Pathogenicity and haemolymph protein changes in *Edessa meditabunda* F. (Hemiptera:Pentatomidae) infected by *Paecilomyces lilacinus*

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ABSTRACT

Adult *Edessa meditabunda* were found to be naturally infected by the entomopathogenic fungus *Paecilomyces lilacinus* (ARSEF 10225) for the first time in Trinidad in the field and consequently laboratory experiments were conducted to determine its efficacy. Mean *in vitro* radial colony growth for *P. lilacinus* was the fastest at 20^0C and slowest at 35^0C. The LC_{50} for adult *E. meditabunda* using *P. lilacinus* was determined as 6.32×10^6 spores/ml. At this concentration, 50% mortality (ie.LT_{50}) of *E. meditabunda* was achieved in 1.95 days. Haemolymph of adult *E. meditabunda* was extracted and used in the identification of proteins by polyacrylamide gel electrophoresis (SDS-PAGE) and the total protein content by the Lowry's method. There were a total of four protein bands present in the haemolymph of control adults, all of which had molecular weights more than 138.7 kDa. There was gradual increase in the number of bands from day 1-3 post infection by *P. lilacinus.* Two new protein bands (104.7 kDa and 49.5 kDa) appeared on Day 3 post infection. The total haemolymph protein concentration was significantly higher in the control compared to all three days post infection.

Key words: *Edessa meditabunda, Paecilomyces lilacinus*, LD_{50}, haemolymph, radial growth, gel electrophoresis

INTRODUCTION

The stink bug *Edessa meditabunda* (F) (Hemiptera: Pentatomidae) is a serious pest of agricultural and non-agricultural crops throughout the world. Most control measures include the use of repeated heavy applications of insecticides. This can lead to the development of insecticide resistance as well increase costs to the farmer (Ihara *et al.*, 2001; Yeo *et al.*, 2003; Hanh Vu *et al.*, 2007). However, there is growing concern over the use of harsh pesticides to produce food for human consumption as well as problems associated with the build up of insect resistance to these pesticides (Yeo *et al.*, 2003; Hanh Vu *et al.*, 2007; Ferron, 1978). Furthermore, there are significant costs attached to the use of agricultural pesticides including monetary cost of the product, build up of toxic residues on food and feed products, possibility of ground water contamination and by extension the destruction of the ecosystem and the development of chemical resistance in insects (Ihara *et al.*, 2001; Yeo *et al.*, 2003; Hanh Vu *et al.*, 2007).

Biological control involves the use of living organisms to kill other organisms by targeting the pest (Evans, 1982; Hanh Vu *et al.*, 2007). Entomopathogenic fungi are effective biological control agents as they help to regulate insect populations (Lacey *et al.*, 2001; Ginsberg *et al.*, 2002). Microbial control

agents are usually characterized by the following: host specificity, safe to natural enemies of the target pest and a high level of virulence with no effect on the environment (Hanh Vu *et al.*, 2007; El- Husseini *et al.*, 2006; Jun Kim *et al.*, 2005). As the demand for more environmentally sound pest control measures increase, biological control of stink bugs can be considered as a viable option (Hiromori and Nishigaki, 2001; Yeo *et al.*, 2003; Jun Kim *et al.*, 2005;).

Over 700 species of fungi have been reported as pathogens but only as few as ten have been developed or are currently being developed and used for insect control (Milner *et al.,*1991; Van Nghiep *et al.*, 1999; Lacey *et al.*, 2001; Ihara *et al.*, 2001; El- Husseini *et al.,* 200). Although entomopathogenic fungi may act slower than chemical insecticides, their effects last longer (Ihara *et al.*, 2001). Since stink bugs are plant sucking insects, entomopathogenic fungi would be a more appropriate agent of control, since, unlike other microbial agents like virus and bacteria that must be ingested to cause infection, entomopathogenic fungi cause infection of the host by directly penetrating the exoskeleton of the insect (Ferron 1978; Storey and Gardner, 1987;Grimm and Guharay, 1998; Milner *et al.,* 1991; Lacey *et al.*, 2001; Ihara *et al.*, 2001).

In the present study, an entomopathogenic fungus (*Paecilomyces lilacinus* (ARSEF 10225) was isolated from

the cadavers of *E. meditabunda* in Trinidad for the first time. The objectives of the present study were to determine the effect of temperature on radial growth of *P. lilacinus* isolated from *E. meditabunda*; the LC_{50} and LT_{50} values for this fungus on adult *E. meditabunda* and the effect of pre- and post infection of *E. meditabunda* by *P. lilacinus* on haemolymph proteins.

MATERIALS AND METHODS

Preparation of Potato Dextrose Agar (PDA) plates

Potato Dextrose Agar (PDA) powder (19.5 g) (Oxoid® Ltd., UK) was suspended in 500ml of distilled water in a 1L media bottle. A stirring magnet was placed in the media bottle and the combination placed on a stirring/heating plate for approximately 30 minutes. The bottle was labeled and autoclaved for approximately one hour at 121^0C. The bottle was removed and cooled in a water bath. The mixture was then carefully poured into labeled sterile 9cm Petri-dishes in an Envair HLF/4/8® laminar flow cabinet and allowed to set (approx. 30 minutes). When cooled, the Petri dishes were covered, inverted and sealed with plastic tape to prevent contamination.

Inoculation

Using an Envair HLF/4/8® laminar flow cabinet, a sample of the fungus was removed from a cadaver of *E. meditabunda* and streaked unto the prepared PDA plates using a sterile inoculating loop to obtain isolated colonies. The plates were covered, labeled and incubated at room temperature ($30\ ^0$C) in an inverted position to prevent condensation on the inoculated surface. The cultures were examined weekly for fungal growth.

Radial growth

Four cardinal radii (North, South, East, and West) were drawn on the outer surface of the Petri dish base using a permanent marker. A small hole (4mm) was made in the centre of the cover of a Petri dish using a hot metal cork borer. The hole was made just large enough to accommodate the loop of an inoculating wand. Fungal spores were taken from the prepared culture plates and inoculated on freshly prepared plates. Agar cores of actively growing cultures were cut from the stock plates and placed in the centre of each plate from which a similarly sized core was removed. The inoculated plates were incubated at 20, 25, 30 and 35 ^{0}C with six replicates for each temperature. The radial growth of the fungus was determined by measuring the growth from the centre along a radius on a daily basis. Hence a mean value could be calculated. This was done for approximately 21 days or until the spores

traversed to the curved end of the plate and was repeated at approximately the same time daily.

Bioassay

Spore count

Spores were harvested from several cultured samples in Petri dishes by gently scraping the surface spores with a sterile spatula into a beaker. Sterile distilled water was added, drop by drop while holding the plate at an angle and the resultant suspension collected into the beaker. A few drops of Tween® 80 were added to the suspension in the beaker while stirring on a stir plate to ensure spores went into suspension. A 1:10 dilution of the sample was made and spores counted using a Neubauer haemacytometer. Based on the original suspension spore concentration of 1.838×10^7 spores/ml, four serial dilutions were made in sterile distilled water. The control was Tween® 80 and sterile distilled water.

Insect treatment and statistical analysis

Ten adult *E. meditabunda* were placed in a small organza bag (5cm x 5cm) and dipped for 10s in one of the constantly stirring dilutions. Insects were dipped in mixture of sterile distilled water and Tween® 80 for the control. Five replicates were used for each concentration. Adults were air dried, placed in labeled plastic containers with a food source post treatment and stored under ambient conditions. Observations on mortality were recorded every 24 hours until complete mortality was observed. Insect mortality data were corrected for control mortality by Abbott's (1925) formula. SPSS 10.0 was used to carry out ANOVA and the LD_{50} and LT_{50} were calculated using the EPA probit analysis program (Version 1.4)

Gel electrophoresis of haemolymph

A 7.5% separating gel was prepared using 4.95 ml de-ionized water, 2.50ml 1.5M Tris-HCl (pH 8.8), 2.5 ml acrylamide-bis (30%), 50µl ammonium persulphate (10%) and 5µl TEMED. The 4% stacking gel was prepared using 3.1ml de-ionized water, 1.25ml 0.5M Tris-HCl (pH 6.8), 0.67ml acrylamide-bis (30%), 25µl ammonium persulphate (10%) and 5µl TEMED.

Protein separation was done in a mini gel apparatus which allowed the use of discontinuous buffers to sharpen the bands. The gel solution was poured into the thin space between the two glass plates separated by spacers which determined gel thickness. A comb was inserted into the top of the gel mould after filling to form the sample wells. Gels were clamped into the apparatus with the top and bottom of the gel in contact with the upper and lower buffer chambers containing cathode and anode electrodes. The comb was

removed, samples loaded and 180V applied for protein separation. Gels were left in the refrigerator overnight. A standard curve was plotted using the distances migrated by each marker protein and the molecular weight extrapolated from the standard curve. The following markers were all prepared at 1mg/200µl De-ionized water: á- Lactalbumin (14.2 kDa), Carbonic anhydrase (29.0 kDa), Albumin from chicken egg (45.0 kDa) and Urease (272-545 kDa). A sample buffer was prepared using 25µl of Sucrose and 25µl of 0.05 % Bromophenol Blue. For each haemolymph sample, 12µl of fresh sample was mixed with 12µl of sample buffer. 10 µl of each marker was mixed with 5µl sample buffer. The gel was run for 1 hour at 180V and 20mA. Gels were stained for 1 hour and then de-stained for 3 hours then washed for 20 minutes in a solution of 50% methanol, 10% acetic acid and 40% water, then rinsed in water and then washed in the amplify solution for 20 minutes after which it was rinsed again in water. The stacking gel was removed and finally vacuum dried. The gel was fixed and stained with Coomasie blue stain. Total haemolymph protein was estimated using the Lowry's method (Lowry *et al.*, 1951).

Haemolymph extraction

Live stink bugs punctured under the pronotum (using a stainless steel pin (size 00)) without destroying the alimentary canal to ensure that the extract did not contain any material from the digestive tract. The haemolymph was collected by inserting a 10ì l capillary tube into the wound and the contents of the capillary tube placed in an Eppendorf tube.

RESULTS

Effect of temperature on radial growth rate of *Paecilomyces lilacinus*

The mean radial colony growth with time was not significantly different from each other at 20^0C and 25^0C (P>0.05). However significantly (P<0.001) higher mean colony radial growth was observed at 20^0C compared to both 30^0C and 35^0C (Table 1). Mean radial colony growth was also significantly higher (P<0.001) at 25^0C compared to that at either 30^0C or 35^0C. There was no significant growth difference (P>0.05) between 30^0C and 35^0C (Table 1).

The *in vitro* radial colony growth rate (K_r) for *P. lilacinus* was highest at 20^0C (62.40 ìm/h) and lowest at 35^0C (3.97 ìm/h) (Figure 1). Radial colony growth rate at the different temperatures decreased linearly as temperature increased (Figure 2).

Insect mortality

The LC_{50} for adult *E. meditabunda* using *P. lilacinus* was determined as 6.32 x 10^6 spores/ml. At this concentration, 50% mortality (ie.LT_{50}) of *E. meditabunda* was achieved in 1.95 days.

Proteins

There were a total of four (4) protein bands present in the haemolymph of adults (control) all of which had molecular weights of >138.7 kDa (Plate 1). On Day 1 post infection protein bands appeared in lower concentration (as indicated by dye intensity). On Day 2 post infection the concentrations

Table 1. Mean (±SE) *P. lilacinus* colony radius over time (450hours) at four temperatures.

Temperature (^{0}C)	Mean colony radius (µm) ± SE*	95% Confidence Interval
20	21498.13 ± 1910.40 [a]	17427, 25569
25	20369.38 ± 1912.40 [a]	16294, 24445
30	3274.63 ± 155.67 [b]	2942.9, 3606.4
35	2208.13 ± 181.73 [b]	1820.9, 2595.4

* Values followed by the same letter are not significantly different from each other at P>0.05 based on Tukey-Kramer Multiple Comparisons Test.

Figure 1. Radial colony growth of *Paecilomyces lilacinus* on Potato Dextrose Agar at four temperatures
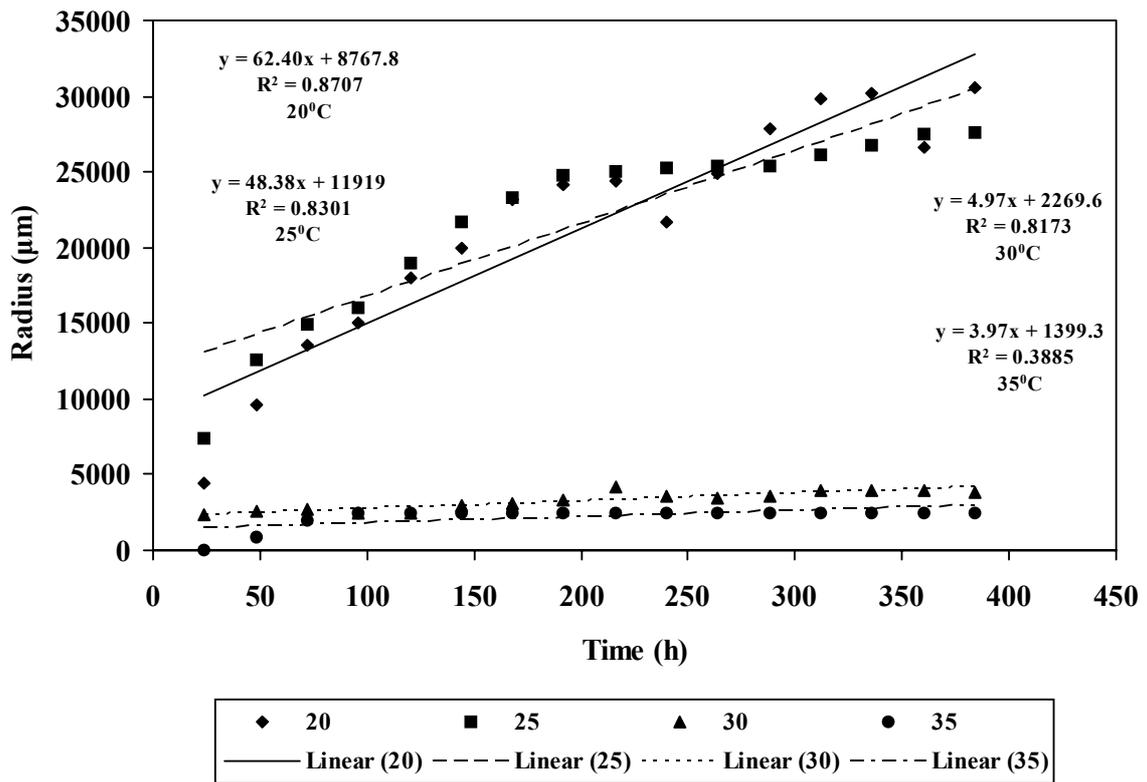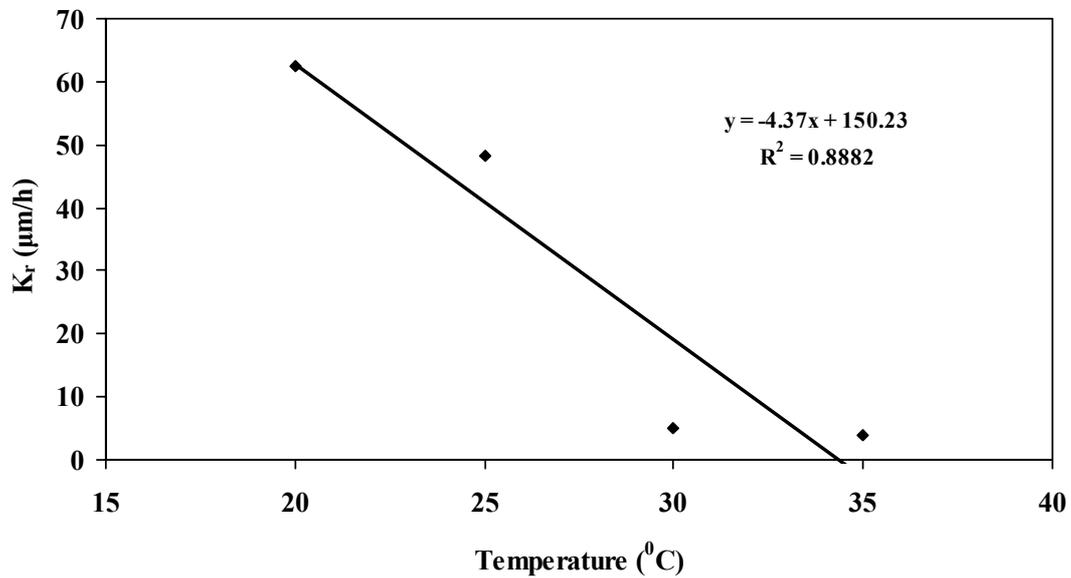

Figure 2. Comparison of the K_r values for *Paecilomyces lilacinus* at four temperatures

of proteins of molecular weights 327.5 kDa and 246 kDa increased slightly compared to the control and Day 1 post infection, while the concentration of protein bands of molecular weights 174.6 and 138.7kDa decreased compared to the control and Day 1 post infection (Plate 1). On Day 3 post infection this trend was reversed, with the concentrations of proteins of molecular weights 327.5 and 246 kDa decreasing and concentration of protein bands of molecular weights 174.6 and 138.7kDa increasing compared to the control and the previous days. Additionally there was the appearance of two new bands of molecular weights 104.7 and 49.5kDa which were not observed in either the control, Days 1 or 2 (Plate 1).

There was a significant difference (p<0.001) between the haemolymph protein levels in all *P. lilacinus* post treated adults compared to the control (14.47±0.25). There was also a significantly higher (p<0.001) total protein concentration between the haemolymph of day 1 (8.27 ± 0.06 ìg/ìl) day 2 (5.19 ± 0.10 ìg/ìl) and day 3 (7.93 ± 0.08 ìg/ìl) treated adults.

DISCUSSION

Colony growth rates of *P. lilacinus* was the fastest at 20⁰C compared to 25, 30 or 35⁰C as determined using a single line model (Figure 1) and growth rates decreased significantly with increasing temperature (Figure 2). Although the radial diameters increased linearly, a decrease in radial growth rate at 30⁰C and 35⁰C was noted. These results are in accordance with Milner *et al* (1991) who noted that entomopathogenic fungi become less active above 20⁰C and this may have implications for the use of *P. lilacinus* under a restricted range of environmental temperatures. However, Yeo *et al* (2003) suggested that not only is the germination behaviour of spores dependent on temperature but also on the presence of water and cuticular components and hence it is difficult to use the results from the *in vitro* studies to predict *in vivo* host–pathogen interactions accurately. Cabanillas *et al* (1989) demonstrated that soil temperature greatly influenced the efficacy of *P. lilacinus* as a biocontrol agent of *Meloidogyne incognita*. In that same study, soil temperatures of 16, 20, 24, and 28⁰C resulted in progressively increased root-knot nematode damage, but this was paralleled by an increase in egg mass infection by *P. lilacinus*. A linear relationship existed between radius colony growth and temperature with the optimal temperature for mycelial growth of *P. lilacinus* being 20°C (Table 1). The radial colony growth rate, K_r and

Plate 1 Gel electrophoresis of haemolymph of *Edessa meditabunda* pre (control) and post infection with *Paecilomyces lilacinus.*
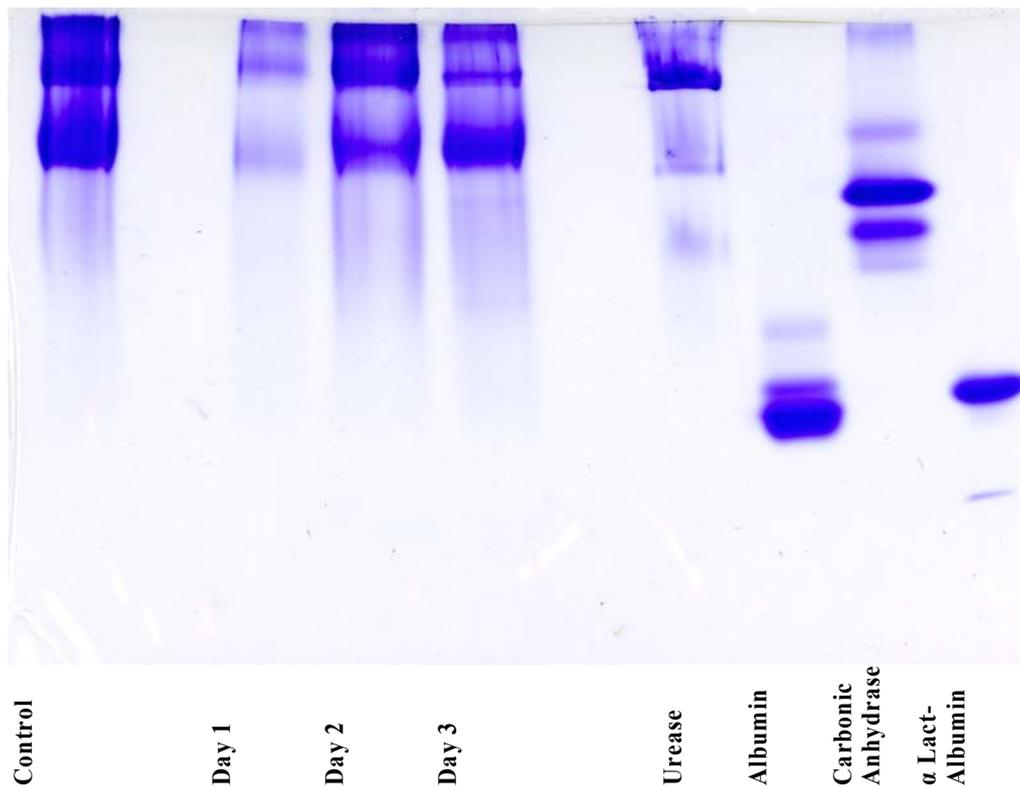
temperature for each replicate was estimated from the slope of the linear regression of the colony radius on time as demonstrated by Yeo *et al* (2003). The linear relationships determined for *P. lilacinus* against *E. meditabunda* at the four temperatures tested are also in agreement with the study conducted by Yeo *et al* (2003) using different entomopathogenic fungi against aphids.

The pathogenicity of *P. lilacinus* was dependent on sufficient fungal spores (dose of conidia) attaching on the cuticle of *E. meditabunda*. This was indicated by the reduced mortality of *E. meditabunda* as the spore concentration decreased. A similar phenomenon has been described for pathogenicity of *M. anisopliae* to immature *I. scapularis* (Gingsberg *et al.*, 2002). The present study suggest that the isolate of *P. lilacinus* can cause high mortality of *E. meditabunda* by topical contact. The LC_{50} for adult *E. meditabunda* was found to be 6.32×10^6 spores/ml and the LT_{50} was achieved in 1.95 days after exposure. These results are in agreement with studies by Jun Kim *et al* (2005) where lower spore concentrations gave relatively low mortality and higher spore concentrations gave nearly 100% mortality. We conclude that *P. lilacinus* has good potential as a microbial agent for *E. meditabunda* on solanaceous crops since the LT_{50} was low. This is in keeping with Ihara *et al* (2001) who suggested that to adopt entomopathogenic fungi for control of stink bugs, specifically *E. meditabunda* in solanaceous crops, fungi which have high pathogenicity against the stinkbugs and the ability to kill them quickly should be used because stink bugs continuously invade crops from other fields.

It is interesting to note that although fungal hyphae did not emerge from the cadavers placed in moist Petri dishes, they were isolated from the internal organs of *E. meditabunda*. While this is unexplained, Zurek and Keddie (2000) in a similar study suggested that that this may be attributed to the possibility that the potential differences in haemolymph composition may affect fungal re–emergence. Additionally, while extracting haemolymph for protein changes post infection it was noted that as time progressed, the quantity of haemolymph present in infected adults decreased. This meant a decrease in moisture quantity as well as possible changes in haemolymph composition may adversely affect the growth of *P. lilacinus* in *E. meditabunda.*

SDS-PAGE indicated the presence of at least 6 protein bands in the haemolymph of *E. meditabunda* pre and post infection. The molecular weights ranged from 49.5 kDa to 327.5 kDa. Additionally there was the appearance of two new bands of molecular weights 104.7 and 49.5kDa on day 3 post infection which were not observed in either the control, days 1 or 2. This indicated changes in haemolymph protein composition

post infection, similar to that observed by Zurek and Keddie (2000).

This study indicates that there is potential for epizootics of *P. lilacinus* to occur in populations of *E. meditabunda* under certain conditions. Although much of the literature pointed to the use of *P. lilacinus* as an existing and effective control for nematodes, the evidence suggests that further research needs to be conducted as an alternative for chemical control of *E. meditabunda*. Further research is required as suggested by Lacey *et al* (2001) on Integrated Pest Management for stink bug control where entomopathogens can provide significant and selective insect control. Research is being conducted on formulations which will improve the effectiveness and viability of *P. lilacinus* in controlling *E. meditabunda,* the compatibility of this entomopathogenic fungus with other control options such as chemical insecticides, host plant resistance and the use of natural enemies.

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