

RESEARCH INTO THE COMMERCIALIZATION OF *Metarhizium anisopliae* (Hyphomycetes) FOR BIOCONTROL OF THE RHINOCEROS BEETLE, *Oryctes rhinoceros* (Scarabaeidae), IN OIL PALM

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ABSTRACT

Using the entomopathogenic fungus, *Metarhizium anisopliae*, to control the rhinoceros beetle, *Oryctes rhinoceros*, was first attempted in 1976. Early screening showed *M. anisopliae* variety major to be highly pathogenic to *O. rhinoceros*. Further work concentrated on using the variety to control *O. rhinoceros* as the pest infestation increased in oil palm plantations. Assessment of isolates from different localities found little variation in pathogenicity between them. Dipping the larvae in a spore solution of 10^8 spores ml^{-1} caused 100% mortality after 12 and 14 days. Analysis of the fungal DNA by RAPD-PCR showed a high similarity in the genetic base among isolates. Two primers can be used for fingerprinting and as a post-release monitoring tool as they were able to differentiate the isolates SE and BP from the others. An initial field study applying the fungus as spore solutions and sporulated substrates infected all stages of the pest, causing up to 84% reduction in its overall population.

The effects of the fungus on the oil palm pollinating weevil and non-target organisms were studied. The fungus did not affect development of the weevil. Toxicity tests showed the fungus to be harmless to rats. Fish exposed to very high spore concentrations of 1000 mg ml^{-1} (eight times higher than the highest rate applied in the field) only caused 25% mortality and at 2000 mg ml^{-1} only 40% mortality. The larvae of the stag beetle, *Aegus chelifer*, were susceptible to *M. anisopliae*, although less so than the larvae of *O. rhinoceros*. At 12 days after treatment, all the tested isolates caused 33.3% - 83.3% mortality to the stag beetle larvae but killed all (100%) of the *O. rhinoceros* larvae.

The spores of *M. anisopliae* were successfully mass produced using solid state fermentation. Fungal mycelia were first produced in liquid medium and then sporulated on a solid medium of maize. Harvesting was done by separating out the spores from the maize by washing in water, collecting them by vacuum filtration and drying at low temperature before finally grinding to powder. The yield of spores was 9.2-10.5 g per 200 g maize bag with a viability of > 80%. The powder formulation was tested in the field, pre-mixed with water and applied to rotting oil palm debris by spraying. *M. anisopliae* infected *O. rhinoceros* in all stages of its life cycle. Application to rotting debris reduced the *O. rhinoceros* population by up to 80%. The field application of *M. anisopliae* did not affect the populations of oil palm pollinating weevil and stag beetle.

Keywords: *Metarhizium anisopliae*, *Oryctes rhinoceros*, powder formulation, non-target organisms, oil palm.

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INTRODUCTION

Biopesticides using entomopathogenic fungi are increasingly being developed, especially based on the genera *Beauveria* and *Metarhizium*, stirred by the successful products using the bacterium, *Bacillus thuringiensis* (Richards and Rogers, 1990; Moore and Prior, 1993). Currently, biopesticides using *M. anisopliae* are being used to control a sugar cane pest, *Mahanarva posticata*, in Brazil, pasture and sugar cane insect pests in Australia and locusts in Africa (Mendonca, 1992; Bateman *et al.*, 1993). There has also been an attempt to control the rhinoceros beetle, *Oryctes rhinoceros*, using *M. anisopliae* in coconut. Latch and Falloon (1976) showed that the beetle was highly susceptible to a long-spored isolate of *Metarhizium*, later identified to be *M. anisopliae* var. *major* (Tulloch, 1979).

O. rhinoceros is a serious pest of oil palm in Malaysia (Bedford, 1980; Norman and Basri, 1997). In areas of severe damage, as much as 15% of the leaf area can be lost (Samsudin *et al.*, 1993), resulting in a yield decline of up to 25% (Liau and Ahmad, 1991). There have been studies to control *O. rhinoceros* in oil palm using *M. anisopliae* by Tey and Ho (1995) and Ramle *et al.* (1999a) who applied sporulated substrates and spore suspensions of the fungus in the breeding sites of the pest. Both formulations were equally effective, significantly reducing the larval population three months after application.

To further pursue the use of *M. anisopliae* as a biocontrol agent against *O. rhinoceros*, the Malaysian Palm Oil Board (MPOB) has been investigating several aspects of it - pathogenicity, characterization, mass production, formulation, safety and, finally, field application methods.

PATHOGENICITY OF *M. anisopliae* AGAINST THE RHINOCEROS BEETLE

Latch (1976) found three out of 27 isolates of *M. anisopliae* which killed 100% of the larvae of *O. rhinoceros* 7 – 16 days after treatment (DAT). The three were identified as *M. anisopliae* var. *major*, with a spore length of 9-14 μm . The others were *M. anisopliae* var. *anisopliae*, which is known to have a wide host range and a spore length of 5 – 8 μm (Tulloch, 1979). Other workers have confirmed that *M. a.* var. *major* mainly infects *O. rhinoceros* (Sivapragasam and Tey, 1995; Ramle *et al.*, 1999a). There was also no difference in the pathogenicity of *M. a.* var. *major* from different localities against *O. rhinoceros* larvae. In this study, four isolates *M. a.* var. *major* (Table 1) were tested by mixing 10^8 spores with rotting oil palm debris in containers. Third instar (L3) larvae of *O. rhinoceros* were then placed

in the containers and their mortality recorded every alternate day. All the isolates killed all (100%) of the larvae at about 14 DAT (Figure 1). A previous study using two isolates of *M. a.* var. *major* against *O. rhinoceros* larvae resulted in about the same lethal time 50% (LT_{50}) for both at 8.9 - 9.1 days (Ramle *et al.*, 1999a).

TABLE 1. THE ORIGIN OF ISOLATES OF *M. anisopliae* var. *major* USED IN BIOASSAY

No.	Name	Origin
1	BP	Bagan Pasir, Perak
2	CI	Carey Island, Selangor
3	SE	Sedenak, Johor
4	ST	Sg Tekam, Pahang

Ho (1996) reported the pathogenicity of *M. a.* var. *major* to different populations of *O. rhinoceros* to be similar with the effective dose 50% (ED_{50}) to be $3.79 - 5.80 \times 10^3$ spores ml^{-1} .

CHARACTERIZATION OF *M. anisopliae* BY RAPD-PCR

The isolates have first to be characterized in order to monitor their progress after release in the field. This is to distinguish them from the endemic populations. Conventional characterization of *M. anisopliae* by their morphological traits (Tulloch, 1979; Rombach *et al.*, 1987), physiological/biochemical properties (Riba *et al.*, 1986; St Leger *et al.*, 1992) and isozyme profiles (St Leger *et al.*, 1987) have often proved wanting due to the variable environmental and physiological conditions. In recent years, more reliable and sensitive molecular techniques, in particular, random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR), have been increasingly used to differentiate *M. anisopliae* isolates (Cobb and Clarkson, 1993; Bidochka *et al.*, 1994; Leal *et al.*, 1994).

A study using RAPD-PCR showed a distinct difference between *M. a.* var. *major* and *M. a.* var. *anisopliae* (Ramle *et al.*, 1999b) - *M. a.* var. *major* has a high degree of similarity in its genetic base but *M. a.* var. *anisopliae* a high degree of variability. This study on four isolates has also indicated that isolates of *M. a.* var. *major* have close similarity in their genetic base. Of five primers tested, primers 3 and 5 produced DNA fragments with only one specific band to differentiate isolates SE and BP from the others (Figure 2). These primers can be used as markers for fingerprinting and for post-release monitoring of the fungus in the field.

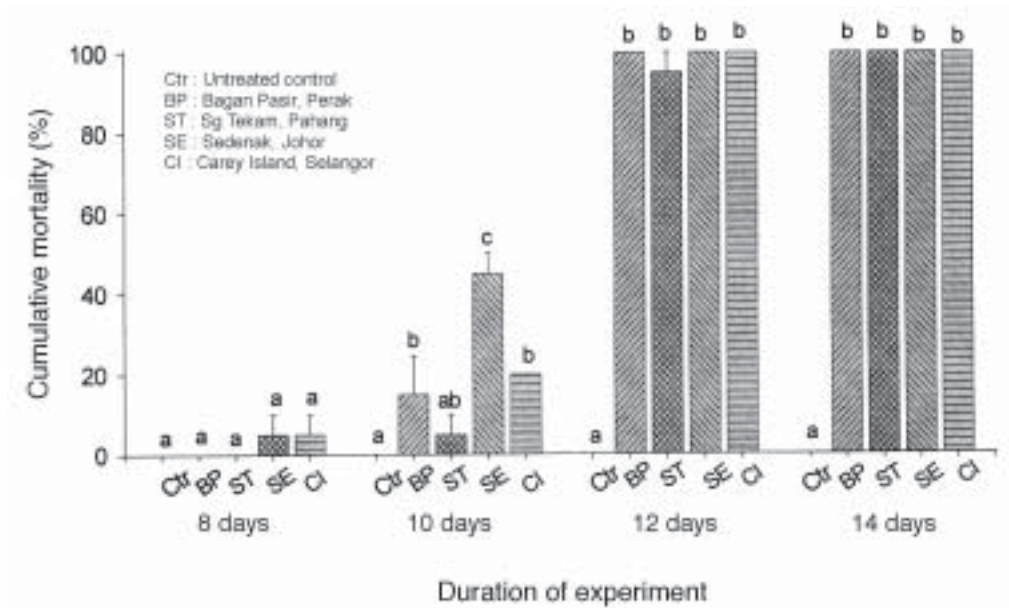


Figure 1. Pathogenicity of four isolates of *M. anisopliae* var. major against the larvae of the rhinoceros beetles. The bars with same letters indicate that the groups are not significantly different ($P > 0.05$) by Duncan's multiple range test.

MASS PRODUCTION OF SPORES

The spores of *M. anisopliae* have been successfully produced using solid state fermentation on broken maize (Ramle *et al.*, 2005). Rice has also been

commonly used to mass produce *M. anisopliae* (Mendonca, 1992). The production process involves two fermentations - propagation of the fungal mycelia in liquid medium and sporulation on solid substrate. The broken maize was placed in

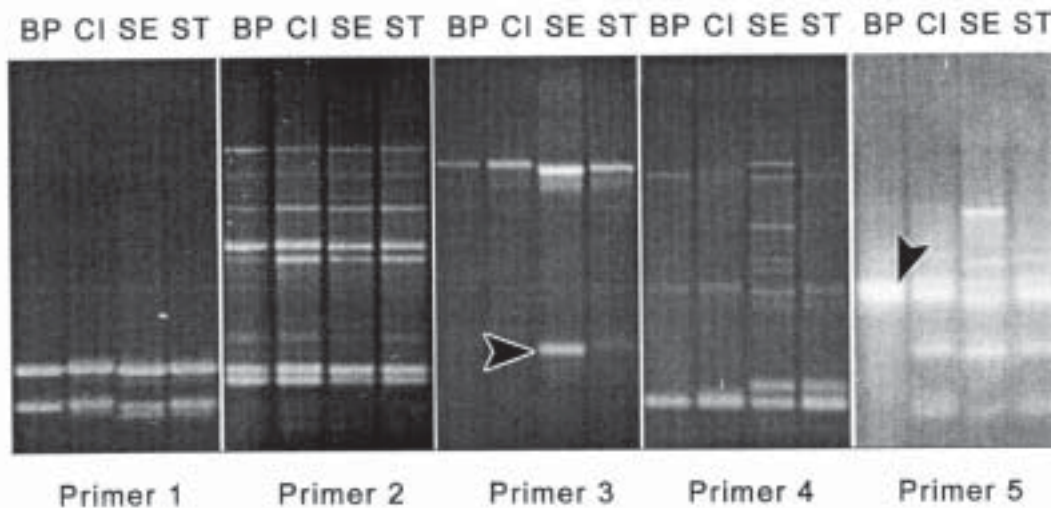


Figure 2. RAPD-PCR products showing DNA fragments from four isolates of *M. anisopliae* var. major amplified using five random primers. The arrows indicate a specific band identifying isolates SE and BP from the others.

autoclavable plastic bags, sterilized, inoculated with the fungal mycelia then incubated at 28°C until maturity. Separation of spores from the maize was by washing in water with 0.0002% Tween 80 to first form a spore solution. The solution was then filtrated and dried at low temperature (10°C - 15°C) for several hours. The spore cakes were then further dried in a safety cabinet for another 1 hr and ground to powder. The processes involved in the mass production of *M. a. var. major* spores are shown in Figure 3.

Ramle *et al.* (2005) have optimized the processes with the following findings:

- wet harvesting produced a higher yield and better quality spores than dry harvesting (Table 2);
- washing with water and a weak concentration (0.0002%) of wetting agent produced a higher yield of spores than washing with water alone;

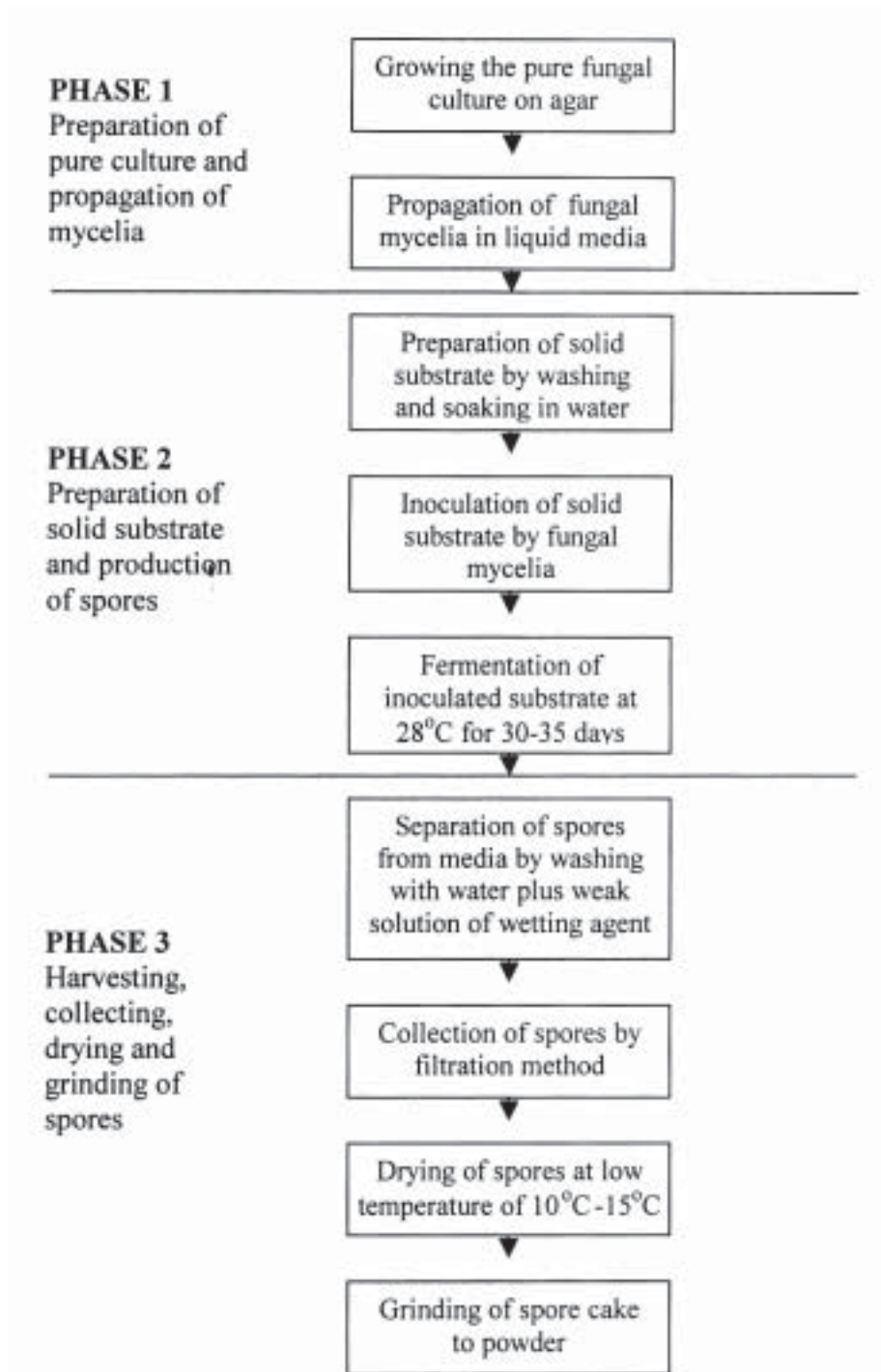


Figure 3. Flow process for mass production of *M. anisopliae var. major* spores using the wet harvesting and filtration method.

- the moisture content influenced the spore viability. High germination of the spores (>80%) was achieved by maintaining the spore moisture level at 40% to 60% (Figure 4);
- drying of the spores in low temperature (10°C -15°C) for about 10 hr is recommended for better quality;
- the age of the culture influenced its yield of spores. Mature cultures of 30-40 days produced 9.2 - 10.5 g spores per bag, higher than young cultures of 20-25 days (Figure 5).

The mass production technology has been transferred to FELDA Agricultural Services Sdn Bhd (FASSB). The plant is at Pusat Perkhidmatan

Pertanian Tun Razak (PPPTR) Jerantut, Pahang in an ex-office named the *Metarhizium* Technology Centre (METEC).

FORMULATIONS

Both the freshly sporulated substrates and spore suspensions were equally effective in controlling *O. rhinoceros* when applied to their breeding sites (Latch and Falloon, 1976; Tey and Ho, 1995; Ramle *et al.*, 1999a). However, both inoculums have disadvantages.

TABLE 2. EFFECTS OF HARVESTING METHODS ON THE YIELD, MOISTURE CONTENT AND VIABILITY OF THE SPORES OF *M. anisopliae* var. *major*

Harvesting methods	Number of bags harvested (N)	Yield of spores/ bag (g)	Moisture content (%)	Viability of spores (%)
Dry	282	2.89a	4.67a	13.34a
Wet	67	4.01b	21.27b	53.09b

Note: Columns with same letters are no difference ($P>0.05$) by Duncan multiple range test.

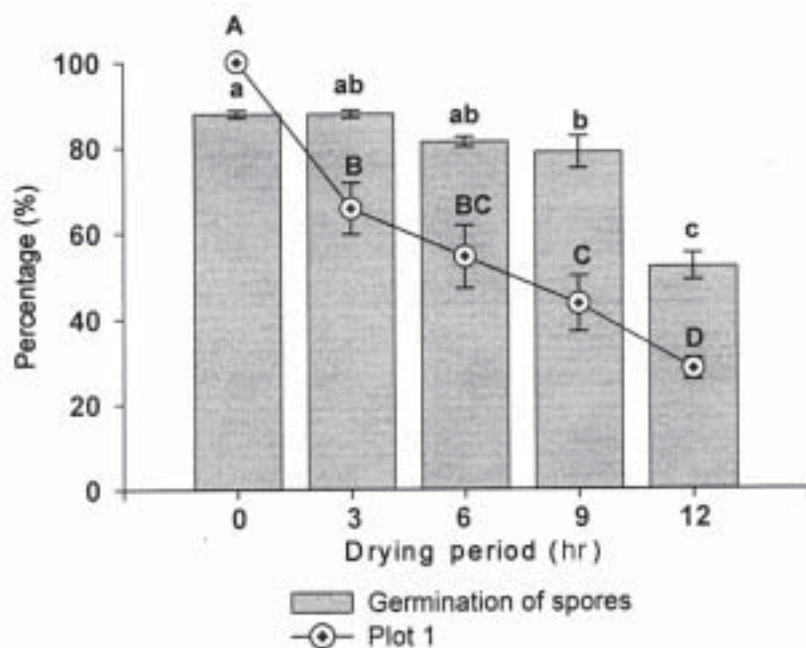


Figure 4. Effects of drying time on the viability and moisture content of *M. anisopliae* var. *major* spores harvested from mature cultures. The means for the bar and line graphs with the same letters are not significantly different ($P>0.05$) by Duncan's multiple range test.

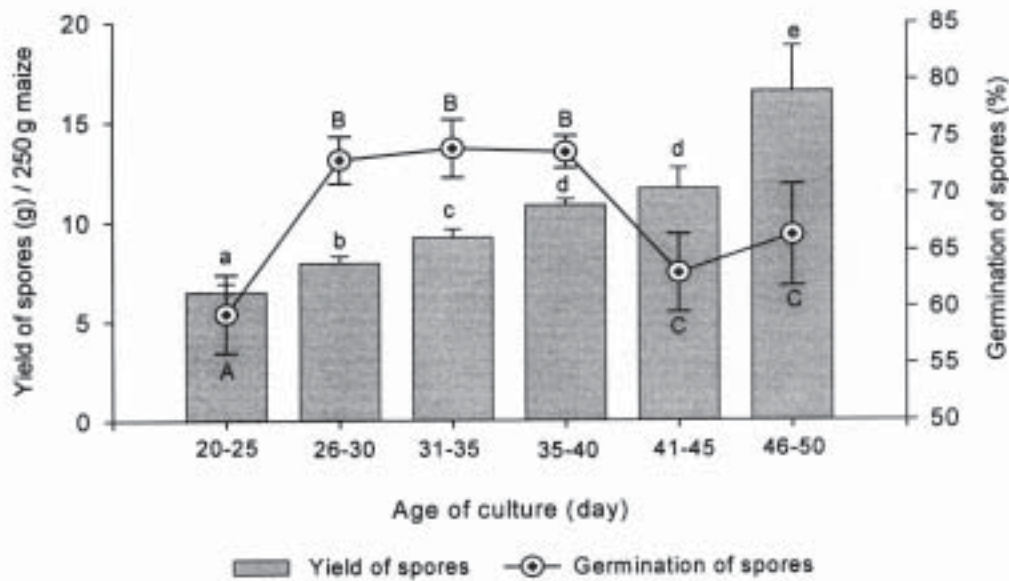


Figure 5. The effects of age of cultures on production and viability of spores of *M. anisopliae* var. major. The means for the bar and line graphs with the same letters are not significantly different ($P>0.05$) by Duncan's multiple range test.

- The spore viability is reduced over time if stored as sporulated substrates or mixed with water. To maintain the spore viability, the inoculums must be kept in low temperature (5°C) which is costly.
- Ultraviolet radiation, high soil temperature, high soil moisture and humidity are detrimental to the spores (Moore *et al.*, 1993; Ekesi *et al.*, 2003). Broadcasting the sporulated substrates will expose the spores to these factors, reducing their viability and effectiveness to *O. rhinoceros*.
- The freshly sporulated substrates are bulky for handling.
- Handling of the raw materials, such as sporulated substrates, may be a problem for those without experience with microorganisms.

To obviate the above constraints, the fungus was formulated in powder form – the pure dry spores (active ingredient) diluted with clay powder (hydrated aluminium silicate, inert material).

Stability and Effectiveness of the Formulation

The stability of the powder formulation stored at 5°C, 15°C and 28°C was determined by thoroughly mixing 0.1 g product with 10 ml water plus 0.02% Tween 80. The spore viability was determined on potato dextrose agar after 3, 7, 9, 12 and 15 months' storage (MAS) (Hisham *et al.*, 2005).

The viability of the spores after 15 MAS largely remained high at about 60% and above (Figure 6). The exception was storage at 28°C which reduced

the spore viability significantly at 9 MAS (to 52.2%) and further with subsequent time – 28.5% at 12 MAS and 1.65% at 15 MAS. However, a bioassay showed the product to be still effective for controlling the L3 larvae, even after 15 MAS. At 14 DAT, the product stored at 15°C (germination 58%) caused the highest (100%) mortality. The product stored at 28°C, even for 15 MAS, was almost as good, causing 90% mortality and 73.3% infection (Table 3); this result was unexpected as its spore viability was only 1.65%.

Stathers *et al.* (1993) found that the spores of *M. anisopliae* formulated in powder stored better at 5°C and 15°C than if formulated in oil. This was even so with higher temperature storage - in oil, at 25°C, the viability was reduced to less than 25% as early as 4.5 MAS, while in powder the germination was still 28.5% at 12 MAS. For commercial use, the bio-pesticide would need to remain viable for 12–18 months, or three to six months if applied at specific times (Couch and Ignoffo, 1981). Walstad *et al.* (1970) found that the optimum storage temperature for the spores of *M. anisopliae* was about 8°C, but in this study the optimum was between 5°C to 15°C.

SAFETY OF *M. anisopliae* TO NON-TARGET ORGANISMS

Oil Palm Pollinating Weevil

The weevil, *Elaeidobius kamerunicus*, was introduced into the country to help pollinate the oil palm for better yield and to save the cost of assisted pollination (Basri *et al.*, 1985; Chan, 1985). It is

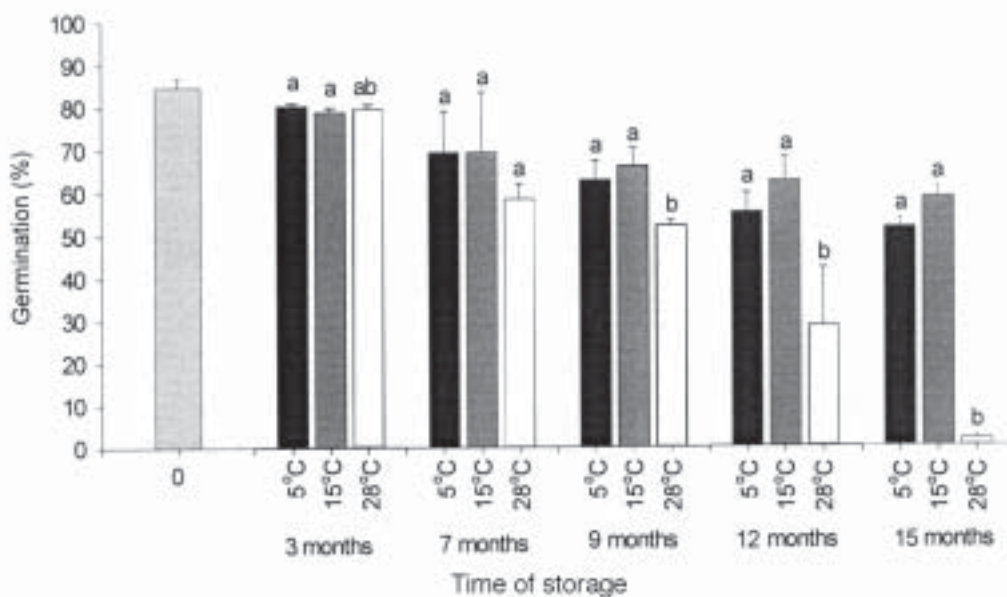


Figure 6. Stability of *M. anisopliae* var. major spores formulated in powder and stored at different temperatures for 15 months. The bars with the same letters indicate that the groups are not significantly different ($P > 0.05$) by Duncan's multiple range test.

TABLE 3. MORTALITY OF *Oryctes* AFTER TREATED WITH STORED PRODUCT OF POWDER FORMULATION OF *M. anisopliae* var. major FOR 15 MONTHS AT DIFFERENT TEMPERATURES

Treatment	Cumulative mortality (%) over time after treatment (day)					Infected larvae (%)
	6	8	10	12	14	
Ctr (-)	0.0 a	0.0 a	6.7 a	20.0 a	26.7 a	16.7 a
Ctr (+)	3.3 a	13.3 a	43.3 b	97.7 b	100.0 b	100.0 c
5°C	10.0 a	10.0 a	20.0 ab	70.0 b	96.7 b	80.0 b
15°C	10.0 a	13.3 a	43.3 b	86.7 b	100 b	90.0 b
28°C	3.3 a	3.3 a	20.0 ab	86.7 b	90.0 b	73.3 b

Notes: Columns with same letters are no difference ($P > 0.05$) by Duncan multiple range test. Ctr (-): untreated control. Ctr (+): larvae were treated by freshly prepared spore suspensions harvested from 30 days cultures.

therefore important to ascertain whether *M. anisopliae* affects it. Although no infection has been reported, the fungus has been used to control other insects in the same family of *Curculionidae*, e.g. the black vine weevil, pecan weevil and other palm weevils (Teddars *et al.*, 1982; Gottwald and Tedders, 1983).

Our assessment showed that *E. kamerunicus* was more susceptible to *M. a. var. anisopliae* than *M. a. var. major* (Ramle *et al.*, 1999a). *M. a. var. anisopliae* caused 58% mortality, 17% - 26% higher ($P < 0.05$) than *M. a. var. major*. Direct application of all the tested isolates of *M. a. var. major* on post-anthesized male inflorescence spikelets of the oil palm did not affect the emergence of new adults. Furthermore, none of the new adults were infected by the fungus.

Rats, Fish and Other Insects

A toxicity study of *M. a. var. major* on rats, fish and a coleopteran insect was carried out by Ramle *et al.* (2004).

On rats, oral administration of *M. a. var. major* at 5000 mg kg⁻¹ did not cause any death to either the males or females. Similarly, all the male and female rats treated dermally at 2000 mg kg⁻¹ survived through the two weeks' study period. In both tests, both the treated and control rats gained the same weight, suggesting that both groups were equally healthy. No toxic symptoms were observed over the study. Nor did post-mortem after the study show any unusual changes from the control animals. It was therefore concluded that the spores of *M. a. var. major* are very safe on rats with oral LD₅₀ > 5000 mg kg⁻¹ and dermal LD₅₀ > 2000 mg kg⁻¹.

On the freshwater fish, *Tilapia nilotica*, treatment with *M. a. var. major* at concentrations of 0.1, 1.0, 10.0 and 100.0 mg spores litre⁻¹ did not cause any

mortality. Only when excessively high concentrations were used did some fish die – 25% at 1000 mg litre⁻¹ and 40% at 2000 mg litre⁻¹.

The stag beetle, *Aegus chelifera*, is commonly found together with *O. rhinoceros* in the same breeding environment of rotting oil palm biomass. Although it is a fruit, flower and leaf feeder (Wood, 1968), very little damage is caused the oil palm.

The beetle was bioassayed together with *O. rhinoceros*, and found to be susceptible to *M. anisopliae* although less so than the latter. At 12 DAT, all the tested isolates caused 33.3% – 83.3% mortality to the stag beetle larvae but killed all (100%) the *O. rhinoceros* larvae.

FIELD APPLICATION

Initial Study

Previously, *M. anisopliae* had been applied as a dry inoculum of sporulated substrates and a wet inoculum of freshly harvested spore suspensions directly to the breeding sites of *O. rhinoceros* (Latch and Falloon, 1976; Tey and Ho, 1995).

Both inoculums effectively reduced the *O. rhinoceros* population, especially the L3 larvae. The number of surviving larvae in the treatment by spraying at 10¹⁰ spores per treatment plot (20 m² rotting) was about the same as that in the treatment by broadcasting at 6 kg sporulated maize per plot, *i.e.*, 5.5 larvae per sampling plot (4 m²) significantly lower ($P < 0.05$) than the surviving population in the control plots (35 larvae). The high reduction in L3 larvae was possibly related to its long (longest developmental stage in the beetle life cycle) duration of 112.5 days (Bedford, 1980). A longer stage would obviously more greatly predispose the larvae to infection by the spores.

The larvae prefer high humidity, a moderate temperature (27°C-29°C) and wet substrate (31% - 73% moisture content) (Bedford, 1980). Such conditions also enhance the longevity and viability of spores and increase the likelihood of infection by the fungus (Latch, 1976). Latch and Falloon (1976) reported that the spores of *M. anisopliae* can survive in the breeding sites of *O. rhinoceros* for at least 24 months.

Due to large amount of maize required to produce the dry inoculum, the wet inoculum is preferred as less maize is required - [180 kg (for dry) *vs.* 1.4 kg (wet) to produce the spores to treat a hectare] (Ramle *et al.*, 1999a).

The study To Date

Preliminary laboratory and small field studies have shown the powder formulation to be effective in controlling *O. rhinoceros*. Application by both a

mist blower and power sprayer were equally good, causing 100% mortality at 10 weeks after treatment (Hisyam *et al.*, 2005).

Further assessment was carried out in a two-year-old replant on 120 ha with rotting heaps of the chipped old palms, fell about 25 months ago. The product was mixed with water to form a solution, then applied by a high volume sprayer. The rate of water used was 0.75 litre m⁻² of rotting heap (the area of all the rotting heaps was 640 m² ha⁻¹), or 480 litres ha⁻¹ (0.75 litre x 640 m²). A single application was applied at T1, 0.2 g, or 2.2 x 10⁷, spores m⁻² of rotting heap (equivalent to 130 g product ha⁻¹) and T2, 0.4 g, or 4.4 x 10⁷, spores per m⁻² of rotting heap (equivalent to 260 g product ha⁻¹), and the population of *O. rhinoceros* monitored for 12 months after treatment (MAT).

The impact of the *Metarhizium* treatment on the *O. rhinoceros* populations could be observed as early as three to five months after application (Figure 7) when they were already depressed below the control population, although not yet significantly so ($P > 0.05$). At 8 MAT, a significant ($P < 0.05$) reduction was obtained. The reduction in *O. rhinoceros* population was due to increased *M. anisopliae* infection (Figure 8). At 8 MAT, the percentage dead and infected *O. rhinoceros* in the treatment plots was 34.2% for T1 and 32.1% for T2, significantly higher ($P < 0.05$) than the control 12.9%. Application of the product at 130 g (T1), therefore, sufficed to significantly reduce the population of *O. rhinoceros*.

The density of viable spores in the rotting biomass and soil were estimated following the method of Ramle *et al.* (1999b). As expected, the density of viable spores **increased** following the increase in beetle infection by *M. anisopliae*.

Effects of the Product on the Stag Beetle

The product also did not affect the stag beetle which populations did not differ between the control and treatment plots (Figure 9). Although there was some infection of both the larvae and adults by *M. anisopliae*, the incidence was very low at 0% - 0.6% at five MAT and 1.7%-2.5% at eight MAT.

CONCLUSION

The entomopathogenic fungus, *M. anisopliae*, especially the variety *major* has been proven to be highly pathogenic against *O. rhinoceros*. The fungus killed 100% of the larvae of *O. rhinoceros* as early as 12 DAT. Analysis of DNA by RAPD-PCR showed that the isolates of *M. a. var. major* have a narrow genetic base compared to the isolates of *M. a. var. anisopliae*. The specific primers for differentiation, especially to differentiate BP and SE from other

isolates, were identified and can be used for fingerprinting and post-release monitoring in field.

Oral and dermal toxicity tests showed that the fungus did not kill nor affect the growth of rats, suggesting it to be safe to mammals. Fish exposed to doses about eight and 16 times higher than the highest rate used in the field (10^9 spores per square meter of breeding site) only caused 25% and 40% mortality, respectively. A laboratory test showed the stag beetle larvae to be susceptible to *M. anisopliae*, causing 33.3%-83.3% mortality. However, in the field, the fungus did not affect the stag beetle much.

An initial field study showed that application of the fungus as both wet and dry inoculums significantly reduced the population of *O. rhinoceros*. The fungus infected all stages in the life cycle of *O. rhinoceros*, with the greatest effect on the L3 larvae. The spores of the fungus were successfully mass produced on a solid substrate of maize. Harvesting the spores by the wet method produced a higher yield and better quality spores. In storage, the viability of the spores decreased with their moisture content. Therefore, excessively drying them is detrimental, and the moisture content should be maintained at 40%-60%. The yield and spore viability peaked at a culture age of 31-35 days.

A higher spore viability was obtained with *M. anisopliae* formulated in powder. Storage at 5°C and 15°C maintained the viability for more than half their initial rate, even after 15 months. With storage at 28°C, the spores still germinated well above half their initial rate after nine months. Laboratory and small field tests showed the fungus to be effective in controlling *O. rhinoceros*.

A single application of the product to oil palm rotting debris using high volume spray sufficed to significantly reduce the population of *O. rhinoceros*. The application did not affect the oil palm pollinating weevil and stag beetle as an example non-target organism.

To use *M. anisopliae* for the control of *O. rhinoceros*, more study would be required, especially in two aspects - improvement of the product and field delivery methods. The first would include a study on the genotype improvement, mass production technology and formulations. The second should seek to determine the timing for field application, better delivery methods and the effects of the product on other organisms and the environment.

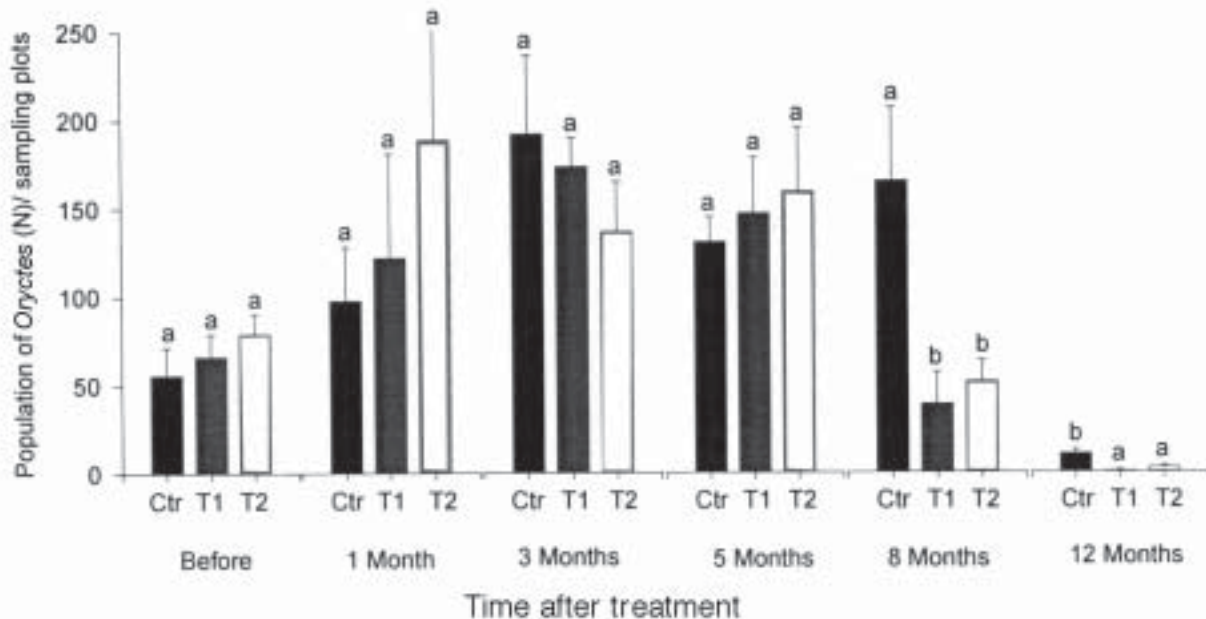


Figure 7. Effect of powder formulation of *M. anisopliar var. major* on the population of *Oryctes* with time. Ctrl: untreated control, T1: 0.2 g product/m² rotting heap, T2: 0.4 g product/m² rotting heaps. The bars with the same letters indicate that the groups are not significantly different ($P > 0.05$) by Duncan's multiple range test.

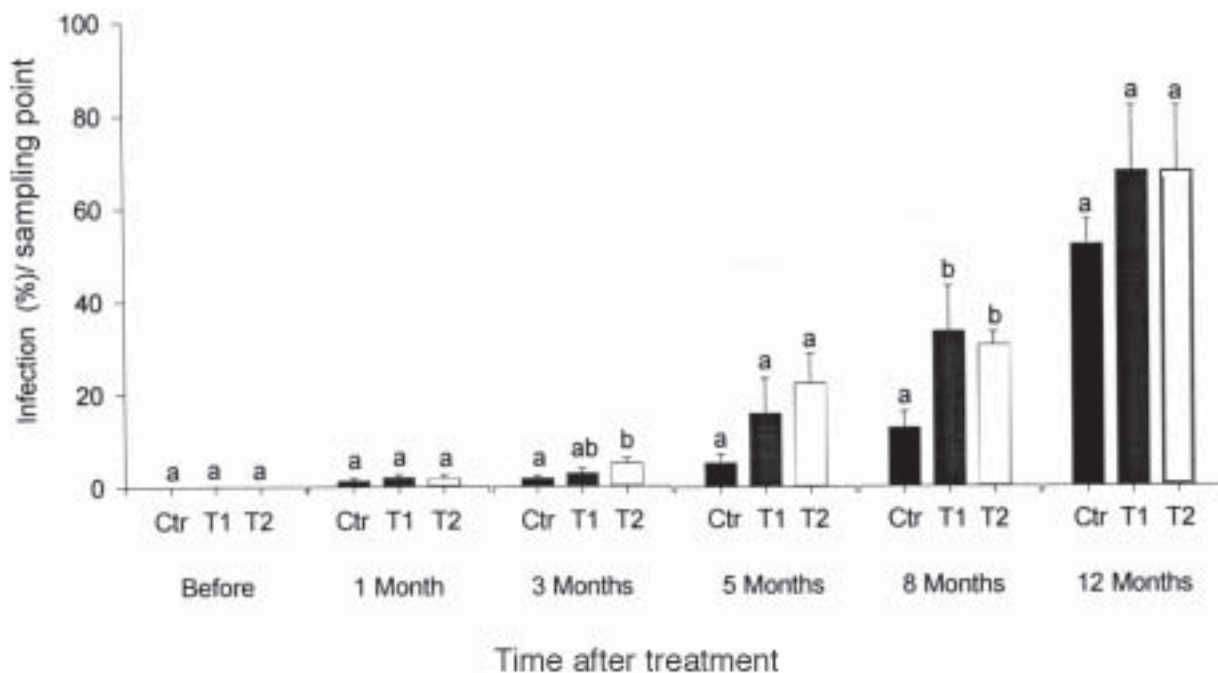


Figure 8. Infection of *Oryctes* in the field by *M. anisopliae* var. major over 12 months after application. Ctr: untreated control, T1: 0.2 g product/m² rotting heaps, T2: 0.4 g product/m² rotting heaps. The bars with the same letters indicate that the groups are not significantly different ($P>0.05$) by Duncan's multiple range test.

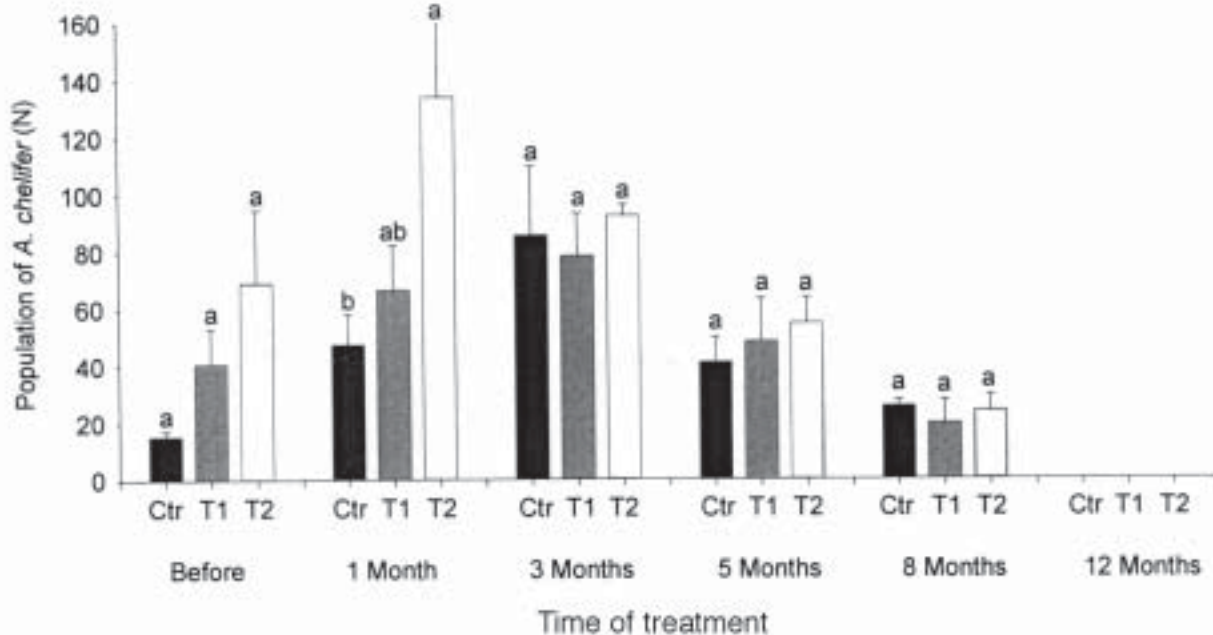


Figure 9. Effect of powder formulation of *M. anisopliae* var. major on the stag beetle, a non-target insect. Ctr: untreated control, T1: 0.2 g product/m² rotting heaps, T2: 0.4 g product/m² rotting heaps. The bars with the same letters indicate that the groups are not significantly different ($P>0.05$) by Duncan's multiple range test.

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