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RESEARCH ARTICLE

Effect of temperature on local and Philippine isolates of *Metarhizium anisopliae* and their virulence on larvae of *Oryctes rhinoceros, a pest of coconut in Sri Lanka*

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ABSTRACT

The effect of temperature on mycelial growth and spore production in Sri Lankan (local isolate) and Philippine isolates (DRC) of *Metarhizium anisopliae* (Metsch.) Sorokin and their virulence to larvae of the black beetle, *Oryctes rhinoceros* L. were compared in the laboratory. Mycelial growth and spore production in each isolate were evaluated at 25^{0}C, 27.5^{0}C, 30^{0}C and 32.5^{0}C. Healthy third instar larvae of black beetle were inoculated with spore suspensions of 10^{6}spores/ml of each isolate to compare the virulence. There was no considerable difference between the two isolates with respect to mycelial growth at the end of the experimental period of 40 days. However, the mycelial growth of the local isolate significantly differed among the four temperatures, with the highest growth at 27.5^{0}C and lowest at 32.5^{0}C. The growth of DRC isolate was similar at all temperatures. The two isolates differed significantly in spore production. Spore production was extremely low at 32.5^{0}C in both isolates. The local isolate produced a significantly higher number of spores at other three temperatures than that of the DRC isolate. In the local isolate spore production was higher at 25^{0}C and 27.5^{0}C and in the DRC isolate it was highest at 25^{0}C. Both isolates caused death in black beetle larvae, the local isolate recorded a significantly higher mortality (67%) than the DRC isolate (42%), 17 days after inoculation. The LT_{50} of the local and DRC isolates were 14 and 18 days respectively. The study reveals that the local isolate has more desirable characteristics than the DRC isolate for use in the management of black beetle larvae in Sri Lanka.

Key words: coconut, *Metarhizium anisopliae* isolates, mycelial growth, *Oryctes rhinoceros*, spore production, temperature effect, virulence

INTRODUCTION

Black beetle, *Oryctes rhinoceros* L. is the most widespread pest of coconut (*Cocos nucifera* L.) which is found across 37 countries in Africa, Asia, Australia and Pacific Islands (Singh and Rethinam, 2005). The attack by the adult beetle causes defoliation leading to yield losses in young and adult palms and severe setback in growth or even death in seedlings. Due to difficulties in effectively managing black beetle by a single method, an integrated management package has been recommended by the Coconut Research Institute of Sri Lanka. It includes destruction of breeding grounds, extraction of beetles using a metal hook, application of used engine oil or coal tar and use of naphthalene balls or carbofuran granules. Due to the necessity of repeated application of these methods for control they are not often practiced by the coconut growers. Although, biological control agents, the *Oryctes* virus and the fungus *Metarhizium anisopliae* (Metsch.) Sorokin are considered prospective agents and are being used in some countries (Singh *et al.*, 2007), they are not widely used in Sri Lanka.

Amongst the three species of *Metarhizium* infecting insects *Metarhizium flavoviridae*, *M. aibum and M. anisopliae* (Sundra babu *et al.*, 1986), *M. anisopliae* commonly known as the Green Muscardine Fungus (GMF) is the most studied species. It is a facultative parasitic fungus, which can grow either as a parasite of insects or as a saprophyte and is the most common soil borne entomofungal pathogen (Brady, 1979). This species has been identified from about 300 species of Lepidoptera, Coleoptera, Orthoptera, Hymenoptera and Hemiptera indicating a wide host range, but most preferred hosts are found in the order Coleoptera (Latch, 1967; Veen, 1968). The different geographical isolates of *M. anisopliae* such as those from Sri Lanka (local), Philippines, India, Malaysia, Indonesia, England bring about different rates of mortality in black beetle larvae. Experiments in the Philippines have revealed very high effectiveness of the DRC isolate (Davao Research Center, Philippines) of the fungus (Singh and Arancon, 2007). This isolate has been provided to other coconut growing countries for testing under respective field conditions.

Entomopathogenic fungi are influenced by a number of biotic and abiotic factors that affect their survival and ability to cause diseases ((Hall and Papierok, 1982; Carruthers and Haynes, 1987; Inglis *et al.*, 2001). An understanding of the environmental variables affecting fungal growth, development and virulence would help to better predict the efficacy of different isolates under field conditions (Kope *et al.*, 2008). Of the different environmental factors temperature, relative humidity and solar radiation are probably the most important (Dimbi *et al.*, 2004). Since the Coconut Research Institute is envisaging the use of a virulent isolate of *M. anisopliae* to be incorporated into the integrated pest management package to enhance its effectiveness, this study was conducted to determine the effect of temperature on the growth and sporulation of the two isolates of *M. anisopliae* and to evaluate the virulence of two isolates on larvae of *O. rhinoceros* in the laboratory.

MATERIALS AND METHODS

Cultures of local and DRC isolates

The local culture of *M. anisopliae* was isolated from an infected larva of *O. rhinoceros* collected from a breeding site in a coconut field in Sri Lanka and the DRC culture was obtained from the Davao Research Center in the Philippines. The two isolates were grown on Potato Dextrose Agar at 27±5^{0}C and stored in a refrigerator. For the experiments the stored cultures were sub cultured in Potato Dextrose Agar and 2-week old cultures incubated at 27±5^{0}C were used.

Effect of temperature on growth of local and DRC isolates of *M. anisopliae* in the laboratory

Spore suspensions of 10^6 spores/ ml were prepared using both isolates and 60µl of each suspension was poured on to a petri-plate with PDA. To obtain a uniform culture, the suspension was spread evenly, on the culture medium using a spatula. Three petri-plates from each isolate were prepared and kept in an incubator at 25±0.5^{0}C.

After seven days, when olive green coloured fungal mat containing spores was produced on the culture medium, plugs of 0.5 cm diameter were cut using a sterile cork borer. Each plug with the fungal mat was placed in a hole of the same size made in the centre of another PDA petri-plate. A total of 32 culture plates were prepared in this manner for each isolate and sealed using parafilm. On the reverse side of the plate, it was divided into four equal quarters by marking two perpendicular lines across the centre of the plug for easy measurement of the radial growth of the fungus colony. Eight petri plates of each isolate were separately incubated at four different temperatures; 25±0.5^{0}C, 27.5 ±0.5^{0}C, 30±0.5^{0}C and 32.5±0.5^{0}C. The plates were kept upside down in the incubator to avoid water vapour accumulating on the culture medium. Radial growth of the mycelium from the centre towards the edge of the colony was measured at four-day intervals using a ruler. Four measurements along the lines were taken at each observation. The measurements were continued until the colony reached the edge of the plate.

The average lengths of mycelium in the eight replicates of each treatment measured at each time interval were analyzed using repeated measures analysis of variance in statistical software SAS.

Effect of temperature on spore production of local and DRC isolates of *M. anisopliae*

Spore suspensions of 10^6 spores/ ml were prepared using local and DRC isolates of *M. anisopliae.* On to each of 15 PDA plates, 60 µl of suspension of each isolate was poured and evenly spread on the medium. Each of 5 plates were incubated at three different temperatures; 25±0.5^{0}C, 27.5±0.5^{0}C and 30±0.5^{0}C. In the experiment, 32.5^{0}C temperature was excluded because in a preliminary study it was revealed that at this temperature sporulation occurred at extremely low levels in both isolates.

The first spore count was done at 4 days after inoculation. A plug of 0.5 cm diameter from 2 cm away from the center of the PDA plate was cut using a sterile cork borer. The plug was shaken in 10 ml of distilled water and the number of spores was counted using a haemocytometer. Spore counts were continued at four-day intervals for one month

using the same procedure. The spore counts of all plates in each treatment at each observation were averaged and were subjected to analysis of variance using GLM procedure of SAS.

Comparison of virulence of local and DRC isolates of *M. anisopliae* against larvae of *O. rhinoceros*

Spores from 2-week old pure fungal cultures of the two isolates grown on Potato Dextrose Agar at 27±5^{0}C were scraped using a spatula and suspended in 1l of distilled water separately. For inoculation of black beetle larvae, spore suspensions were shaken well to produce a homogeneous suspension and made to 10^6 spores/ml by adding sterilized distilled water.

Healthy third instar black beetle larvae used in the study were collected from the field and were individually kept in separate plastic containers and were provided with sterilized, moist cow dung medium and maintained for two weeks to ascertain that larvae were disease free. A total of 88 healthy larvae were inoculated with each isolate by allowing each of them to crawl on 500 ml of the spore suspensions for 20-30 minutes. The same numbers of healthy larvae were exposed to 500 ml of sterilized water for 20-30 minutes as the control. The inoculated larvae were transferred into clean clay pots (1250 cc) and half filled with sterilized, moisten cow dung. Four inoculated larvae were introduced into each pot and covered with another inverted clean clay pot. . Pots were kept at room temperature, 28±3^{0}C and 70±10% RH and moistened every two days with sterilized water to maintain an optimum moisture level. The larvae were examined on 7, 10, 13 and 17 days after inoculation and dead larvae were removed. The larva was considered infected by *M. anisopliae* if the body appeared creamy white and hardened. These larvae were kept separately under same conditions until olive green spores developed on them to ascertain infection. The number of larvae dead due to fungal infection and other causes were recorded separately. At each observation, after removing the dead larvae remaining live larvae in each pot were placed in a fresh medium of sterilized cow dung.

Cumulative numbers of dead larvae due to *M. anisopliae* infection were subjected to analysis of variance using the GLM procedure of SAS (SAS, Release 8.2, SAS Institute Inc., U.S.A.).

RESULTS

Effect of temperature on mycelial growth of local and DRC isolates of *M.anisopliae*

Growth of both isolates of *M. anisopliae* was observed at all four temperatures tested, but at varying rates. The growth of mycelium of local and DRC isolates at the end of the experimental period of 40 days were 2.2cm – 4.2cm and 3.5cm – 3.9cm respectively (Figures 1 and 2). Mycelial growth of the local isolate was significantly differed at different temperatures ($P<0.05$). The highest growth was observed at 27.5^{0}C and lowest at 32.5^{0}C, which is nearly half that at 27.5^{0}C (Figure 1). In the DRC isolate significant differences in mycelial growth was not observed at the different temperatures.

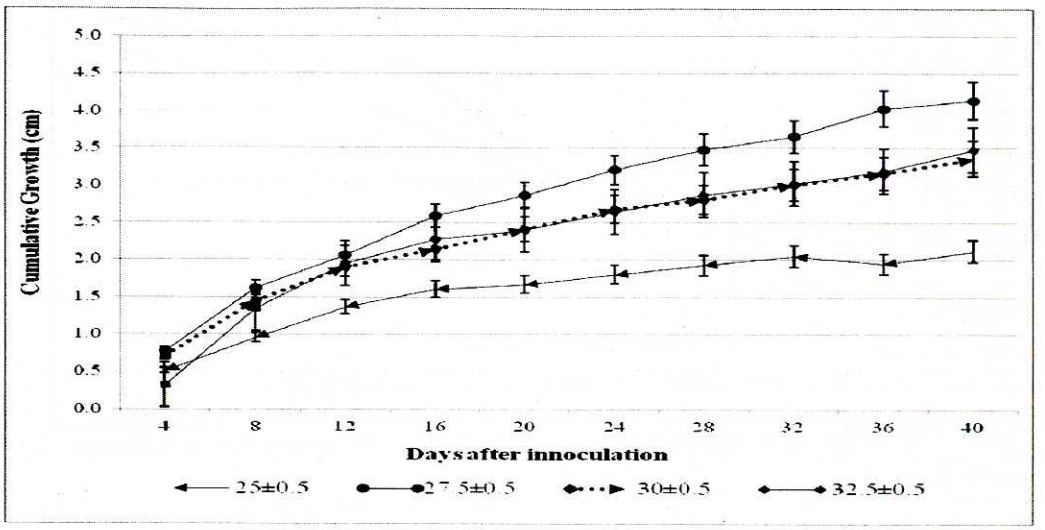

Figure1 Growth of mycelium (±S.E.) of the local isolate of *M. anisopliae* at different temperatures over time

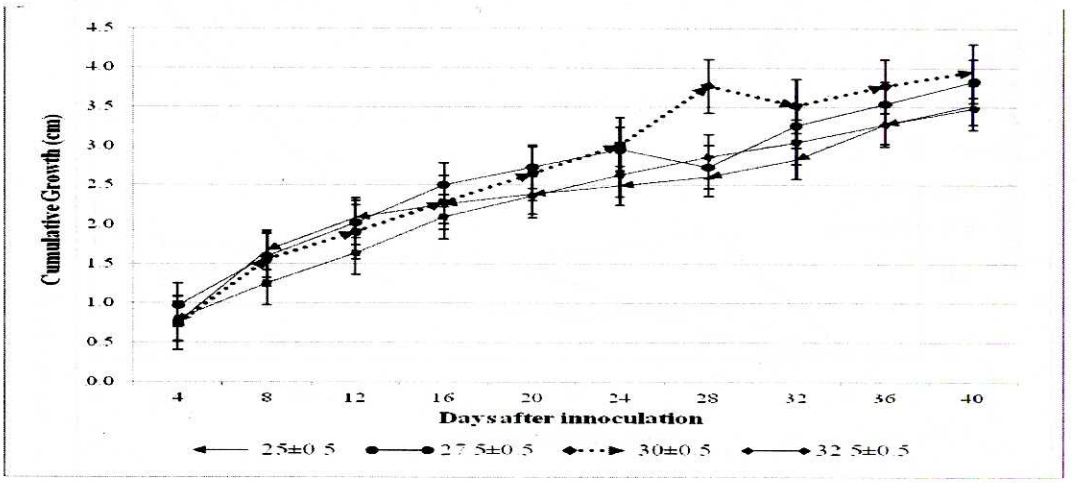

Figure 2 Growth of the mycelium (±S.E.) of DRC isolate of *M. anisopliae* at Different temperatures over time

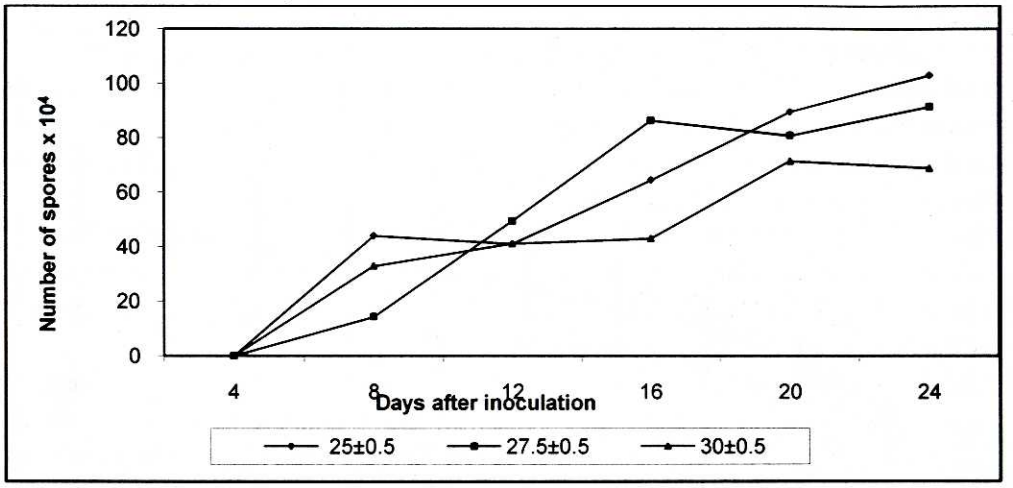

Figure 3 Average spore production of local isolate of *M. anisopliae* at different temperatures

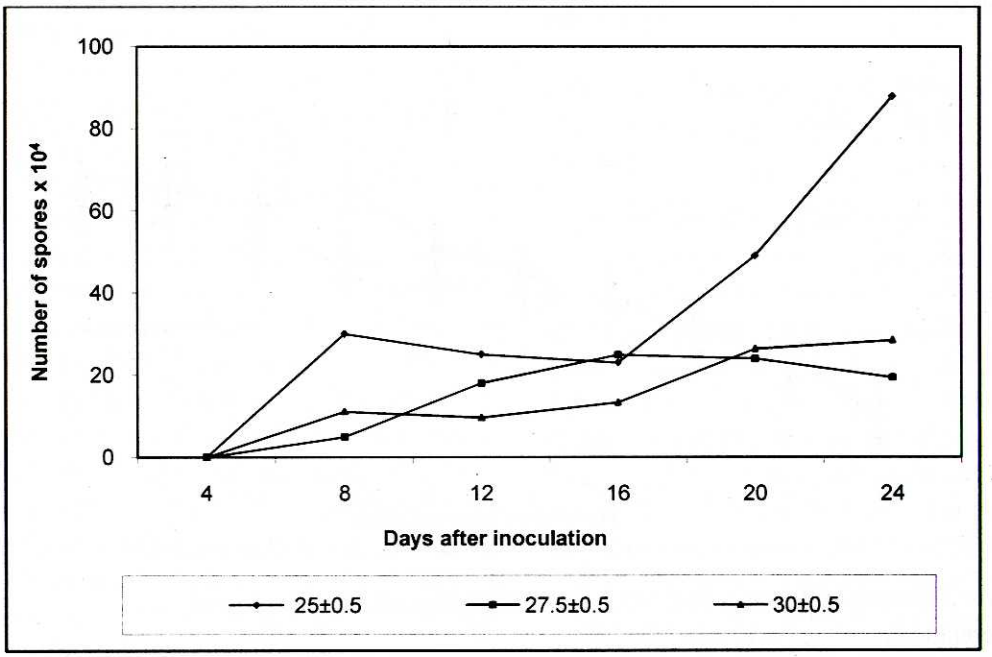

Figure 4 Average spore production of DRC isolates of *M. anisopliae* at different temperatures

Effect of temperature on spore production in local and DRC isolates of *M. anisopliae*

The number of spores produced in the local and DRC isolates were significantly different ($P<0.05$). At all temperatures, the local isolate produced significantly higher number of spores ($P<0.001$) than the DRC isolate (Figures 3 and 4). In the local isolate spore production was higher at 25^{0}C and 27.5^{0}C than at 30^{0}C (Figure 3). Spore production in DRC isolate was considerably higher at 25^{0}C than at other two temperatures (Figure4).

Comparison of virulence of local and DRC isolates of *M. anisopliae* against of *O. rhinoceros*

All the larvae of *O. rhinoceros* used in the study were killed by the 17th day of the experimental period either due to infection by *M. anisopliae* or other causes. Both local and DRC isolates caused mortality in larvae, but the mortality different significantly in the two fungi ($P<0.05$). At each time interval, the cumulative mortality of larvae due to the local isolate was higher than that of the DRC isolate (Figure 5). Overall, local isolate resulted in a cumulative mortality of 67% compared to 42% in the DRC isolate. The larvae in the untreated control group also showed low level of *M. anisopliae* infection (4.5%), which is likely due to the presence of already infected larvae being used in the study as they were collected from their natural breeding sites. These larvae did not show any symptoms during the two week testing period. A considerable larval mortality was observed in all the treatments either due to infection by the *Oryctes* virus or other factors resulting from collection and handling.

The time taken to 50% mortality of the larvae (LT_{50}) was 14 days for local and 18 days for DRC isolate respectively (Figure 5).

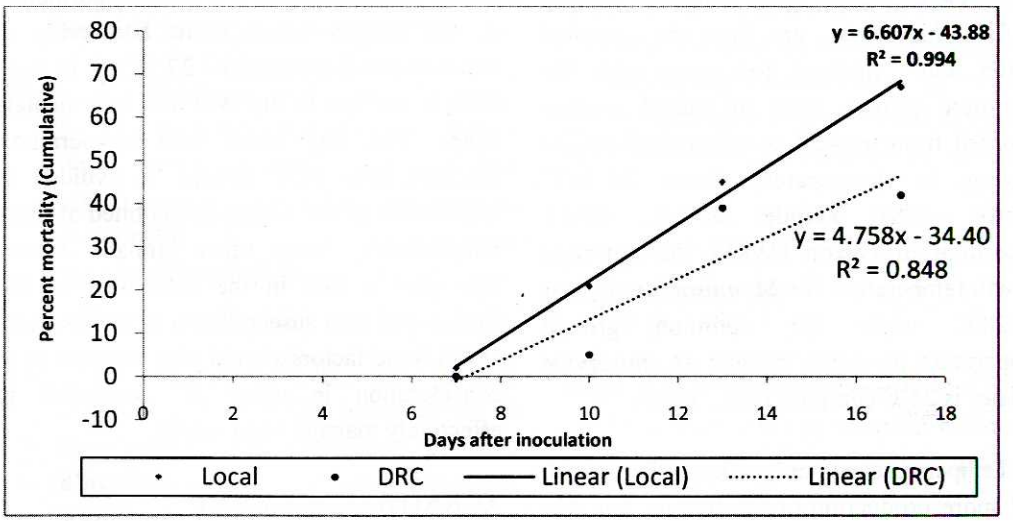

Figure 5 Cumulative mortality of local and DRC isolates of *M. anisopliae* and estimation of LT_{50}

DISCUSSION

The effect of temperature on mycelial growth and spore production differed in the two isolates. Mycelial growth in DRC isolate occurred more or less similarly at all the temperatures, but in the local isolate highest growth occurred at 27.5^{0}C. Local isolate produced a higher number of spores than the DRC isolate at all the temperatures. Overall, the isolates performed better at 25^{0}C – 27.5^{0}C temperature range.

Vidal *et al.* (1997) demonstrated that the potential of isolates to tolerate high or low temperatures is normally related to the climate of their geographic origin. A relationship between thermal tolerance and climate of origin has been shown for other isolates of entomopathogenic fungi such as *Beauveria bassiana, B. brongniartii* (Saccado) Petch, *Metarhizium anisopliae* (*flavoviride*) var. *acridum*, *Paecilomyces fumosoroseus* (Wize) Brown and Smith and *Nomuraea rileyi* (Samson) (Fargues *et al.*, 1992; Vidal *et al.*, 1997). Since the isolates used in this study are from the tropical region, our findings too agree with the maximum growth rates of fungal isolates recorded from tropical or subtropical origins ranging in temperature from 25–30^{0}C (Ferron, 1981; Fargues *et al.*, 1992). According to Ferron (1981), the optimum growth temperature for *M. anisopliae* is near 27–28^{0}C while the optimum growth temperature for some tropical *M. anisopliae* isolates is 25^{0}C (Fargues *et al.*, 1992).

Temperature not only affects the growth and spore production of the fungus, but also regulates the physiology of the fungus and the host insect, as well as the ability of the fungus to infect the host (Dimbi *et al.*, 2004). Different isolates of *M. anisopliae* showed different mortalities in the host insect at different temperatures, but for most tropical isolates 25–30^{0}C were the optimum temperatures for the infection process (Ekesi *et al.*, 1999; Dimbi *et al.*, 2004). In the study virulence of the fungal isolates was tested at room temperature of 28±3^{0}C, which is in the optimum range for tropical *M. anisopliae*. At this temperature the local isolate showed a higher virulence resulting in a higher mortality in black beetle larvae (67%, LT_{50} of 14 days) than the DRC isolate (42%, LT_{50} of 18 days) indicating that the local isolate would performs better in the field than the DRC isolate. Hence the local isolate could be considered suitable for the management of black beetle. This would also avoid any risks due to use of an exotic fungal isolate.

The study sheds light on an important aspect of *M. anisolpliae* use in the field. It is evident that the fungus could not be used under all environmental conditions in Sri Lanka. Since mycelial growth and sporulation of the fungus occur more favourably at temperatures between 25 - 27.5^{0}C, it is more suitable for use in the Wet and Intermediate zones. The Dry zone with temperatures reaching over 30^{0}C should be avoided as sporulation of the fungus is inhibited at these temperatures. Since other climatic factors also play a role in the infectivity of the fungus and host susceptibility (Zimmermann, 1982) these factors should also be taken in to consideration in using *M. anisopliae* to effectively manage black beetle.

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