceipes* are very active parasitoids and can be observed mating and searching for hosts within only a few hours after eclosion from their mummified aphids. This potential for rapid reproduction and the ability to parasitize a large number of hosts early in its life makes ARSEF 954 the safest isolate tested for use in IPM programmes with *L. testaceipes*.

Isolate ARSEF 954 gave the lowest LC_{50} = 7.00 x 10^5 spores/ml with a corresponding LT_{50} valve of 5.32 ± 1.10 days for *B. thurberiphagae*. The results of the isolates of *M. anisopliae* tested against *B. thurberiphagae* revealed that ARSEF 932, IMI 152222 gave LC_{50} values of 7.59 x 10^6 spores/ml and 1.72 x 10^7 spores/ml respectively while the highest LC_{50} value (7.00 x 10^7 spores/ml) was recorded by isolate IMI 299982. From these LC_{50} values, it can be concluded that all four isolates of *M. anisopliae* are relatively pathogenic to *B. thurberiphagae*. However, of the four isolates ARSEF proved to be the safest. Despite the fact that ARSEF 932 gave a low LC_{50} value it had the lowest LT_{50} of 8.10 ± 1.06 days. This indicates that the safest isolate with respect to length of insects' survival (post inoculation) is ARSEF 932. The length of survival of an insect after being infected by the fungi will have a direct implication on the size of the next generation.

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