LARGE SCALE PRODUCTION OF NEW ZEALAND STRAINS OF *BEAUVERIA* AND *METARHIZIUM*

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ABSTRACT

Beauveria and *Metarhizium* spp. are currently under development around the world as biocontrol agents for a variety of pest insects. Use of these fungi in practical biocontrol programmes will require production of large amounts of inoculum. Although production techniques on solid substrates have been developed overseas, the production characteristics of New Zealand strains were unknown. The effects of solid media (rice, wheat and barley) with additives (glucose and yeast extract), temperature and length of incubation on conidial production were investigated. Maximum conidial yields achieved were 4.38×10^9 conidia/g rice for *B. bassiana*, 3.02×10^9 conidia/g dry substrate for *B. brongniartii*, and 1.42×10^9 conidia/g dry substrate for *M. anisopliae*. In all cases, maximum yield was achieved when fungi were grown on rice for 3 weeks at 23°C, under natural daylight.

Keywords: *Metarhizium*, *Beauveria*, production, conidia, fermentation

INTRODUCTION

Among the insect pathogenic fungi, the genera *Beauveria* and *Metarhizium* contain strains which kill a number of pest species (Glare *et al.* 1993). Consequently they are under investigation in New Zealand as control agents for insects such as Argentine stem weevil, grass grub and porina. Selection of highly virulent strains against specific pests does not guarantee a biological control solution. One of the major impediments to developing microbes for large scale application is lack of suitable mass production techniques. In this respect, *Beauveria* and *Metarhizium* have many advantages. These asexual fungi are easily cultured on media, producing large quantities of conidia and have been economically produced for commercial applications in other countries (Soper and Ward 1981).

Although methods have been developed for the large scale production of these fungi, the New Zealand isolates of *M. anisopliae* differ genetically from other *M. anisopliae* (Curran *et al.* 1994). Similarly, the New Zealand *Beauveria* isolates are different strains than those previously studied elsewhere in the world (Glare unpublished). Consideration of the New Zealand isolates for potential use as biological control agents requires development of methods that will allow optimal production of an efficacious, cost effective product.

Conidia are probably the most appropriate propagule for field use, as they are the infectious units and show greater stability under dry conditions and after application than hyphae or blastospores (Soper and Ward 1981; Feng *et al.* 1994). Herein we report on an investigation into the conditions for optimal production of conidia on solid substrates by strains of both genera.

MATERIALS AND METHODS

Fungal isolates and culture

Three isolates from the AgResearch Insect Pathogens Culture Collection (Lincoln) were used in this study: *Beauveria brongniartii* strain F156, originally isolated from a grass grub larva in Waikato; *Beauveria bassiana* strain F186, originally isolated from a grass grub larva from Matangi, Hamilton; *Metarhizium anisopliae* strain F204, originally isolated from a grass grub larva from Canterbury. Isolates were maintained

on potato dextrose agar (PDA)(GibcoBRL) at 25°C and subcultured weekly. To produce inoculum for solid substrate production experiments, conidia from 1.5-3 week old PDA plate cultures were collected into 1 litre of sterile 0.05% Triton x-100 to a final concentration of 10^6 conidia/ml. Conidial viability was determined by plating serial dilutions on PDA and a modified selective medium based on streptomycin and tetracycline (JC) (Townsend *et al.* 1995). The resulting colony-forming units (cfu) were counted after 4-7 days incubation at room temperature.

Production on solid substrates

The standard replicate was an autoclave bag (300 x 450 mm) containing 200 ± 0.5 g long grain white rice (or other grain) and 40 ml H₂O. A metal tube (55 mm long x 5 mm diameter) was taped into each bag opening and a cotton wool bung inserted. Bags were sterilised by autoclaving at 121°C, 15 psi for 15 mins. Any clumps of rice were separated manually on cooling. Bags were inoculated with 40 ml of 10^6 conidia/ml, prepared as above, and mixed thoroughly. The standard growth condition was $23 \pm 2^\circ\text{C}$ under natural photoperiod for 3 weeks. For all experiments, five replicates of each treatment were used.

Quantification of conidial yield and viability

Conidia were removed from rice grains by adding approximately 200 ml of sterile 0.05% Triton x-100 (Sigma) to each bag; bags were shaken, then the suspension was filtered into sterile containers. Grains were then rinsed with another 200 ml of Triton x-100. The total volume containing conidia was measured. Haemocytometer counts of the recovered spore suspension gave estimates of the total conidial yield and serial dilutions plated on PDA or JC were used as estimates of the viable conidial yield.

Factors affecting yield

The effect of various factors on yield of conidia was determined in a series of growth experiments.

- 1) Growth period - all isolates were grown under standard conditions on rice, and harvested after 1.5, 3, 4.5 and 6 weeks incubation. Total conidial yields were determined by haemocytometer counts.
- 2) Temperature - yield of *B. brongniartii* (F156) and *M. anisopliae* (F204) was determined at 20°C, 23°C and 30°C after 3 weeks growing on rice in the dark (due to lack of light facilities in incubators).
- 3) Substrate - the effect of substrate on yield and viability of *B. brongniartii* (F156) conidia was investigated under standard temperature and time conditions. Treatments were: rice, rice + 5% glucose, rice + 5% yeast extract, kibbled wheat and barley (ground to approximately the same size as kibbled wheat). Conidial yield and viability were measured after 3 weeks incubation at 23°C.

Statistical Analysis

Analysis of data was carried out using analysis of variance through Genstat 5.3.3

RESULTS

Growth period

When one isolate of *Metarhizium* and two of *Beauveria* were grown under standard conditions on rice and harvested at 1.5, 3, 4.5 and 6 weeks, significant differences in yield were found between isolates. Highest total conidial yields were obtained after 3 weeks for all isolates (F186: 4.38×10^9 /g rice; F156: 1.10×10^9 /g rice; F204: 1.42×10^9 /g rice). Due to the distribution of the data, a log transformation was used prior to statistical comparison. Length of growth period had a significant effect on total conidial yield (haemocytometer count) independent of isolate ($P < 0.001$) (Fig. 1).

Temperature

Conidial yield on rice for *B. brongniartii* (F156) and *M. anisopliae* (F204) was measured at three temperatures (Fig. 2). For F156, significantly more conidia were produced at 20°C than at 23°C or 30°C ($\text{LSD} = 1.83 \times 10^8$, $P < 0.05$). For F204, conidial yields at 20°C and 23°C were not significantly different at the 5% significance level. While growth occurred, no sporulation was observed at 30°C for F204 (Fig. 2). Due to some bacterial and fungal contamination, conidia production levels observed for F204 were reduced in comparison with all other experiments.

FIGURE 1: Mean conidial yield from *Metarhizium* (F204) and *Beauveria* (F156 and F186) when incubated for 1.5-6 weeks (23°C with natural daylight).

FIGURE 2: Total conidial yield of *Metarhizium* (F204) and *Beauveria* (F156) when incubated at three temperatures without light.

Data from the effect of temperature experiment showed no significant difference ($P < 0.433$) between total and viable conidial yields at three weeks. It appeared that under these conditions, haemocytometer counts were a good estimate of viable conidial yields.

Substrate

The effect of substrate on conidial yield was investigated for *B. brongniartii* (F156). Of the three grains and two grain plus additives (yeast extract and glucose) investigated, rice supported the highest total yield of conidia (Fig. 3). This was significantly greater ($P < 0.05$) than all other treatments, except rice plus yeast. Overall, substrate had a very significant effect on total conidial yield ($P < 0.001$). The highest yield of viable conidia was achieved on kibbled wheat.

FIGURE 3: Production of conidia by *B. brongniartii* (F156) (as estimated by haemocytometer and viable plate counts) on various substrates (23°C with natural daylight).

Comparisons between total (haemocytometer) and viable (PDA or JC) counts showed a significant effect ($P < 0.001$) of substrate on the viability of conidia. There appeared to be an inverse relationship between quantity and viability for rice and rice plus yeast after 3 weeks (Fig. 3).

DISCUSSION

Production of conidia was influenced by substrate, temperature and duration of incubation for *Metarhizium* and *Beauveria* strains. Optimal incubation time for a strain of *M. anisopliae* has been previously reported as two weeks (Dorta *et al.* 1990).

The yields achieved here are comparable to those previously reported for similar specifications. Using methods similar to those described herein, Alves and Pereira (in Feng *et al.* 1994) achieved 2.6×10^{11} conidia/g conidial powder for a strain of *B. bassiana*, which equates to 7.8×10^9 conidia/g dry rice. Aregger (1992) reported production varying from 1×10^8 conidia/g barley after 24 days, to 2×10^9 conidia/g barley after 42 days with *B. brongniartii*.

Temperature had a significant effect on the total conidial yield of *B. brongniartii* with an optimal temperature of around 20°C. No conidial production for *M. anisopliae* was recorded at 30°C.

Of the substrates investigated, rice alone sustained the highest total yield of *B. brogniartii* (3×10^9 conidia/g dry substrate). However, it seems that while quantity of total conidia was high on rice, the viability of the conidia was reduced. The maximum viable conidia measured was on wheat when grown at 23°C for three weeks, with natural daylight. In Brazil, *M. anisopliae* has been found to produce conidial yields of 5-15 times higher using rice bran/rice husk substrate mixtures than yields usually obtained for rice grains, with viabilities of higher than 85% (Dorta *et al.* 1990).

As viable conidia are the most desirable end product, production of *B. brongniartii* on wheat or barley seems justified. Production on rice did produce a higher total yield, but only 50% of that was viable conidia. However, substrate preferences depend largely on availability and cost. For each new strain and species of entomopathogenic

fungi, the optimal conditions required for large scale production may differ from those found previously and need to be tested to produce a high level of an infective inoculum at minimal cost.

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