PATHOGENICITY OF GRANULE FORMULATIONS OF *Metarhizium anisopliae* AGAINST THE LARVAE OF THE OIL PALM RHINOCEROS BEETLE, *Oryctes rhinoceros* (L.)

RAMLE MOSLIM*; NORMAN KAMARUDIN* and MOHD BASRI WAHID*

ABSTRACT

Granule formulations consisting of mycelia and spores of Metarhizium anisopliae *var.* major *as the active ingredient were produced and tested against the larvae of* Oryctes rhinoceros. *The effect of the medium pH on the production of mycelia was investigated, and the granule compositions were optimized. The fungus produced higher yields of mycelial pellets (0.58 g) at pH=8 as compared to pH=5, 6 or 7. Granules prepared from mycelia with the growing medium (G+MM) improved fungal growth (100%) and sporulation (87.2%) as compared to granules prepared from the mycelia alone (G+M) (growth and sporulation, 62.4% and 47.6%, respectively). The amounts of ingredients, such as kaolin and rice bran used in making the granules, were then optimized. The weight of granules increased as the amount of kaolin and rice bran increased, but granule quality was reduced. The highest quality granules (with growth 98.5%, sporulation 88.6% and dry weight 1249 g) were prepared with 925 g kaolin and 400 g rice bran. The pathogenicity of the G+MM granules was tested against the third instar larvae of* O. rhinoceros. *The test showed that at 20 days after treatment (DAT), treatment with rates of 1.0 g and 2.0 g granules/box caused 90% mortality, which was as high as with the treatment using pure spore solutions (96%). The G+MM granules produced more spores and more quickly than granules made from spores (G+Sp). Both types of granules produced 0.42-6.60 x* 10^6 *spores/granule. Results of the bioassay indicated that application of G+MM and G+Sp at rates of 3 g, 6 g and 9 g killed 100% the third instar larvae as early as at 18 DAT. Infection level increased as the application rate increased. G+MM and G+Sp applied at 9 g/box caused the highest infection of 93.3% in the larvae. The potential use of the granule formulation to control* O. rhinoceros *in the field was also discussed.*

Keywords: *Metarhizium anisopliae*, oil palm pest, *Oryctes rhinoceros*, alginate granule formulation, biological control.

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INTRODUCTION

The entomopathogenic fungus *Metarhizium anisopliae* (Metcsh.) Sorokin has been intensively studied to control a wide species of insect pests, including the oil palm rhinoceros beetle, *Oryctes rhinoceros*. *M. anisopliae* var. *major* has proven to be highly pathogenic against the beetle, killing 100% of the larvae of the beetle as early as 14 days after treatment (DAT) (Sivapragasam and Tey, 1995; Ramle *et al.*, 1999). In the early establishment stage of oil palm, the larvae develop in the rotting oil palm residues that are left to rot naturally in the fields. Application of the fungus by spraying of spore solutions and broadcasting of sporulation media into the habitat has significantly reduced the larval population (Ramle *et al.*, 1999). The fungus was then mass produced and formulated into a powder form, which extended the product life span, made

* Malaysian Palm Oil Board,
P. O. Box 10620,
50720 Kuala Lumpur,
Malaysia.
E-mail: ramle@mpob.gov.my

it easy to handle, store and apply in the field (Noor Hisham *et al.*, 2005; Ramle *et al.*, 2006). A field trial on the application of the product onto the rotting oil palm residues significantly reduced not only the larvae, but also the pre-pupae and pupae (Ramle *et al.*, 2007).

For the application of the powder formulation, it must be mixed with water to form spore solutions. Application of this formulation is hence restricted in hilly areas where a water source is limited. As an alternative, the potential use of a granule formulation for controlling the pest was studied. The granule formulation of *M. anisopliae* has been used to control the black vine weevil, *Otiorhynchus sulcatus*, an insect that has a similar life cycle to *O. rhinoceros*, which spends a part of its life cycle in the soil. In a previous study, the granules were developed from pure dehydrated mycelial pellets (Andersch *et al.*, 1990; Andersch, 1992; Stenzel, 1992). In this study, the fungus was formulated in an alginate granule formulation as this is more resistant to high temperature and low humidity. In other fungi, the alginate granules have been successfully used to control a carrot disease caused by *Rhizoctonia solani* (Fravel *et al.*, 1985; Lewis and Papavizas, 1987). The alginate granules can be prepared from mycelia, spores or mixtures of the two (Fravel *et al.*, 1995).

In this study, the preparation and the pathogenicity of the alginate granule formulation against the larvae of the *O. rhinoceros* beetle are described. The granules were made by incorporating a nutrient carrier to provide the food base, necessary to initiate growth which later promoted sporulation (Shaban and El-komy, 2000). In the field, as the granules absorbed water, the mycelia will grow and then produce infectious spores, contaminating the surroundings and later infecting healthy larvae or adults, *O. rhinoceros*.

MATERIALS AND METHODS

Source of Fungal Isolate

The fungus *M. anisopliae* var. *major* was isolated from field-infected larvae using *Metarhizium* Selective Media (Ramle *et al.*, 1999). To maintain a high fungal pathogenicity level, a single spore isolation method was performed. The pure single spore cultures were maintained on potato dextrose agar supplemented with 0.02% choramphenicol to inhibit bacterial growth. The culture plates were then incubated at 28°C for 30 days. Spore suspensions were prepared by adding to the plates 10 ml of distilled water plus 0.02% Tween 80. The spores were separated from the media by scraping them off the media surface using an L-shaped inoculation needle. The spore mixture was then filtered through glass fibres into a 25-ml universal bottle. The concentration of the spore suspension was then determined by a hemocytometer, and then diluted to a final concentration of 10^7 spores per ml.

Preparation of a Liquid Medium for Propagation of Mycelia

Mycelia of *M. anisopliae* were propagated in a liquid medium containing 2% (w/v) dextrose, 0.25% (w/v) peptone and 1% (w/v) yeast extract. Chloramphenicol at 0.02% (w/v) was added into the medium to inhibit the growth of bacteria. Liquid medium (500 ml) were then placed in a 1 litre conical flask, sterilized at 121°C for 20 min, and inoculated by adding 3 ml of spore suspension (10^7 spores per ml) as prepared previously. The flasks were then incubated in a refrigerated orbital shaker at 28°C and shaken at 166 rpm for four consecutive days.

Basic Method for the Preparation of Alginate Granules

An alginate solution was first made by dissolving 10 g alginic acid (sodium salt, Sigma) in 20 ml absolute alcohol. Once the homogenous alginate solution was formed, it was then poured into a 1.5-litre beaker filled with 1-litre sterilized distilled water (ddH_2O). The solution was mixed until the alginic acid was completely dissolved in the water. The process of preparing the fungal mycelia can then be initiated. In a 1-litre flask, the mycelia were broken into small fragments by directly homogenizing them in a high speed homogenizer at 1000 rpm for 30 s. The homogenated mycelia were then poured into the 1 litre alginate solution and mixed with a magnetic stirrer until homogenous. Kaolin (200 g w/v, particle size 1-40 μ, moisture content < 5%) and rice bran (200 g w/v) were then slowly added into the mixture. Prior to that, the kaolin and rice bran were sterilized at 121°C for 20 min. The mixture was then slowly mixed using a top-headed stirrer machine until a homogenous mixture was formed.

The granulation process was manually performed by using a 10 ml micropipette with a tip. The mixture was gently dropped into a 3 M calcium chloride ($CaCl_2$) solution. Round-shaped granules were formed immediately as the drops submerged in the solution. These granules were then collected and rinsed by transferring them into distilled water in a container for about 2-3 min; they were then dried in a laminar flow cabinet at a temperature of 28°C for 24 hr. The granule size depended on the size of the orifice of the pipette tip. Adjustments can be made by cutting the tip to get the desired granule size. In this study, the size of the dried granules ranged from 1 mm to 3 mm (*Figure 1*). The weight of the dried granules and their quality were also determined.

Figure 1. The alginate granule formulation of M. anisopliae *containing rice bran as a base nutrient source and kaolin as an inert material.*

Determination of Granule Quality

Granule quality was determined at two granule sizes: small (diameter 1.5-2.0 mm) and big (diameter 2.0-2.5 mm). For each size, the granules were individually placed inside a 96-well ELISA plate. Two plates were used for each size, giving four plates for each preparation. Each granule was wetted by adding 40 µl ddH_2O and the plates were wrapped using a thin plastic film, then placed in an incubator at 28°C for 10-14 days. The number of granules observed with growth of whitish hyphae and the number of granules producing spores were counted. Good granule quality was identified when a high number of granules were producing spores of *M. anisopliae*.

The number of spores produced on each granule was determined by placing a sporulated granule into a 1.5-ml microtube containing 1.0 ml ddH_2O plus 0.02% Tween 80. The tubes were vigorously shaken to separate the spores from the granule. The number of spores was then estimated using the Neubauer hemocytometer. Twenty granules were used in the estimation.

Experiment 1: Effects of pH on the Production of Mycelia

The effects of the medium pH on the production of *M. anisopliae* mycelia were determined. Preparation of the medium followed the previously described method. Four pH values, pH 5, 6, 7 and 8, were tested. The pH 5 medium was prepared in four flasks and the pH 6, 7 and 8 media were prepared in three flasks, respectively. Each flask contained 250 ml of the liquid medium. The pH of the medium was adjusted as soon as all the ingredients were completely dissolved in the water. The flasks were then closed by wrapping up the mouths with aluminum foil and sealing with autoclavable tape. The flasks were then sterilized by autoclaving at 121°C for 20 min. The effect of pH on the growth of the fungus was estimated from the yield of mycelia. Harvesting fungal mycelia was conducted by vacuum filtration through a cotton cloth. The wet mycelial mats were peeled from the cloth and oven-dried at 50°C overnight. The mycelia dry weight was then recorded.

Experiment 2: Quality of Granules Prepared Using Mycelia Alone and Mycelia with the Growing Medium

Two types of granules were prepared: granules made from only the mycelia (G+M) and granules made from the mycelia with the growing medium (G+MM). The G+MM granules were prepared by the process described above for the basic preparation of the granules. For G+M, granules were prepared from the harvested mycelia using the vacuum filtration method. Two rates (wet weight) of mycelia, 13.6 g and 16.2 g, respectively, were used to make the granules. The wet mycelial mat was transferred into a 1.5-litre beaker filled with 1 litre of 2% (w/v) sucrose solution. The mycelia were then homogenized using a high-speed homogenizer. Once the homogenous mycelial solution was formed, alginic acid solution (10 g dissolved in 20 ml alcohol) was added and mixing was continued until homogenous. A total of 200 g kaolin and 200 g rice bran was then added and again mixed until homogenous. Granules were then produced by releasing drops of the mixture into a 3M $CaCl_2$ solution, rinsed in ddH_2O and dried at 28°C for 24 hr.

The quality (based on percentages of mycelial growth and sporulation) and the yield of both granule types from three preparations were compared. In each preparation, the percentages of growth and sporulation of granules were determined

for two sizes of granules: small (diameter 1.5-2.0 mm) and big (diameter 2.0-2.5 mm), following the method described previously.

Experiment 3: Optimization of Granule Ingredients

The granules were prepared from four-day-old mycelia grown in 500 ml liquid medium. Six recipes were evaluated to determine the optimum composition of the granule ingredients. Each recipe contained different volumes of distilled water and different amounts of kaolin and rice bran (*Table 1*).

Recipe 1 was prepared five times and the others three times. For each preparation, the percentages of mycelial growth and sporulation were determined from two granule sizes: small (diameter 1.5-2.0 mm) and big (diameter 2.0-2.5 mm). For each size, the granules were individually placed inside a 96-well ELISA plate. Two plates were used for each size, giving four plates for each preparation. The weight of dried granules produced in each preparation was also recorded.

Experiment 4: Pathogenicity of Different Rates of Granules Against the Larvae of *O. rhinoceros*

The pathogenicity of the highest quality granules produced in Experiment 3 was tested against field-collected third instar (L3) larvae of *O. rhinoceros*. The L3 larvae were inspected for any *Metarhizium* infection at the time of collection. Only larvae free from the fungal infection were collected and habituated in the laboratory for a week. For each treatment, five larvae were placed into a plastic container of dimensions 15 cm (length) x 10 cm (width) x 8 cm (height), half-filled with rotting oil palm trunks (about 200 g, moisture content 20%-40%). The containers were then treated separately at four rates of granules at 0.5 g, 1.0 g, 1.5 g and 2.0 g. Two control treatments, positive and negative, were used in the experiment. For the positive control, the rotting materials were mixed with a spore suspension of 10^6 viable spores (cfu). For the negative control, the larvae were placed in a container only added with 50 ml ddH_2O. Each treatment was replicated five times. The treatments were placed in the laboratory at 28°C ± 5°C. Mortality of larvae was monitored every alternate day until 20 days after treatment (DAT).

Experiment 5: Pathogenicity of Granules Made from Mycelia and Spores Against the Larvae of *O. rhinoceros*

The pathogenicity of granules made from mycelia (G+MM) and spores (G+Sp) was evaluated against the L3 larvae of rhinoceros beetle. The highest quality G+MM granules produced in Experiment 3 were used in this study. The G+Sp granules were prepared by the same method used to prepare G+MM granules, but with the mycelia replaced with spores. The amount of spores used was 4 g spores per litre. The spores were produced on a solid medium and harvested by washing the sporulated medium with water plus a weak solution of Tween 80 as described by Ramle *et al.* (2006).

Quality of granules. The quality of both types of granules was evaluated based on their capability of producing the spores of *M. anisopliae*. Granules with sizes between 2.0 and 2.5 mm were selected and individually placed in two 96-well ELISA plates. The granules were then wetted by adding 40 µl ddH_2O per well, and the plates were wrapped using a thin plastic film, then placed in an incubator at 28°C for 30 days. The number of spores produced on the granules was estimated every five days for 30 days after incubation (DAC). At each time of assessment, six granules, three each from plate 1 and plate 2, were randomly selected and placed individually into a microtube that was filled with 1.0 ml ddH_2O plus 0.02% Tween 80. The tubes were then vigorously shaken using a vortex mixer for 3-5 min to separate the spores from the granules. The number of spores in the solution was then counted by the Neubauer hemocytometer.

TABLE 1. AMOUNTS OF INGREDIENTS USED TO OPTIMIZE THE PRODUCTION OF GRANULE FORMULATIONS OF *M. anisopliae*

Recipe No.	Number of preparations	Amount of alginic acid (g)	Volume of mycelial solution (ml)	Volume of distilled water (ml)	Amount of kaolin (g)	Amount of rice bran (g)
1	5	10	500	1 000	300	200
2	3	10	500	1 500	450	300
3	3	10	500	1 500	500	300
4	3	15	500	2 000	925	400
5	3	15	500	2 000	950	400
6	3	15	500	2 000	1 000	400

Pathogenicity test. The pathogenicity of both types of granules was tested against the field-collected L3 larvae which were prepared as in Experiment 4. To ensure that the granules acted as sources of spores, (capable of contaminating substrates which later infected the larvae), the test was conducted in a larger experimental box with dimensions 30 cm (length) x 25 cm (width) x 20 cm (height). Each box was quarter-filled with rotting oil palm materials as a food source (about 1 kg, moisture content 20%-40%). For each granule type, three rates were tested: 3 g, 6 g and 9 g granules per box. The granules were homogenously mixed with the rotting oil palm materials. In the control, the rotting materials were mixed only with granules made without any fungal inocula. The experiment was replicated five times, and in each replication, five L3 larvae were used. Data on larval mortality and infection were recorded every alternate day for 20 DAT. The cadavers were placed in a closed, damp container (relative humidity 80%-90%) for a week. Those cadavers showing the growth of whitish hyphae, which later sporulated with dark green spores were confirmed to be infected by *M. anisopliae*.

Statistical Analysis

Data on the yield of mycelia in Experiment 1, the weight of dried granules produced in Experiments 2 and 3, and the number of spores produced on granules in Experiment 5 were analysed by ANOVA using PROC GLM (SAS System 1997). The means were separated by the Least Significant Difference (LSD) Test at P=0.05. The percentage of granules with growing hyphae and producing spores in Experiments 2 and 3, and the percentages of cumulative mortality and infection on *O. rhinoceros* in Experiment 4 were arcsine-transformed before performing ANOVA. The percentage cumulative mortality of L3 larvae in Experiment 5 was corrected by Abbot's Formula, arcsine-transformed and then ANOVA was performed.

RESULTS

Experiment 1: Effects of pH on the Production of Fungal Mycelia

The yield of mycelia increased as the pH of the medium increased (*Figure 2*). There was no statistical difference (F=3.71, df: 6,6, P>0.05) in the yield of mycelia produced in the medium at pH=5 (0.24 g), pH=6 (0.40 g) and pH=7 (0.31 g). However, the highest yield of mycelia produced at pH=8 (0.74 g) was significantly higher (P<0.05) than the yields produced in the media at the other pHs.

Experiment 2: Quality of Granules Prepared from Mycelia Alone and Mycelia with the Growing Medium

Higher quality granules were produced from mycelia with the growing medium (G+MM) as compared to granules made from the mycelia alone (G+M) (*Figure 3*). The fungus grew on 100% of the G+MM granules, significantly higher (F=16.70, df: 6,5, P<0.05) than the fungus growing on the G+M granules (62.2%). An average of 87.2% of the G+MM granules produced spores, significantly higher (F=20.08, df: 6,5, P<0.05) as compared to only 48.0% for the G+M granules. However, the yield of the G+M granules was significantly higher (F=182.93, df: 3,2, P<0.05) than for the G+MM granules, being 394.9 g *vs.* 353.9 g.

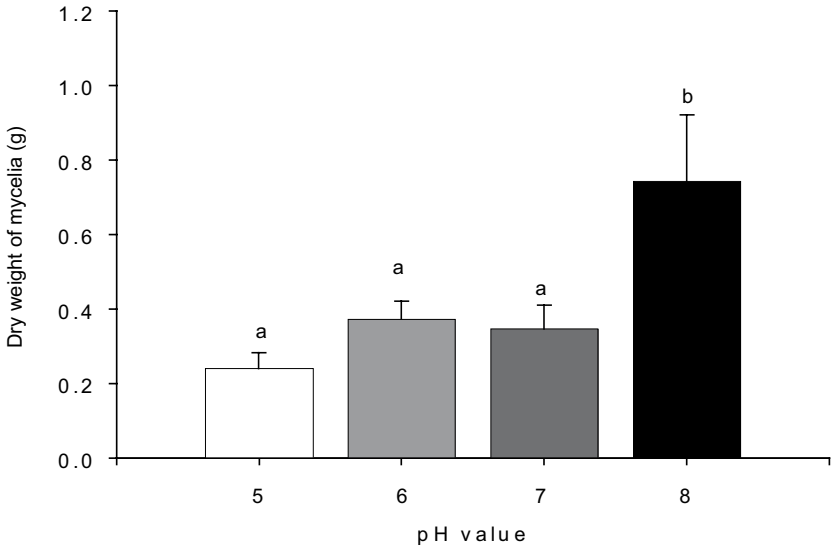

Figure 2. Production of mycelia of M. anisopliae *grown in a liquid medium with pH value ranging from 5 to 8 (bars with the same letter are not significantly different by LSD test at P=0.05).*

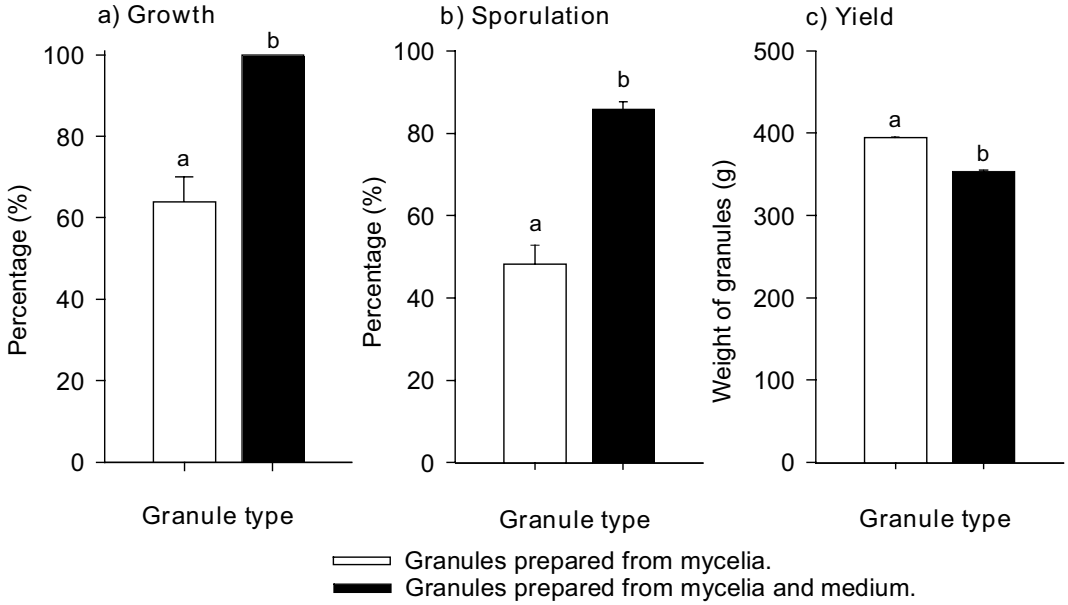

Figure 3. Quality of granules prepared from mycelia with the growing medium (G+MM) and from mycelia alone (G+M). a) Growth of fungus on granules, b) sporulation of the fungus on granules, and c) dry weight of granules (bars with the same letter are not significantly different by LSD test at P=0.05).

Experiment 3: Optimization of Granule Ingredients

The growth of *M. anisopliae* on granules was higher at between 93% and 100% for Recipe 5 (950 g kaolin, 400 g rice bran, 2 litres ddH_2O). The growth was then reduced to 75.1% on granules prepared in Recipe 6 (1000 g kaolin, 400 g rice bran, 2 litres ddH_2O (*Figure 4*). The best granule quality was produced by Recipe 4. Using this recipe, the fungus grew on 98.5% of the granules and 88.6% of them produced spores. Granule dry weight was 1249 g. The optimum amounts of ingredients were determined not only based on the capability of the fungus to grow and produce spores, but also based on the weight of the granules. An accepted good granule quality was for granules with more than 80% of them producing spores, and granules with this quality were only produced with Recipe 4 (925 g kaolin and 400 g rice bran) and Recipe 5 (950 g kaolin, 400 g rice bran) (*Figure 4*). Fungal sporulation on granules produced with Recipe 4 was not significantly different ($p>0.05$) from those granules produced by Recipe 5 (80.3%).

Based on the weight of dried granules, granule production increased following increases in the amounts of kaolin and rice bran (*Figure 5*). The weight increased from 484.3 g in Recipe 1, to 737.0 g (Recipe 2), 789.3 g (Recipe 3), 1249.1 g (Recipe 4), 1353.1 g (Recipe 5) and finally to 1391.2 g in Recipe 6. Although Recipes 5 and 6 produced similar weights (not significantly different at $P>0.05$), the sporulation level for Recipe 5 was significantly higher ($P<0.05$) than with Recipe 6 (*Figure 4*). Therefore, Recipe 5 had the optimum amounts of ingredients for granule composition. The number of spores produced with this preparation was estimated at between 0.52×10^6 and 1.53×10^6 spores per granule.

Experiment 4: Pathogenicity of Different Rates of Granules Against the Larvae of *O. rhinoceros*

The mortality of L3 larvae in treatments with granules at different rates is shown in *Table 2*. Over time after application, larval mortality increased as the granule rate increased. At the highest rate of 2.0 g, the granules caused the highest mortality on L3 larvae (91.0%), followed by granule rates of 1.0 g (90.0%), 1.5 g (81.0%) and 0.5 g (67.0%). At 20 DAT, mortality due to the granule formulation was as high as the mortality caused by the spore solutions (96.0%) ($P>0.05$).

Experiment 5: Pathogenicity of Granules Made from Mycelia and Spores Against the Larvae of *O. rhinoceros*

Granules made from mycelia with the medium (G+MM) were quicker in producing spores as compared to granules made from the spores (G+Sp) (*Figure 6*). Furthermore, the G+MM granules produced significantly more spores as compared to the G+Sp granules in all the assessments ($P<0.05$), except at 30 DAC. At 5 DAC, the G+MM granules produced as many spores as 3.81×10^6 spores per granule. Five days later, the spores increased significantly ($P<0.05$) to 6.28×10^6 spores per granule, peaking at 6.60×10^6 spores per granule at 15 DAC, then reduced slowly in number until 30 DAC. For the G+Sp granules, production of spores was only recorded at 10 DAC. The number of spores increased

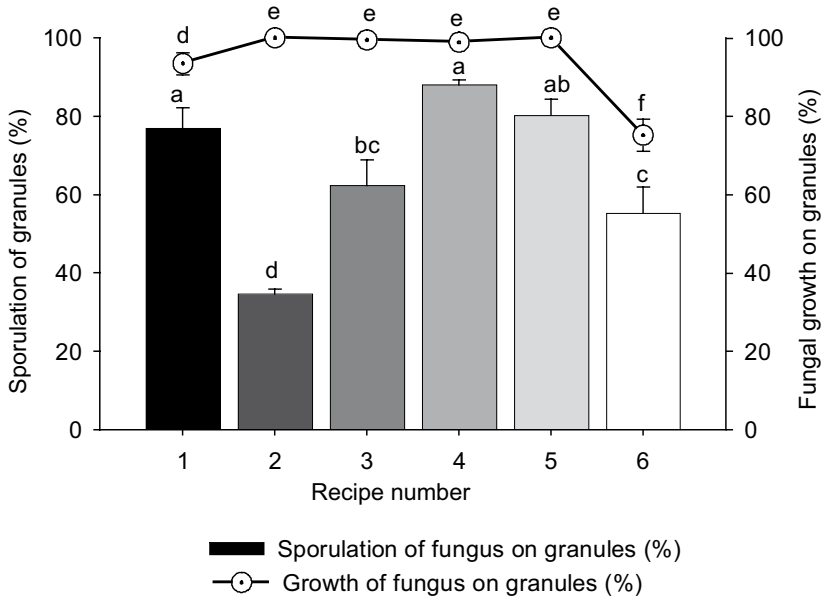

Figure 4. Quality of granules produced by six recipes, each containing different amounts of kaolin and rice bran (line and bars with the same letter are not significantly different by LSD test at P=0.05).

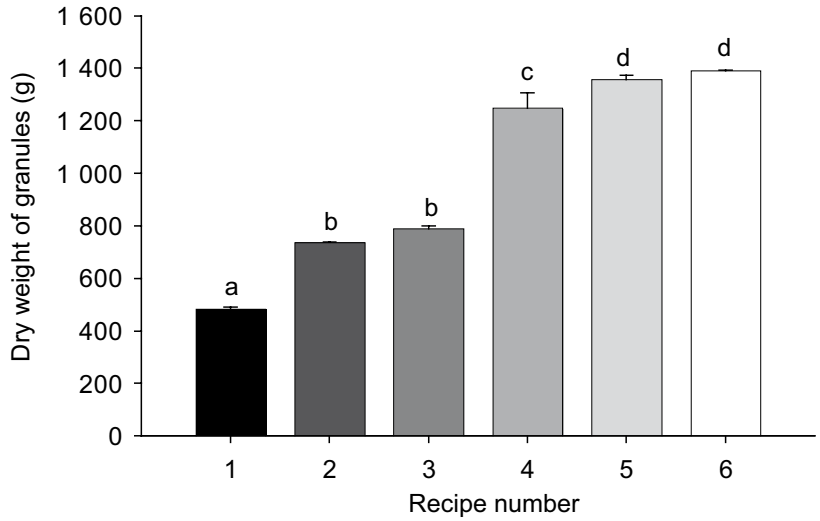

Figure 5. Yield of granules (dry weight) produced from six recipes containing different amounts of kaolin and rice bran (bars with the same letter are not significantly different by LSD test at P=0.05).

TABLE 2. PERCENTAGE CUMULATIVE MORTALITY OF THE LARVAE OF *Oryctes rhinoceros* AFTER TREATMENT AT DIFFERENT RATES WITH GRANULES MADE FROM MYCELIA AND THE GROWING MEDIUM

Treatment	Percentage cumulative mortality (mean ± SE) at days after treatment (DAT)			
	8 DAT	12 DAT	16 DAT	20 DAT
Control	20.0 ± 12.6a	28.0 ± 17.4b	32.0 ± 19.6b	41.0 ± 19.2b
Spore suspension (10^6 cfu)	40.0 ± 14.1a	64.0 ± 17.2ab	84.0 ± 11.7a	96.0 ± 4.0a
Granule (0.5 g)	30.0 ± 7.6a	55.0 ± 8.9ab	63.0 ± 12.8ab	67.0 ± 14.6ab
Granule (1.0 g)	40.0 ± 6.9a	63.0 ± 10.1ab	90.0 ± 6.1a	90.0 ± 6.1a
Granule (1.5 g)	41.0 ± 12.1a	63.0 ± 10.1ab	77.0 ± 8.0a	81.0 ± 9.3a
Granule (2.0 g)	45.0 ± 15.7a	76.0 ± 11.2a	91.0 ± 5.6a	91.0 ± 5.6a

Note: Means within a column with the same letters are not significantly different by LSD test at P=0.05.

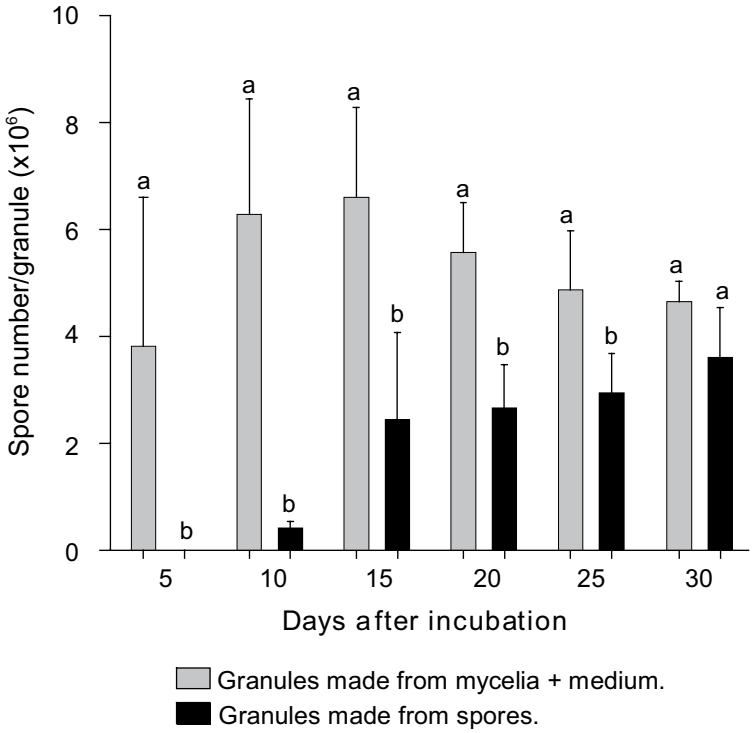

Figure 6. Number of spores produced on granules made from mycelia plus medium (G+MM) and from spores (G+Sp) at different incubation periods (bars in groups with the same letter are not significantly different by LSD test at P=0.05).

significantly ($P<0.05$) from 0.42 x 10^6 spores per granule to 2.45 x 10^6 spores per granule at 15 DAC. Thereafter, the number of spores gradually increased at a slower rate, ending at 3.61 x 10^6 spores/granules at 30 DAC.

Use of both types of granules has proven effective in controlling the L3 larvae of *O. rhinoceros*. Application of both types of granules at all rates killed 100% of the L3 larvae, as early as 18 DAT, significantly higher ($P<0.05$) as compared to the control (*Figure 7*). None of the L3 larvae in the control died because of *M. anisopliae* infection. The percentages of dead L3 larvae infected by *M. anisopliae* at all rates of the G+MM and G+Sp granules are shown in *Figure 8*. For the G+MM granules, the infection by *M. anisopliae* increased in tandem with the application rate, although infection in the G+MM treatment at 6 g per box was no different ($P>0.05$) from the application rate of 9 g per box. For the G+Sp granules, the percentage of infection reduced from 73.3% (for the rate of 3 g per box) to 60.0% (at 6 g per box), but then increased significantly ($P<0.05$) to 93.3% (at 9 g per box). Application of either of the G+MM or the G+Sp granules at the highest rate caused the highest infection level (93.3%).

DISCUSSION

The use of a liquid medium containing 2% (w/v) dextrose, 0.25% (w/v) peptone and 1% (w/v) yeast extract and 0.02% (w/v) chloramphenicol was successful in producing mycelia of *M. anisopliae.* Growing *M. anisopliae* in the liquid medium will usually produce three types of inocula – blastospores, mycelial pellets and submerged spores (Jenkins *et al.*, 1998). The production of these inocula is largely dependent on the composition of the medium, the medium pH, the growing temperature and chemical additives (Kleespies and Zimmermann, 1998). In this study, only effect of medium pH on the yield of fungal mycelia was evaluated. The other medium components, such as the medium composition, growing temperature and chemical additives were not studied.

The blastospores produced by *M. anisopliae* in a liquid medium are suitable for producing spores as they can be evenly distributed on a solid medium to promote sporulation (Jenkins *et al.*, 1998). This inoculum has been used in the mass production of *M. anisopliae* spores for the biological control of *O. rhinoceros* beetle in oil palm plantations (Ramle *et al.*, 2006). In this study, higher yields of mycelial pellets were produced as compared to blastospores. This finding supports results from a previous study by Kleespies and Zimmermann (1992), who found that propagation of *M. anisopliae* in a liquid medium at pH 7 to pH 8 produced more mycelial pellets than blastospores. For the development of granule formulation, mycelial pellets are preferable as they are highly resistant to mechanical stress, tolerant to high temperatures and have a longer life span during

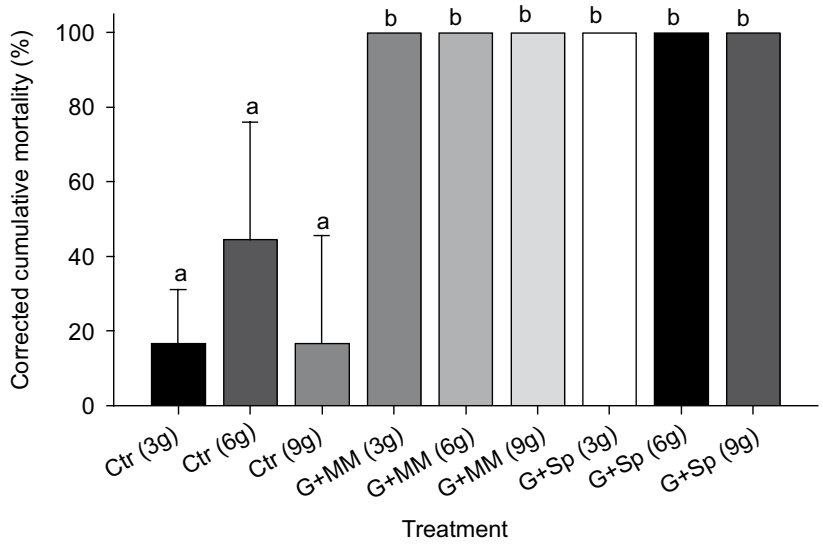

Figure 7. Corrected cumulative mortality of L3 larvae of O. rhinoceros *using granules made without mycelium (Ctr), with mycelia (G+MM) and spores (G+Sp) applied at different rates at 18 days after treatment (bars with the same letter are not significantly different by LSD test at P=0.05).*

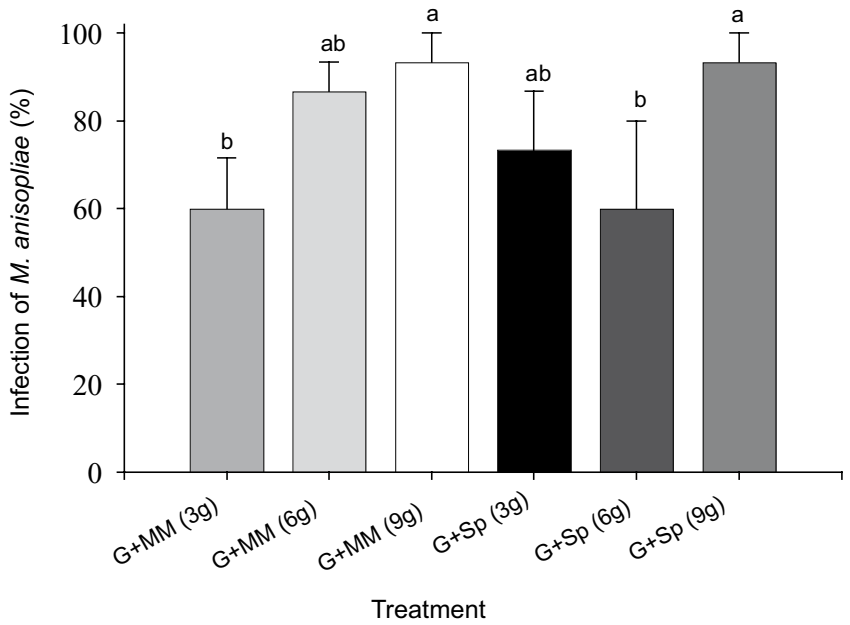

Figure 8. Percentage of infection on L3 larvae of O. rhinoceros *by* M. anisopliae *after treatment with different rates of granules made from mycelia (G+MM) and granules made from spores (G+Sp) (bars with the same letter are not significantly different by LSD at P=0.05).*

storage. Therefore, mycelial pellets had been used as a base for the granule formulation of *M. anisopliae* for the control of the black vine weevil, *Otiorhynchus sulcatus* (Reinecke *et al.*, 1990).

Data from Experiment 2 showed that granules made from mycelia with the medium (G+MM) were better in encouraging mycelial growth and sporulation as compared to the granules made using mycelia alone (G+M). On the G+MM granules, fungal growth was 100% with 87.2% of the granules were producing spores, while on the G+M granules, fungal growth was only 62.2% with 48.0% of them producing spores. This improvement in the quality of the G+MM granules was expected as this preparation was rich in carbon and nitrogen sources derived from dextrose, yeast extract and peptone, as well as from rice bran. By contrast, for the G+M granules, the carbon and nitrogen sources were only from rice bran and sucrose. *M. anisopliae* grows and sporulates well in a medium containing sufficient amounts of these two nutrients (Jenkins *et al.*, 1998).

Experiment 3 showed that the granule composition was optimized by gradually increasing the volume of water, kaolin and rice bran in the mixture. The results indicate that Recipe 5 (2000 litres water, 950 g kaolin and 400 g rice bran) produced the highest amount of granules (1353.1 g) with a sporulation level of 80.3%. The pathogenicity study in Experiment 4 indicate that the G+MM granules at the highest rate of 2.0 g killed 91.0% of the L3 larvae, as high a mortality rate as that caused by the treatment with spore solutions (96.0%).

In Experiment 5, the granule formulations were produced from mycelia and spores as the active ingredients, respectively. However, the G+MM granules produced spores much earlier and significantly more than the G+Sp granules. The mycelia in the G+MM granules grew immediately as they rehydrated, which led to quick formation of new spores. However, for the G+SP granules, the mycelial development and production of new spores must first be initiated by spore germination. This process generally took a few days to complete.

A bioassay demonstrated that the granules prepared by using either of the fungal inocula were effective in killing the L3 larvae. The current study shows that treatments with the G+MM and G+Sp granules at all rates (3 g, 6 g and 9 g granules per box) killed 100% of the L3 larvae of *O. rhinoceros* at 18 DAT. Larval cadavers due to fungal infection also increased in tandem with the application rate. At the highest rate, both the G+MM and G+Sp granules resulted in the highest level of infection (93.3%) compared to other treatments.

This study demonstrates that the time required to achieve 100% mortality by both granule types was 18 DAT, slightly longer than the killing time for spore solutions at 12-14 DAT (Ramle *et al.*, 1999). The delay in killing time was because the granules needed to form infectious spores before initiating the infection process. In the assessment of granule quality, it was found that the time taken for sporulation of *M. ansiopliae* on the granules was between 6 and 10 days. A much shorter sporulation time of five to six days was reported on *M. anisopliae* granules that were freshly prepared from mycelial pellet granules (Andersch, 1992).

Applying granules is easier than applying the powder formulation to control the *O. rhinoceros* in oil palm plantations, especially in the hilly areas and in areas where the water source is limited. The granules can be applied manually by broadcasting them onto the potential breeding sites of the beetle. Absorption of water by the granules from the environment, such as wet soil, rain or dew, will initiate the growth of the fungus, which then leads to the production of spores. In the long run, supplementing the nutrient food base in the granules, with ingredients such as rice bran in this study, will make the formulation last longer in the pest habitats. In addition, the granules themselves will serve as source of infectious spores, without depending only on the cadavers of the infected host as the secondary source of inoculum.

CONCLUSION

Propagation of *M. anisopliae* in a liquid medium consisting of 2% dextrose, 0.25% peptone, 1% yeast extract and 0.02% antibiotic at pH 8 produced a high yield of mycelial pellets that are suitable for the production of granule formulations. Preparation of the granules by mixing the fungal mycelia with the growing medium (G+MM) improved the quality of the granules. The pathogenicity study shows that the G+MM granules killed the L3 larvae of *O. rhinoceros*, as effectively as a spore solution. A further study indicates that granules can be made from mycelia and spores of *M. anisopliae*. Both types of granules were equally effective in controlling the L3 larvae of *O. rhinoceros*. At the highest application of 9 g per box, both formulations killed 100% of L3 larvae, with 93.3% of the larvae infected. Although, the killing time of the larvae was longer (18 DAT) than with spore solutions (12-14 DAT), this formulation was easier to apply as compared to a powder formulation. The granules can be applied by directly broadcasting onto the breeding sites of the *O. rhinoceros*. As the granules are supplemented with a nutrient source, the fungus will grow immediately after water absorption and remain viable in the pest habitats for a longer period of time. Although this study produced convincing results, a field study is still needed to further determine the effectiveness of the granule formulation of *M. anisopliae* in controlling *O. rhinoceros*.

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