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IMPACT OF *Metarhizium anisopliae* (DEUTEROMYCOTINA: HYPHOMYCETES) APPLIED BY WET AND DRY INOCULUM ON OIL PALM RHINOCEROS BEETLES, *Oryctes rhinoceros* (COLEOPTERA: SCARABAEIDAE)

Keywords: ***Metarhizium anisopliae,*** rhinoceros beetles, ***Oryctes rhinoceros,*** wet and dried inoculum, field application.

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Four local isolates of Metarhizium anisopliae *(Bp, MO, Ma and Ml) were bioassayed against larvae of* Oryctes rhinoceros *in the laboratory. The larvae were surface-sterilized in 0.3% sodium hypochlorite and dipped into spore suspensions containing* 10^8 *conidia* ml^{-1}. *Of the four isolates, two long-spored isolates (Bp and MO) were highly pathogenic against the beetles. Both isolates caused 100% mortality within 12 days of treatment and caused 71% to 75% mycosis. The times required to kill 50% (*LT_{50}*) of Oryctes larvae for isolates Bp (*LT_{50} *= 9.1 days) and MO (*LT_{50} *= 8.9 days) were shorter than short-spored isolates Ma (*LT_{50} *= 10.0 days) and Ml (*LT_{50} *= 14.0 days). The mortality rates (regression slopes) showed that isolate Bp (5.32) killed* Oryctes *larvae slightly faster than isolate MO (5.17); therefore, isolate Bp was selected for a field experiment.*

A field trial was conducted in seven-month-old heaps of chipped oil palm trunks, using a single application of wet and dry inoculum, Using wet inoculum, each plot was drenched separately with 10 litres plot-" of water containing 10^8, 10^9 *and* 10^{10} *conidia. For dry inoculum, the plots were broadcasted with inoculated maize at 3 and 6 kg* $plot^{-1}$. *In the field,* Metarhizium *infects adults, pupae and all larval stages of* Oryctes *beetles. At three months after treatment, pooled mortality for all stages of* Oryctes *beetles at the highest application rate was between 37% (dry inoculum) and 51% (wet inoculum). A great impact of* Metarhizium *was found only on third*

instar larvae, where it was able to reduce the number to about 5.5-5.8 larvae / sampling plot. This was significantly lower than the control which had 34.8 larvae/sampling plot.

The densities of viable spores in plots treated with wet inoculum at 10^{10} conidia (158.6 cfu) and dry at 6 kg (169.0 cfu) inoculum were significantly higher than the other application rates, especially at S-month of treatment. A higher density of viable spores in breeding materials enhanced the disease development and subsequently reduced the beetle population. Wet inoculum had more advantages over dry inoculum. It was easily prepared using water from readily available sources such as field drains, river and ex-tin mining pools. This study showed that wet inoculum was effective for control of Oryctes beetle and was more economical to apply than dry inoculum.

INTRODUCTION

The rhinoceros beetle, *Oryctes rhinoceros* (Linnaeus), is a pest of coconut (Cocos *nucifera*) but now adapted to oil palm (*Elaeis guineensis)* (Bedford, 1980; Norman and Basri, 1997). The beetle breeds in decaying vegetative materials including oil palm tissues such as empty fruit bunches and rotting trunks (Samsudin *et al., 1993). The* current methods for replanting oil palm restrict the burning of palm residues [Environmental Quality (Clean Air) Regulations 19781. From past experience, without burning or only partial burning, oil palm biomass is a conducive breeding site for *Oryctes* (Liau and Ahmad, 1991). In 1995, a survey by Norman and Basri (1997) showed that 25% of 180 068 ha, or 45 610 ha of young palms were attacked by the pest. Repeated attacks by *Oryctes* adults can kill young palms and may predispose palms to *Rhynchophorus schach* infestation (Liau and Ahmad, 1991; Sivapragasam et *al.,* 1990). Damage can also result from the reduction of leaf area of up to 15% (Samsudin *et al.,* 1993) which can result in a 25% crop loss (Liau and Ahmad, 1991).

Various control measures of this pest have been well documented, including application of chemical insecticides, planting leguminous cover crops, pheromone trapping, cultural practices like winkling and application of biological control agents (Norman and Basri, 1995). The use of natural pathogens such as *Baculouirus oryctes* and *Metarhizium anisopliae* has not been fully exploited, despite being recorded since 1912 (Friederichs, 1920). Due to its ability to infect a wide range of insect pests, ease of production on simple substrates, easy storage and longer persistence of conidia in soil (Goettel, 1992), *M. anisopliae* has been commercially used as an environmental mycoinsecticide for controlling many insect pests (Prior, 1992; Mendoza, 1992).

M. anisopliae has two varieties - var. *anisopliae* and var. *major* (Tulloch, 1979). The spore length for var. *anisopliae* is 5.0-8.0 pm and for var. *major* 9-15 µm. *M. anisopliae* var. *anisopliae* is a cosmopolitan pathogen of innumerable insect pests (Veen, 1968), while var. *major* is largely restricted to the *Oryctes* beetle (Coleoptera: Scarabaeidae: Dynastinae) (Tulloch, 1979). Natural infection of *M. anisopliae on 0. rhinoceros* has been reported by Friederichs (1920) in 1912 at Western Samoa. Since then, attempts have been made to control *0. rhinoceros* with *M. anisopliae* with some success in Pacific regions (Bedford, 1980) and New Zealand (Latch, 1976). Laboratory bioassay has shown *Oryctes* larvae to be more susceptible to the long spored than short-spored isolate (Ferron et al., 1975; Latch, 1979; Sivapragasam and Tey, 1995).

Selection of highly virulent isolates by previous workers was by direct comparison of mortality or dead larvae with mycosis (Latch, 1976; Sivapragasam and Tey, 1995). In this study, besides mortality and percent mycosis, the highly virulent isolate was selected based on the time taken to kill 50% of larvae (LT_{50} value). Field delivery of this pathogen still requires some work, although Tey and Ho (1995) have demonstrated the potential of broadcast. ing dry inoculum, as they did not estimate the

optimum level of inoculum to use. Therefore, the objectives in this study were to evaluate the potential of applying wet (drenching) and dry (broadcasting) forms of *M. anisopliae* inoculum and estimate the optimum inoculum level to use.

MATERIALS AND METHODS

Assessment of *M. anisopliae* in Laboratory

Source of isolates. Four isolates of *M. anisopliae* were used (Table 1) – Bp and Mo from Universiti Kebangsaan Malaysia and Ma and Ml from adults and larvae of 0. *rhinoceros* collected in Teluk Merbau Estate, Sepang, Selangor. These isolates were maintained on malt extract agar (MEA) (Oxoid, Unipath. Ltd. England) at 28°C ± 1°C (Joan Maxi Artic) in the dark. The spore dimensions of the isolates were measured. The spore length for Bp and Mo were between 12-14 pm, and for Ma and Ml 6-8 µm. According to the system of Tulloch (1979) and Rombach et al. (1987), Bp and Mo were identified as *M. anisopliae* var. *major,* and Ma and Ml as *M. anisopliae* var. *anisopliae.*

Source of insect. Various stages of 0. *rhinoceros* larvae were collected from the Jendarata Estate, United Plantations Bhd., Teluk Intan. The larvae were maintained in plastic containers of 48.0 cm x 33.0 cm x 26.0 cm (length x widthxheight), each containing 3.0-3.5 kg (50%-60% moisture content) of decayed oil palm tissues. Forty to 50 larvae were placed in each container and kept at 25°C ± 5°C for a week prior to the experiment. The larvae were surface sterilized by washing in 0.3% (v/v) sodium hyphoclorite for 30 sec, rinsing twice with sterilized distilled water, and air drying for 5-10 min prior to treatment.

Inoculation and bioassay. Spore suspensions were prepared by adding sterilized distilled water containing 0.2% (v/v) Tween 80 (BDH, Lab. Supp. England) to two-week-old MEA plates. Spores were scraped off from the surface of the medium using a sterilized 'L' shaped inoculation needle, and transferred into a screw-capped universal bottles. After vortexing for 2 min, the spores were separated from the debris by filtering through sterilized glass wool. The spore concentration was estimated using an improved Neubauer hemocytometer and adjusted to 10^8 conidia ml^{-1} by dilution with 0.2% (v/v) Tween 80.

0. rhinoceros larvae were inoculated by dipping in the spore suspensions for 30 sec. Inoculated larvae were transferred into plastic containers of 37 cm x 26.5 cm x 10.5 cm (length x width x height), containing a sterilized mixture of 1.5 kg (50%-60% moisture content) cowdung, 0.5 kg (dry weight) frond fibre and 0.5 kg (dry weight) chipped frond of oil palms. The fibre and chipped frond were soaked in distilled water for 30 min before use. All containers were kept at 25°C ± 5°C. To maintain the moisture content in the containers, 20 ml of sterilized distilled water were sprayed onto the surface of the mixture daily. One hundred larvae of *0. rhinoceros* were used to bioassay each isolate *of M. anisopliae.* The larvae were divided into

TABLE 1. SOURCES AND DIMENSIONS OF SPORES OF *Metarhizium anisopliae* ISOLATES USED IN THE STUDY

Isolate	Origin	Host	Length of spore (µm)
Bp	UKM	Soil	12 - 14
Mo	UKM	Unknown	12 - 14
Ma	T. Merbau, Sepang.	0. *rhinoceros* (Adult)	6 - 8
Ml	T. Merbau, Sepang.	0. *rhinoceros* (Larvae)	6 - 8

Note: UKM = Universiti Kebangsaan Malaysia, Bangi, Selangor.

five containers (20 per container) prepared as above. The first three containers were allocated third instars (L3), and the fourth and fifth containers second instars (L2) and first instars (L1), respectively. The numbers of dead larvae were recorded daily until one of the treatments achieved 100% mortality. All dead larvae *were* collected for confirmation of *Metarhizium* infection.

Confirmation of infection. The dead larvae were washed with distilled water to remove soil and air dried for 5 min. They were then surface sterilized with 0.3% (v/v> sodium hyphoclorite, followed by double rinsing with sterilized distilled water before being air dried again for 10 min. Ten to 15 larvae were transferred into a transparent plastic container of 17.5 cm x 12.0 cm x 6.5 cm (length x width xheight) and kept at 25°C ± 5°C. Wetted paper towel (Scott paper, Malaysia) was placed at the bottom of the containers. This was to maintain high humidity in the containers, so that mycosis can develop. The numbers of larvae with mycosis symptoms were recorded daily.

Assessment of *M. anisopliae* in Field

Sites and experimental design. A field experiment was conducted in December 1996, in an oil palm to oil palm replanting area with seven-month-old heaps of chipped oil palm trunks at Jenderata Estate, United Plantations Bhd., Teluk Intan. The experiment was a randomized complete block design (RCBD). The heaps were divided into four blocks in a row. Each block contained six plots of 10.0 m x 2.0 m (length x width) or 20 m^2. Between blocks and within plots were separated by removing the heaps at 2.0 m and 1.0 m, respectively. Plots were equally divided into 20 subplots, giving each subplot an area of a square metre. In each plot, four subplots (4 m^2) were sampled each month, with the number of live and dead larvae, pupae and adults recorded by sifting through the oil palm heaps. The percentage mortality and population of 0. *rhinoceros* were estimated using this approach.

Production of inoculum. *Metarhizium* inoculum was produced on semi-ground maize. Maize (200 g) was soaked in water for 15-20 min, air dried for 5 min and transferred into a poly propylene bag of 356 mm x 229 mm (length x width). The filled bags were autoclaved at 121°C for 20 min before inoculation with 3 ml *Metarhizium* conidia (10^6 conidia ml-l). The inoculated bags were maintained at 25°C in complete darkness. Two- to three-week-old inoculum was used in this experiment.

Fungal inoculum and delivery methods, Fungal conidia were applied as wet and dry forms ofinoculum. For wet application, inoculum was harvested by thoroughly mixing well-sporulated bags with 250 ml 0.2% Tween 80 solution. Spore suspensions were filtered through a 250 μm sieve before adjusting to the desired concentrations for stock solution. The stock inoculum was kept in a refrigerator at 5°C before the experiment. Fungal inoculum was delivered by drenching using a hydraulic sprayer (Solo 475, Germany) equipped with a 5/64-inch fan nozzle. Each experimental plot was drenched separately with 10 litres ofwater that was added with 10^8 (Dch-10^8), 10^9 (Dch-10^9) and 10^{10} (Dch-10^{10}) conidia. Water used was from the field drains. For dry inoculum, plots were broadcasted with inoculated maize at 3 kg plot-l (Brd-3 kg) and 6 kg $plot^{-1}$ (Brd-6 kg). Only a single application was applied.

Density of viable inoculum in soil. The density ofviable inoculum was estimated monthly for three months after treatment. A 100 g ofsoil mixture (topsoil with some decayed oil palm tissues) was sampled from subplots recorded. The samples were mixed thoroughly and placed individually in plastic vials. One gramme was placed in a screw capped universal bottle, 10 ml of sterilized distilled water added, and then vortexed for 1 min at 8 revolutions sec^{-1}. The soil aliquot was diluted 10 times with sterilized distilled water. Three hundred microlitres of the aliquot were streaked onto 90 mm selective medium (modified from Mohan *et al.*, 1982) plates using a sterilized 'L' shaped glass rod. The medium compositions were peptone, 1.00 g (Difco); yeast extract, 0.50 g (Difco); K_2HPO_4, 0.58 g (Univar); KH_2PO_4, 1.00 g (Hamburg Chemical GmbH); $MgSO_4.7H_2O$, 1.00 g (BDH); rose' bengal, 0.07 g (Sigma); chloramphenicol,

0.20 g (Sigma); mycological agar, 25.50 g (Difco) anddistilled water, 1 litre. The pH ofthe medium was adjusted to 6.4. The medium was then autoclaved at 121°C for 20 min. Before pouring, an antibiotic mixture of cycloheximide, 0.20 g (Sigma); streptomycin sulphate, 0.10 g (Sigma); chlorotetracycline, 0.05 g (Sigma), and hexadecyl , tri-methyl ammonium bromide (CTAB), 0.19 g (ICN) was incorporated into the medium. Plates were incubated at 28°C ± 1°C. The density of viable spores was estimated in numbers of colony forming units (cfu) at 7-10 days after incubation.

Effects of water on spore viability. To study the above effect, samples of water were collected from field drains in the experimental area, a river and an ex-tin mining pool. The pH was determined and 10 ml of unsterilized water was used to adjust spore suspensions to a final concentration of 10^6 conidia ml^{-1}. For the control, sterilized distilled water was used. Two hundred microlitres of each sample were streaked onto four plates of potato dextrose agar (PDA) containing 0.02% antibiotic (chloramphenicol). Spore germination was estimated after 18 hr incubation at 28°C ± 1°C. In each plate, 15 observations were made, with more than 100 eonidia per observation. This gave a total counted conidia of more than 1500 per water sample. The spore viability was estimated at 3, 24 and 48 hr after preparation.

Data analysis. The percentage cumulative mortality, percentage infected larvae in the laboratory assessment, percentage mortality in field experiment, and percentage germination of conidia and densities ofthe viable spores were angular-transformed before analysis by PROC GLM (SAS, 1991). Data on field populations of rhinoceros beetles (adults, pupae and all larval stages) were analysed without transformation. The lethal time 50% (LT_{50}) values were probit analysed based on percent mycosis following the method of Finley (1971) and Wigley and Kalmakoff (1977). The means for all parameters were compared by Duncan's multiple range test at P=0.05 (SAS, 1991). Correlations between spore density and mortality, and total population of *Oryctes* were carried out using SigmaSTAT software (SigmaSTAT, 1995).

RESULTS

Assessment of *M. anisopliae* in Laboratory

The percentage cumulative mortality of *O. rhinoceros* larvae treated with 10^8 eonidia ml^{-1} is shown in ***Table*** 2. At 8 to 12 days after treatment (DAT), all isolates caused significantly higher ($P<0.05$) mortality than the control. At this stage, the long-spored isolates caused significantly higher ($P<0.05$) mortality than short-spored isolates. Bp caused 100%

TABLE 2. CUMULATIVE PERCENTAGE MORTALITY OF LARVAE OF *Oryctes* rhinoceros AFTER TREATMENT WITH FOUR ISOLATES OF *Metarhizium anisopliae*

Isolate	Cumulative percentage mortality (Mean ± SE)		
	4 DAT	8 DAT	12 DAT
Control	3.0 ± 1.2 a	7.0 ± 2.6 a	10.0 ± 1.6 a
Bp	22.0 ± 8.6 bc	53.0 ± 13.4 b	100.0 ± 0.0 c
MO	12.0 ± 6.4 ab	64.0 4 11.2 b	97.0 ± 2.0 a
Ma	22.0 ± 2.6 c	56.0 ± 4.3 b	70.0 ± 3.2 b
Ml	25.0 ± 7.2 c	51.0 ± 11.3 b	68.3 f14.4 b

Notes: means in columns with the same letter are not significantly different by Duncan's multiple range test (P>0.05).

DAT = days after treatment, SE = standard error.

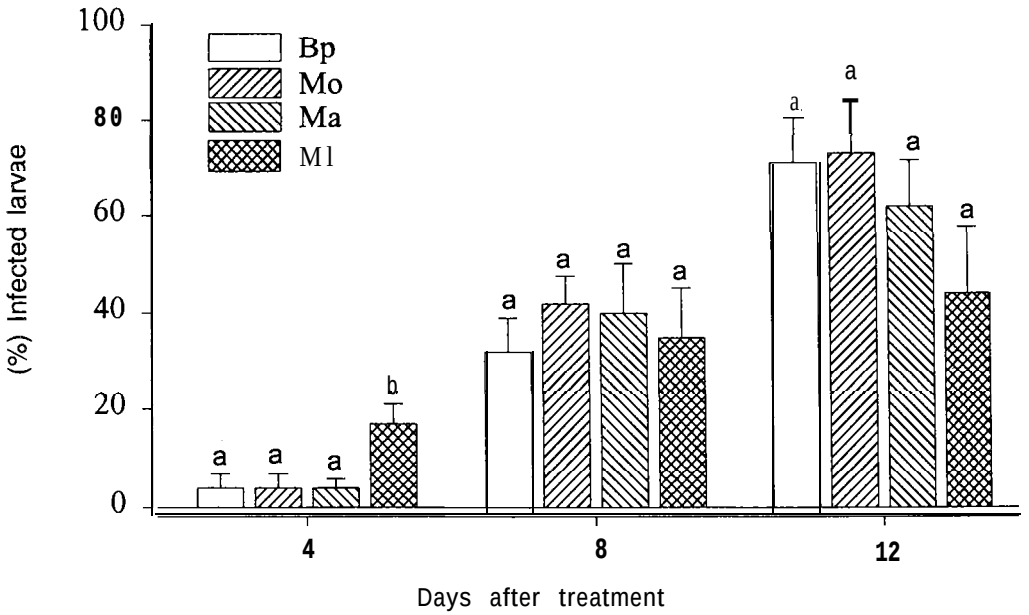

Figure 1. Percentage of infected larvae of Oryctes rhinoceros *after exposure to different isolates of* Metarhizium anisopliae. *Bars with the same letter are not significantly different (P>0.05) by Duncan's multiple range test.*

mortality 12 DAT, followed by MO at 14 DAT. The percentages of infected larvae with *M. anisopliae* at 4, 8 and 12 DAT are shown in *Figure 1*. At 12 DAT, MO (75%) and Bp (71%) caused slightly higher infection than Ma (62%) and Ml (44%). No infected larvae were recorded in the control treatment.

Most of the dead larvae produced brownish lesions before death. But in a few cases the dead larvae did not showed any symptoms. Such larvae hardened two to three days after death before turning whitish. One to two days later, whitish mycelia emerged around the spiracles at the thoracic and abdominal regions. At these regions, mycelia were also found on the intersegmental membranes. Soon after that, the cadavers became covered with mycelium. Sporulation on the cadavers was normally observed after complete colonization by the mycelium. At this stage, the cadavers became yellowish green before finally turning into a dark green,

Table 3 estimates the $ET_{,,}$ values for the isolates of *Metarhizium* against the larvae of *Oryctes*. The $LT_{,,}$ values for Bp and MO were about the same – 9.1 and 8.9 days. The values

TABLE 3. PATHOGENICITY OF FOUR ISOLATES OF *Metarhizium anisopliae* AGAINST THE LARVAE OF *Oryctes rhinoceros* BEETLE

Isolate	LT_{50} (Fiducial limits) (Days)	Regression	Chi-square value	S.E slope
Bp	*9.1 (8.6 - 9.7)*	*Y = 0.11 +* 5.32x	5.67*	*0.0640*
Mo	*8.9 (8.4 - 9.4)*	*Y =* 0.01 + 5.17x	0.15*	*0.0640*
Ma	10.0 (9.3 - 10.8)	*Y = 0.72 +* 4.28x	*0.80"*	*0.0633*
Ml	14.0(10.4 -18.8)	Y = 3.01 + 1.68x	0.29*	0.0589

Notes: y=log of days, x=empirical probit of mortality.

* Regression significantly representing the experimental results at P=0.05, df=3 (x2=7.81).

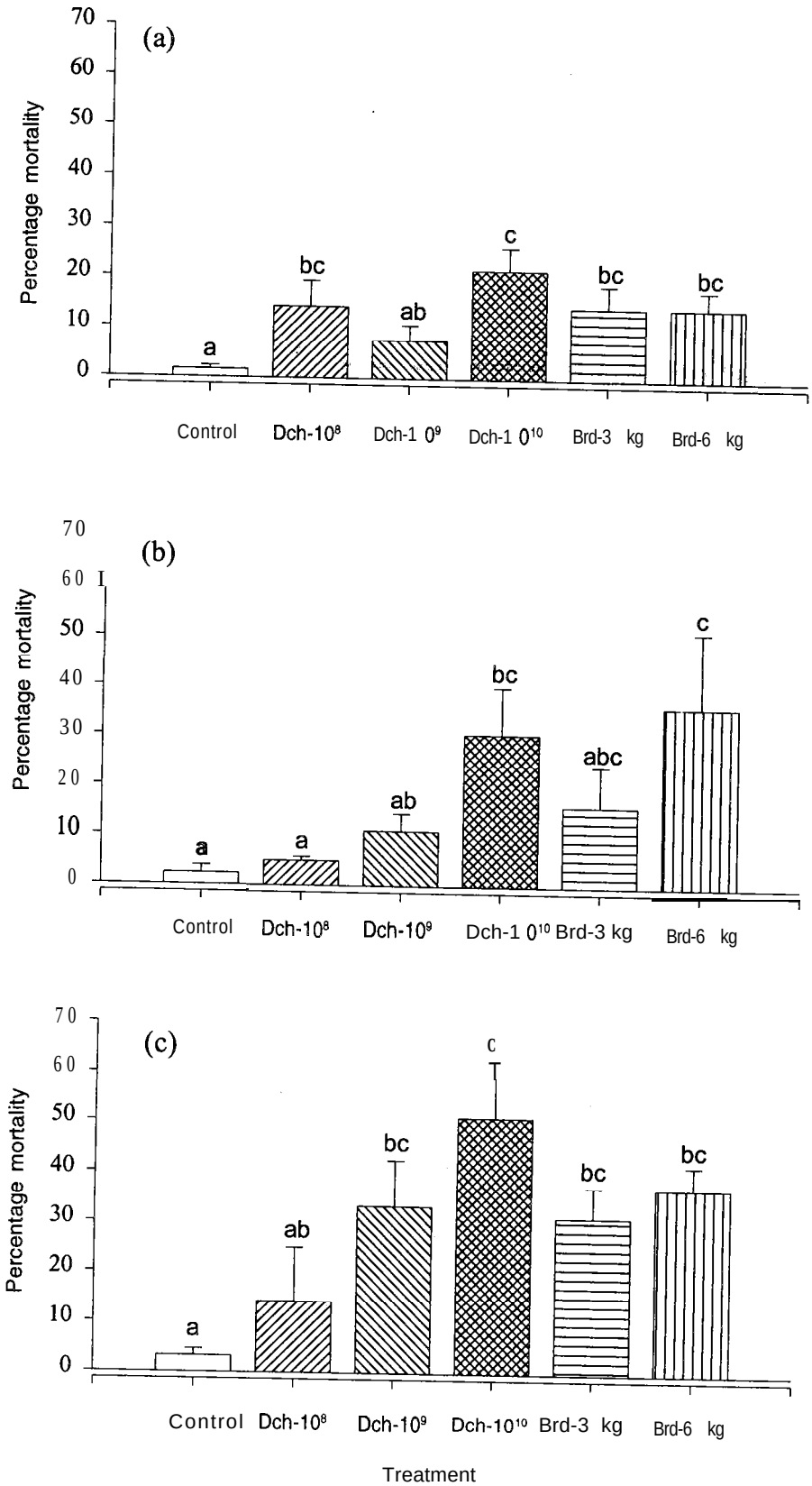

Figure 2. Percentage mortality ***of*** Oryctes rhinoceros *after exposure to* Metarhizium anisopliae *at (a) 1 MAT, (b) 2 MAT and (c) 3 MAT. Bars with the same letter are not significantly different (P>0.05) by Duncan's multiple range test.*

for Ma (10.0 days) and Ml (14.0 days) were slightly longer. From the mortality rates (regression slopes), Bp (regression slope = 5.32) was slightly more virulent than Mo (regression slope = 5.17). Therefore, Bp was selected for the field experiment.

Assessment of *M. anisopliae* in Field

The effects *0% M. anisopliae* on total mortality (larvae, pupae and adults) of rhinoceros beetle could be seen as early as one month after treatment *(Figure 2a)*. At two-month after treatment (MAT), mortality with drenching (30.8%, N = 216) and broadcasting (36.6%, N = 270) were significantly higher ($P<0.05$) at the highest application rate than in the control (2.3%, N = *240) (Figure 2b)*. However, there was no increased mortality ($P>0.05$) from drenching with 10^8 and 10^9 conidia plot-l, and broadcasting at 3 kg plot-l. At three MAT, mortality in all the treatments was higher than the control, except for wet at 10^8 conidia $plot^{-1}$ *(Figure 2c)*. *The* mortality in drenching at 10^{10} conidia $plot^{-1}$ and

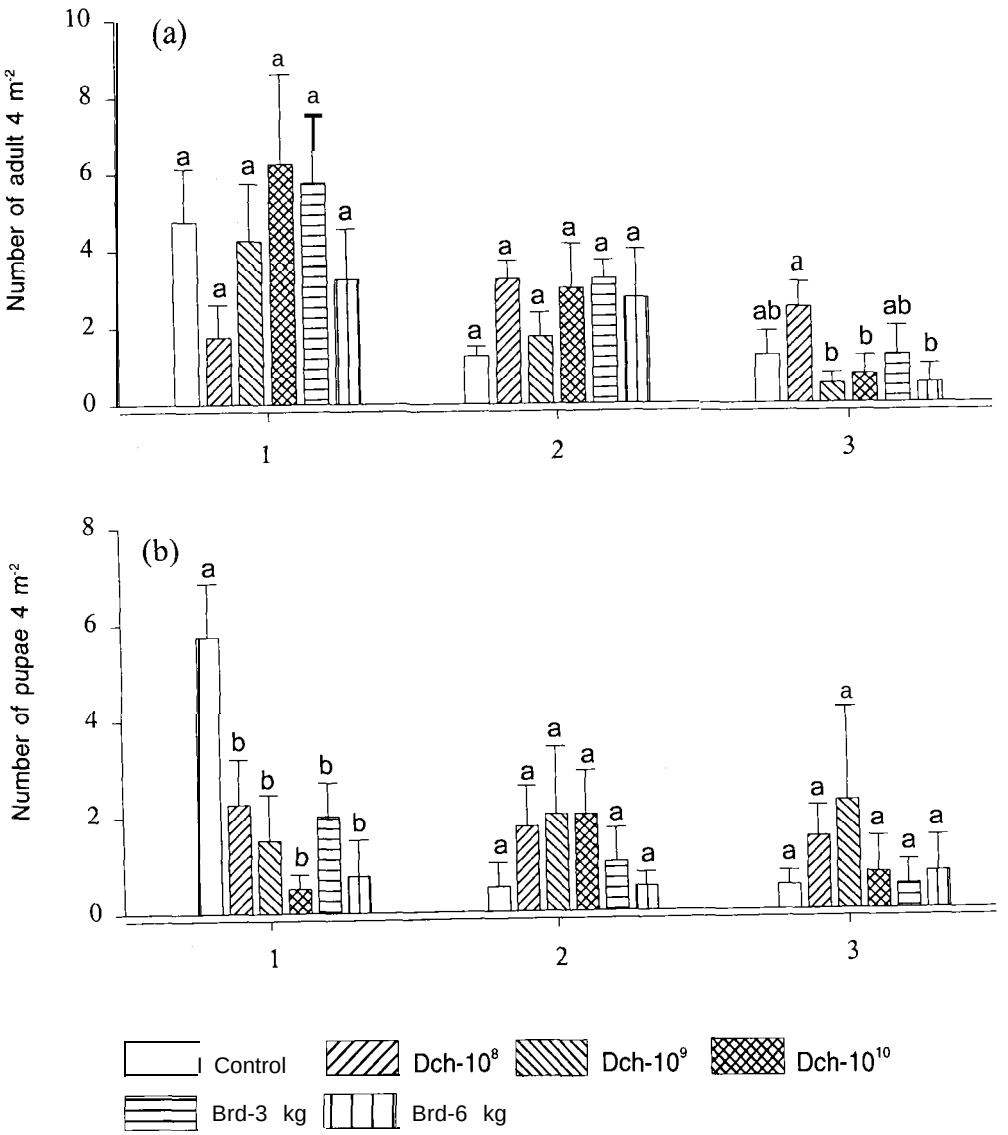

Figure 3. Effects of Metarhizium anisopliae *on population of (a) adults and (b) pupae of* Oryctes rhinoceros. *Bars with the same letter are not significantly different (P>0.05) by Duncan's multiple range test.*

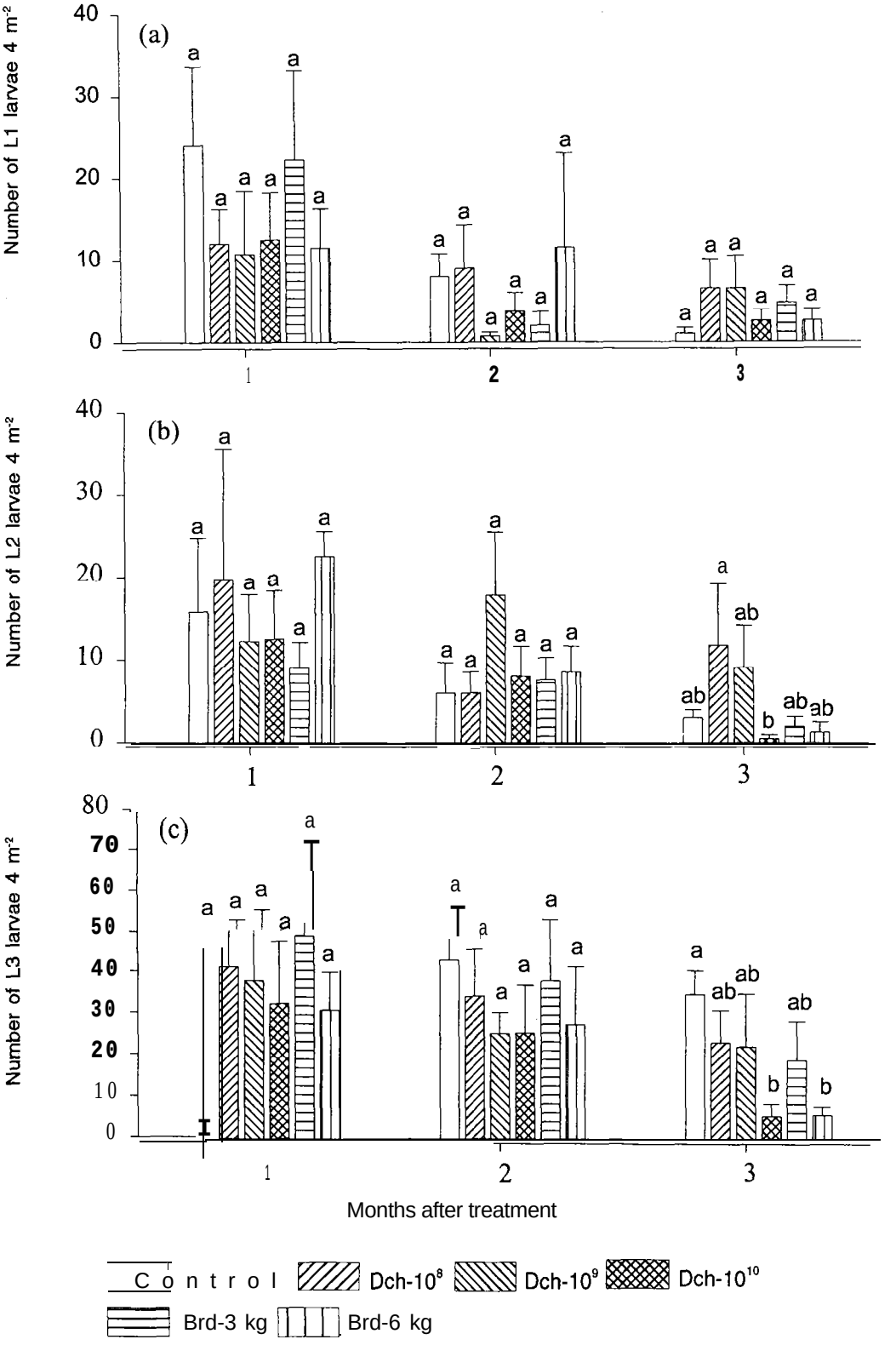

Figure 4. *Effects of* Metarhizium anisopliae *on different larval stages of* Oryctes rhinoceros. *(a) L1, (b) L2 and (c) L3. Bars with the same letter are not significantly different (P>0.05) by Duncan's multiple range test.*

broadcasting at 6 kg $plot^{-1}$ was 51.0% (N = 72) and 37.5% (N = 72), respectively. The mortality was slightly higher (P>0.05) than drenching at 10^9 conidia plot-' (33.4%, N = 215) and broadcasting at 3 kg plot-l (31.4%, N = 156).

Figure 3 estimates the impact of *M. anisopliae* on the adult and pupal stages of the beetle. In general, *Metarhizium* reduced the adult *(Figure 3a)* and pupal *(Figure 3b)* populations, but the trends were inconsistent. This resulted in no overall difference (P>0.05) in the treated plots from the control. The effects of *Metarhizium* on L1 *(Figure 4a)* and L2 *(Figure 4b)* larvae were similar to those for adults and pupae. The greatest effect was on L3 larvae, especially at three MAT *(Figure 4c)*. The populations of L3 larvae after drenching at 10^{10} conidia $plot^{-1}$ and broadcasting at 6 kg plot-l were significantly lower (P<0.05) than that of the control. The average number of larvae per sampling plot-" ($4m^2$) for both treatments was similar (5.5 - 5.8 larvae), and significantly lower (P<0.05) than that of the control (34.8 larvae $4m^{-2}$).

The densities of viable spores in the experimental plots are shown in *Table 4*. At two MAT, the spore densities in drenching and broadcasting at the highest rates were significantly (P<0.05) higher than in the control and other treatments. At three MAT, spore densities in drenching at 10^{10} conidia $plot^{-1}$ and broadcasting at 6 kg $plot^{-1}$ had substantially increased (P<0.05) to averages of 158.6 cfu and 169.0 cfu, respectively. A highly positive correlation (r^2 = 0.650, P<0.001, N = 69) was obtained between spore density and *Oryctes* mortality *(Figure* 5). Similarly, a highly significant negative correlation (r^2 = 0.437, P<0.001, N = 69) was obtained for spore density and *Oryctes* population *(Figure 6)*.

Germination of *M. anisopliae* spores prepared with water sampled from the various sources is shown in *Figure* 7. The water from field drain (pH = 5.37) was more acidic than the other sources - pH of 6.31 for both ex-tin mining pool and river water-while the pH of the control (distilled water) was 6.37. At three hours after preparation (HAP), germination in all water samples were high at 93.0% - 95.0%. In general, there was a reduction in spore germination with increased incubation period. At 24 and 48 HAP, germination from the three sources of water was significantly (P<0.05) lower than in the control.

DISCUSSSION

Based on percentage mortality, long-spored isolates (Bp and Mo) killed larvae within the period reported by Latch (1976). Of 36 isolates, he found that long-spored isolates killed *Oryctes* larvae within 7-16 days, while in our study total mortality was recorded within 12-14 DAT (Table

TABLE 4. DENSITY OF VIABLE SPORES OF *Metarhizium anisopliae* IN PLOTS TREATED WITH WET AND DRIED INOCULUM

Treatments and rate of application/ plot	Colony forming unit (cfu) of *M. anisopliae* (Mean ± SE)		
	1 MAT	2 MAT	3 MAT
Control	0.9 ± 0.6 a	1.9 ± 0.4 a	3.8 ± 1.2 a
Drenching, 10^8 conidia	4.9 ± 2.3 a	1.6 ± 1.0 a	10.5 ± 3.0 a
Drenching, 10^9 conidia	7.4 ± 2.5 a	2.9 ± 0.9 a	23.8 ± 5.5 a
Drenching, 10^{10} conidia	21.5 ± 9.0 b	16.9 ± 5.5 b	158.6 ± 47.1 b
Broadcasting, 3 kg	2.3 ± 0.6 a	2.5 ± 1.0 a	17.5 ± 1.6 a
Broadcasting, 6 kg	4.8 ± 2.3 a	21.4 ± 9.7 b	169.0 ± 98.9 b

Notes: means in columns with the same letter are not significantly different by Duncan's multiple range test (P>0.05).

MAT = month after treatment, SE = standard error.

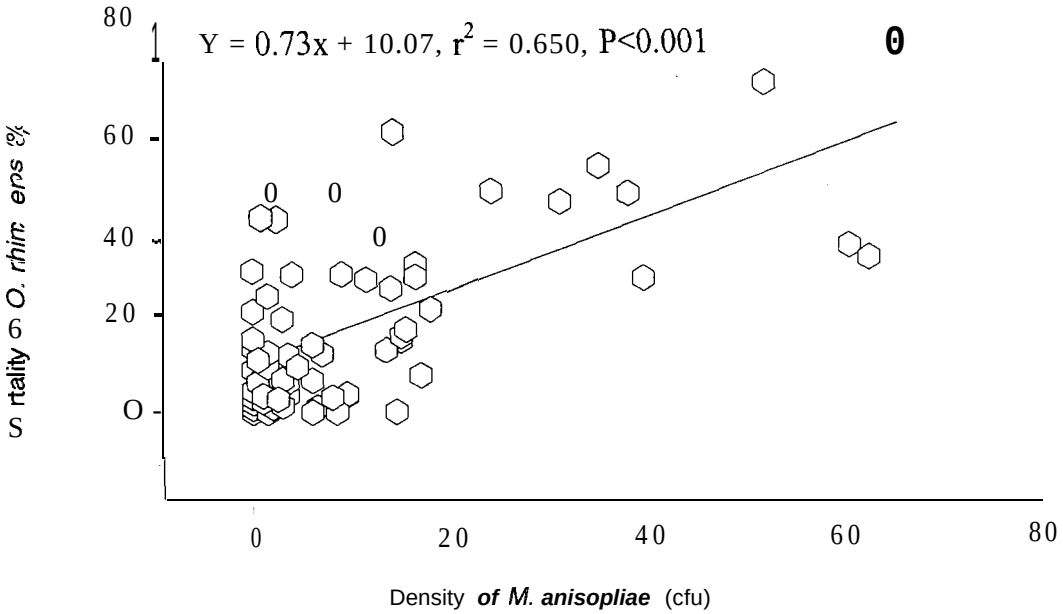

Figure 5. Relationship between density of viable spores ***of*** Metarhizium anisopliae *and mortality* ***of*** Oryctes rhinoceros *beetles.*

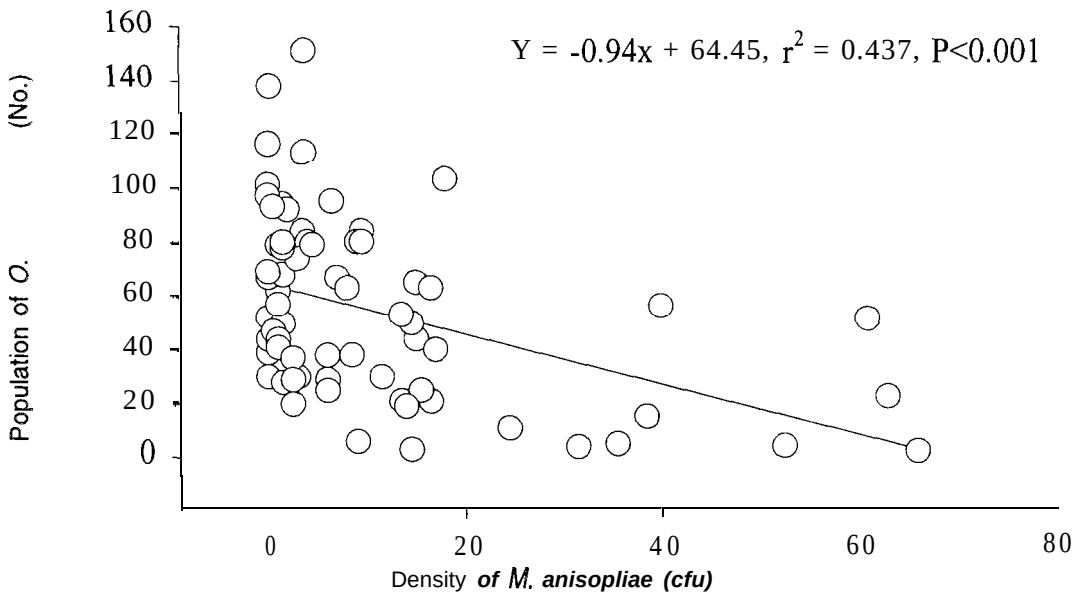

Figure 6. Relationship between density ***of*** *viable spores* ***of*** Metarhizium anisopliae *and population* ***of*** Oryctes rhinoceros *beetles.*

2). Similar studies were conducted by Latch (1976), Sivapragasam and Tey (1995), but the time taken for each isolate to kill 50% larvae (LT_{50}) was not estimated. The LT,, values for long-spored isolates were 9.1 days (Bp) and 8.9 days (MO), shorter than short-spored isolates, which were 10 days (Ma), and 14 days (Ml) (Table 4).

The percentage infections by isolates Bp and MO were 75% and 71%, respectively *(Figure I).* These were slightly higher than that reported by Sivapragasam and Tey (1995) who had only one out of three long-spored isolates causing over 70% infection. The higher infection in this study could have been due to the stage of larvae used as only L3 larvae were used by Sivapragasam and Tey (1995). This study used the early

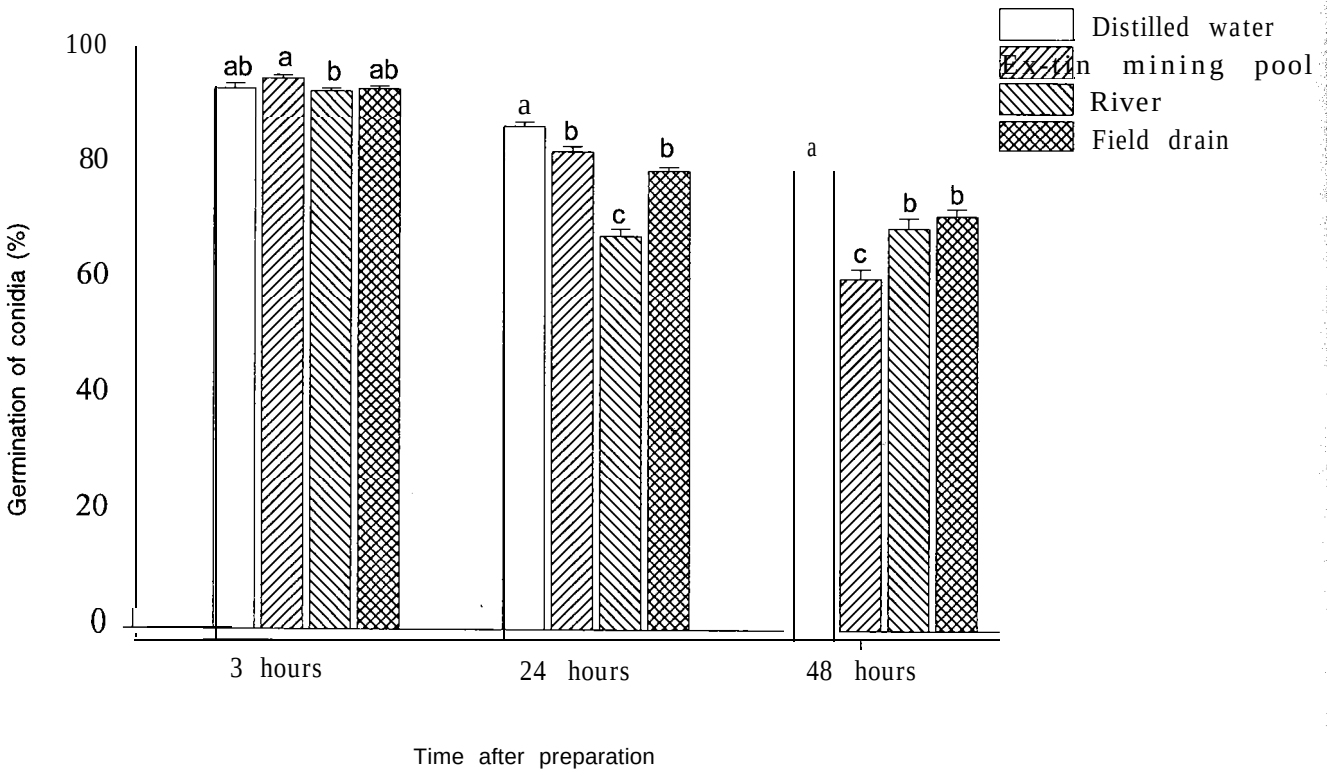

Figure 7. *Percentage germination* ***of*** Metarhizium anisopliae *prepared in water collected* ***from various*** *sources. Bars with the same letter are not significantly different (P>0.05) by Duncan's multiple range test.*

instars as well and the early instars are more susceptible than the later ones (Latch, 1976).

The infection of *Metarhizium* is normally via spores that adhere to the cuticle. Under suitable conditions, the spores germinate and penetrate the cuticle (St Leger *et al.,* 1991). At the site of penetration, brown or black lesions will appear as an early sign of mycosis (Charnley, 1992). In our study, lesions were scattered on the cuticle of the larvae and death usually ensured shortly thereafter. In a few cases, the larvae died without symptom, but were later covered by green spores of *M. anisopliae.* In such larvae, penetration by spores may have been through the gut. Although this remains uncertain, it has been reported on the subterranean termites, *Reticulitermes* sp., by Kramm and West (1982).

In the field trial, some infection by *Metarhizium* also occurred in the control plots, although the incidence was very low *(Figure 2).* Most of the infected *Oryctes* were adults and L3 larvae – they were, therefore, possible migrants from the neighbouring treated areas. In the treated plots, healthy adults from other areas or newly emerged adults may have got infected as they foraged for food, or bred and laid eggs. The adults could also have been infected as pupae as Latch (1976) found that all infected pupae successfully pupated, and that the emerging adults died within 9 to 15 days from inoculation. After death, the diseased adults became a secondary source of inoculum, infecting *Oryctes* breeding in the area. In our trial, migration of infected adults into the control plots is suspected because of the close proximity of the plots (2 m apart). This view is supported by the results of Latch and Falloon (1976) who found infected adults to have migrated to control plots as far as 300-400 m away.

With both wet and dry inoculum, larval mortality increased with application rate and time after treatment *(Figure 2).* At one MAT, larval mortality in all treatments, except drenching at 10^8 conidia plot-l, were between 14% to 21%. At two MAT, mortality in drenching at 10^{10} conidia $plot^{-1}$ and broadcasting at 6 kg $plot^{-1}$ were 30% and 35%, respectively. At three MAT, mortality with drenching at 10^{10} conidia $plot^{-1}$ was 51.0%, 13.5% higher than broadcasting at

6 kg plot^{-1} (Figure 2c). Within the same period, larval mortality in study by Tey and Ho (1995) was only 42.0%, 9.0% lower than in our drenching at 10^{10} conidia plot^{-1}, but 4.5% higher than in broadcasting at 6 kg plot-l. The estimated larval mortality by Tey and Ho (1995) was the total from monthly, two-month and three-month applications. They found that frequent application' of Metarhizium had resulted in a higher mortality.

The effects of wet and dry inoculum on adult *Oryctes* were varied and inconsistent amongst the plots. For example, at one and three MAT, the adult population in control was only slightly higher than in some of the treated plots, but the reverse was observed at two MAT (Figure 3a). Nevertheless, due to migration, the effect of *Metarhizium* on the adults was possibly underestimated. Inconsistent effects of Metarhizium were also observed in the pupae (Figure 3b). In our trial, infected pupae were hardly seen. Of 87 pupae from all treated plots, only one was infected by the fungus. The pupae develop deep in the soil and therefore generally escape direct contact with the spores or contaminated substrates. Even if the pupae were infected, the fungal infection may only appear after they turn adults (Latch, 1976).

L1 larvae exposed to wet and dry inoculum at all application rates were unaffected (Figure 4c). For L2 larvae, drenching *of Metarhizium* at 10^{10} conidia plot^{-1} and broadcasting at 6 kg plot-' reduced the population, although the differences with the control were not significant (Figure 4b). Similar results were reported by Latch and Falloon (1976), who found that most healthy larvae in the field to be L1 and L2 larvae. No explanation can be offered for this and research is required to unravel this mystery.

The greatest effect of *Metarhizium* was on L3 larvae, especially at three MAT, when application with both wet and dry forms at the highest rates reduced the population significantly *(Figure 4c)*. The bigger effect could be due to the length of the life stage. Bedford (1980) estimated the average duration for the L3 larvae to be 112.5 days, the longest developmental stage in the Oryctes life cycle. A longer life stage would give a longer infective period for the larvae. Similar requirements for survival and development for both the larvae and *Metarhizium* are possibly another factor that contributed to the higher ***effect*** of ***Metarhizium on*** the L3 population. The larvae prefer a high humidity, moderate temperature (27°C-29°C) and wet substrate (31% to 73% moisture content)(Bedford, 1980). Such conditions also enhance the longevity of conidia and increase the incidence of infection by the fungus (Madelin, 1963; Latch, 1976).

Latch and Falloon (1976) reported that spores *of Metarhizium* can survive in the breeding substrates for at least 24 months, but did not estimate the density of viable spores. In our study, at three MAT, the density of viable spores was significantly higher ($P<0.05$) in the wet and dried inoculum plots at the highest application rates than in the other treatments (*Table* 4). The higher densities of viable spores in both plots were believed hue to the large number of dead diseased insects acting as a secondary source offungal inoculum. Our results also showed that besides longevity, the density of viable spores in breeding substrates played an important role in the effective control of ***Oryctes.*** As expected, a significant positive correlation ($r^2 = 0.650$, $P<0.001$, N = 69) was found between spore density and ***Oryctes*** mortality *(Figure 5)*, and a negative correlation between spore density and *Oryctes* population ($r^2 = 0.437$, $P<0.001$, N = 69). This suggests that maintaining viable spores at a higher density will reduce the beetle population *(Figure 6)*.

This study shows that at the highest rates of application, *Metarhizium* in wet and dry inoculums was equally effective in controlling ***0. rhinoceros.*** Each inoculum has its advantages and disadvantages. Wet inoculum is easily prepared in water available in the field or from other sources such as rivers and ex-tin mining pools. Our test showed that water from all these sources can be used to prepare wet inoculum, but the inoculum has to be applied immediately before the spore viability declines *(Figure 7)*. Spores from fresh inoculum can possibly infect ***Oryctes*** larvae as soon as they are deposited in the breeding sites. The presence of water can facilitate deeper conidial distribution in soils and decayed oil palm tissues. This will increase the exposure of ***Oryctes*** larvae to *Metarhizium*. Furthermore, spores that distributed deep in the breeding materials are less exposed to

abiotic factors such as low humidity, high temperature and ultraviolet radiation (Zimmermann, 1982; Walstad et al., 1970; Moore et al., 1993) which reduce their viability.

Due to the substantial quantity of maize required to produce dry *Metarhizium* inoculum, wet inoculum may be preferable as less maize is required. Based on our production rate' of 4.4 x 10^{10} conidia 200 gof autoclaved maize, wet inoculum requires only 1.4 kg maize ha^{-1} (estimated area of chipped oil palm trunks is 600 m^2), while dry inoculum requires 180 kg. At a maize price RM 1.00 kg-l, the cost of application based on maize alone is RM 1.40 for wet inoculum versus RM 180.00 for dry inoculum. However, the main advantage of using dry inoculum is that it can be stored at room temperature for several months before use.

CONCLUSION

Our study has proved that *M. anisopliae* var. *major* or the so-called long-spored isolate is highly virulent against the larvae of *0. rhinoceros* beetle. In the field, this isolate could infect all stages of the pest, with particular effectiveness on L3 larvae. Both wet and dry inoculum by drenching were equally effective in controlling *Oryctes* in the field. At the highest application rate (drenching at 10^{10} conidia $plot^{-1}$ and broadcasting at 6 kg plot-l) both methods drastically reduced the L3 population. For wet inoculum, the amount of maize needed to treat one hectare is 1.4 kg (equal to 3.08 x 10^{11} conidia ha-l), and for dry inoculum 180 kg (equal to 3.96 x 10^{13} conidia ha-l). Therefore, this pathogen is more economically applied in wet form using water easily available in the field.

The results show that *M. anisopliae* is effective in reducing the larval population of the *Oryctes*. Further reduction in the population of *0. rhinoceros*, especially adults, can be achieved by combining *M. anisopliae* with *Baculovirus oryctes*. The virus will shorten the life span of adults and has proven effective in suppressing the beetle population in several Pacific regions (Zelazny *et al.*, 1992). Application of these two pathogens for *Oryctes* control is cheap, and environmentally friendly and helps reduce the over dependence on chemical insecticides. Therefore, further research on the subject is vital for long term sustainability of the sil palm industry.

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REFERENCES

BEDFORD, G O (1980). Biology, ecology, and control of palm rhinoceros beetles. *Annual Review of Entomology, 25:*309-339.

CHARNLEY, A K (1992). Mechanisms of fungal pathogenesis in insects with particular reference to locust. In Lomer, C J and Prior, C (eds) *Biological Control of Locust and Grasshoppers.* Proceedings of workshop held at the International Institute of Tropical Agriculture, Cotonou, Republic of Benin. April-May 1991. CAB International. Wallingfort. Oxon. p. 181-190.

ENVIRONMENTAL QUALITY (CLEAN AIR) REGULATIONS 1978. (ACT 127). ENVIRONMENTAL QUALITY ACT 1974.

FERRQN, P; ROBERT, P H and DEOTTE, A (1975). Susceptibility of *Oryctes rhinoceros* adults to *Metarhizium anisopliae. Journal of Invertebrate Pathology, 25:*335-356.

FINLEY, D J (1971). *Probit Analyses.* 3rd edition, Cambridge University, Press. England.

FRIEDERICHS, K (1920). Uber pleophagie des insektenpilzes, *Metarhizium anisopliae* (Metsch.). *Centralbl. Bakt. Parasit. Infektionskrankh., 2:*335-356.

GOETTEL, M S (1992). Fungal agents for

biocontrol. In Lomer, C J and Prior, C (eds) *Biological Control of Locust and Grasshoppers.* Proceedings of workshop held at the International Institute of Tropical Agriculture, Cotonou, Republic of Benin. CAB International. Wallingfort. Oxon. p. 122-132.

KRAMM, K R and WEST, D F (1982). Termite pathogens: effects of ingested *Metarhizium, Beauveria,* and *Gliocladium* conidia on worker termites *(Reticulitermes* sp.). *Journal of Invertebrate Pathology, 40:* 7-11.

LATCH, G C M (1976). Studies on the susceptibility *of Oryctes rhinoceros* to some entomogenous fungi. *Entomophaga, 21(1):*31-38.

LATCH, G C M and FALLOON, R E (1976). Studies the use of *Metarhizium anisopliae* to control *Oryctes rhinoceros. Entomophaga, 21(1): 39-48.*

LIAU, S S and AHMAD, A (1991). The control of *Oyctes rhinoceros* by clean clearing and its effect on early yield in palm to palm replants. *Proc. of the 1991 PORIM International Palm Oil Development Conference. Module II-Agriculture.* Palm Oil Research Institute of Malaysia, Bangi. p. 396-403.

MADELIN, M F (1963). Diseases caused by hyphomycetous fungi. In Steinhaus, E A (ed.) *Insects Pathology: An Advanced Treatise. New* York, Academic Press. p. 233-271.

MENDOZA, A F (1992). Mass production, application and formulation of *Metarhizium anisopliae* for control of sugarcane froghopper, *Mahanarva posticata* in Brazil. In Lomer, C J and Prior, *C* (eds.) *Biological Control of Locust and Grasshoppers,* CAB International. Wallingfort. Oxon. p. 239-244.

MOHAN, K S; PILLAI, G B; PEREIRA, J C R; DHINGRA, 0 D and CHAVES, G M (1982). A selective medium for isolation of *Metarhizium anisopliae* from cattle dung. *Transactions of the British Mycological Society, 78(1):*181-182.

MOORE, D; BRIDGE, P D; HIGGINS, P M; BATEMAN, R P and PRIOR, C (1993). Ultra violet radiation damage to *Metarhizium flavoviride* conidia and protection given by vegetable and mineral oils and chemical sunscreens. *Annual Applied Biology, 122: 605-*616.

NORMAN KAMARUDIN and MOHD BASRI WAHID (1995). Control methods for rhinoceros bettles, *Oryctes rhinoceros* (L) (Coleoptera: Scarabaeidae). *PORIM Occasional Paper No. 35:*30pp.

NORMAN KAMARUDIN and MOHD BASRI WAHID (1997). Status of rhinoceros beetles, *Oryctes rhinoceros* (Coleoptera: Scarabaeidae) as a pest of young oil palm in Malaysia. *The Planter, 73 (850):* 5-20.

PRIOR, C (1992). Discovery and characteristics of fungal pathogens for locust and grasshoppers control. In Lomer, C J and Prior, C (eds.) *Biological Control of Locust and Grasshoppers,* CAB International. Wallingfort. Oxon. p. 159-180.

ROMBACH, M C; HUMBER, R A and EVANS, H *C* (1987). *Metarhizium album,* a fungal pathogen of leaf and planthopper of *rice. Transactions of the British Mycological Society, 88(4):* 451-459.

SAMSUDIN, A; CHEW, P S and MOHD, M M (1993). *Oryctes rhinoceros:* breeding and damage on oil palms in an oil palm to oil palm replanting situation. *The Planter, 69(813): 583-591.*

SAS (1991). *SASISTAT User Guide Statistics, Version* 6, SAS Institute Inc., North Carolina.

SIGMASTAT (1995). *Jandel Scientific, Version* 2.0, Jandel Corporation.

SIVAPRAGASAM, A; ARIKIAH, A and RANJIT, CA (1990). The red striped weevil, *Rynchophorus schach* Oliver (Coleoptera: Curculionidae): an increasing menace to coconut palms in Hilir Perak. *The Planter, 66:*113-123.

SIVAPRAGASAM, A and TEY, C C (1995). Susceptibility of *Oryctes rhinoceros* (L.) larvae

to three isolates of *Metarhizium anisopliae* (Metsch.) Sorokin. *MAPPS Newsletter, 18(2):* 13-14.

ST. LEGER, R J; GOETTEL, M; ROBERTS D W and STAPLES, R C (1991). Prepenetration events during infection of host cuticle by *Metarhizium anisopliae. Journal of Invertebrate Pathology, 58:*168-179.

TEY, C C and HO, C T (1995). Infection of *Oryctes rhinoceros* (L) by application of *Metarhizium anisopliae* (Metsch.) Sorokin to breeding sites. *The Planter, 71(837):*563-567.

TULLOCH, M (1979). The genus of *Metarhizium. Transactions of the British Mycological Society, 66:*407-411.

VEEN, K H (1968). Researches sur la maladie, due a *Metar(r)hizium anisopliae* chez le criquet pelerin. Thesis. H. Veenman and N. V. Zonen. Wageningen.

WALSTAD, J D; ANDERSON, R F and STAMBAUGH, W J (1970). Effects of environmental conditions on two species of muscardine fungi *(Beauveria bassiana* and *Metarhizium anisopliae). Journal of Invertebrate Pathology, 16:*221-226.

WIGLEY, P J and KALMAKOFF, J (1977). Practical assessment of dose mortality data bioassay of NPV to *Heliothis armigera.* In UNESCO/UNEP/ICRO (eds.) *Microbial Control of Insects Pests, A Record of Lectures and Practical Classes for Regional Training Course.* Dunedin, New Zealand, August 1977. p.39-42.

ZELAZNY, B; LOLONG, A and PATTANG, B (1992). *Oryctes rhinoceros* (Coleoptera: Scarabaeidae) populations suppressed by a baculovirus. *Journal of Invertebrate Pathology, 59:*61-68.

ZIMMERMANN, G (1982). Effects of high temperature and artificial sunlight on the viability of conidia of *Metarhizium anisopliae. Journal of Invertebrate Pathology, 40:*36-40.