ENTOMOPHAGA, 33 (3), 1988, 315-324

PRODUCTION OF *BEAUVERIA BASSIANA* [*DEUTEROMYCOTINA : HYPHOMYCETES*] IN DIFFERENT LIQUID MEDIA AND SUBSEQUENT CONIDIATION OF DRY MYCELIUM

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Mycelium of *Beauveria bassiana* can be grown in liquid culture, filtered, and the mycelium dried. After rehydration the mycelium sporulates.

Two carbohydrate sources (sucrose and maltose), and one nitrogen/vitamin source (yeast extract) were tested for mycelium growth and subsequent conidial production. Maximum mycelium growth (12.31 mg/ml), in liquid culture, was in the sucrose (3.5 %)/yeast extract (3.5 %) medium, but mycelium from a maltose (2 %)/yeast extract (0.75 %) medium produced the maximum of 4.62×10^6 conidia/mg dry mycelium after incubation in moist Petri dishes.

Using the data on mycelium yield (in liquid culture) and conidial production (by dry mycelium) it is calculated that the sucrose (3.5 %)/yeast extract (3.5 %) and the maltose (2 %)/yeast extract (0.75 %) media produce most conidia per media volume (an equivalent of $3.52\text{-}3.72 \times 10^7$ conidia/ml).

KEY-WORDS : *Beauveria bassiana,* marcescent process, mass production, fermenter, dry mycelium, conidiation.

Various species of entomogenous fungi are presently being tested for use against important agricultural and medical insect pests (**Burges & Hussey,** 1971 ; **Burges,** 1981 ; **Hoy & Herzog,** 1985). For large scale field experimentation and commercialization of the fungi development of efficient mass production techniques is necessary.

Several techniques for the mass production of entomopathogenic Hyphomycetes are available, mostly designed to yield infective conidia; the conidia are harvested and formulated for storage and field use. These methods are mostly variations on production techniques using solid substrates — e.g. production of *Metarhizium anisopliae* (Metsch.) Sorok. (**Aquino** *et al.,* 1977), *B. bassiana* (Bals.) Vuill (**Bajan** *et al.,* 1975) and other entomopathogenic Hyphomycetes (**Goettel,** 1984). In techniques using the relatively

labor intensive di-phasic fermentation the mycelium is grown in liquid media followed by inoculation of a solid medium or an inert carrier on which conidia are produced (**Ferron,** 1981 ; **Soper & Ward,** 1981). Mycelium can also be incubated in shallow, aerated vessels to produce conidia directly on the surface of the mycelium pellets (**Kybal & Vlcek,** 1976 ; **Samsinakova** *et al.,* 1981). However, for less expensive mass production a single phase, liquid fermentation process is desirable, allowing existing engineering, fermentation, and production routines to be utilized. With this technology a product containing dry blastospores of *Verticillium lecanii* Viégas (**Hall,** 1981) was produced. Also, techniques were developed to produce *B. brongniartii* (Delacr.) dry blastospores (**Blachère** *et al.,* 1973) and, recently, *B. bassiana* conidia which are produced submerged (**Thomas** *et al.,* 1987 ; **Rombach,** 1988). The latter production method might revolutionize *B. bassiana* mass production, submerged production of conidia being the most desirable single phase process. However, the drying-, storage-, field application properties and virulence of submerged produced *B. bassiana* conidia are not known yet.

Production of *B. bassiana* dry mycelium is a single phase fermentation process, not unlike production of Entomophthoralean mycelium as patented by **McCabe & Soper** (1985). The mycelium is produced by liquid fermentation, stabilized with additives, dried, and milled. This mycelium can be formulated and stored. The material can be applied in the field with conventional spray equipment ; however, with large mycelium particles (>0.3 mm) the use of a spinning disc applicator is advisable (**Rombach** *et al.,* 1987). After field application the mycelium sporulates on the plant — and these conidia infect the insects.

Media composition is of key importance for growth and sporulation of *B. bassiana* (e. g. **Ferron,** 1981) — but, when grown on agar media, nutrients from the medium sustain growth *and* sporulation. However, after the marcescent process is applied virtually no media remains in the product to sustain conidiation — and sporulation depends totally on inherent qualities of the mycelium.

In this study the results of tests on growth of *B. bassiana* mycelium in liquid media of different composition, and subsequent sporulation of dry mycelium are reported.

MATERIALS AND METHODS

ORIGIN OF THE ISOLATE AND INOCULATE PREPARATION

The isolate [ARSEF 714 (2)] of *B. bassiana* originated from the brown planthopper, *Nilaparvata lugens* (Stål) (*Homoptera : Delphacidae*) from China. It is a multi-spore isolate, which was stored in liquid nitrogen up to 3 months prior to the experiment. Before the experiment the isolate was maintained on SD[-agar (Sabouraud dextrose agar with 1 % yeast extract) on which it was routinely transferred every 2 weeks.

Inoculum for the experiment was grown in 1 l Sabouraud dextrose broth in 2 l Erlenmeyer flasks on a rotary shaker (150 rpm) for 3 days at room temperature (24-28 °C). The liquid culture consisted mainly of mycelium pellets and blastospores. The culture was harvested by vacuum suction in a sterile Buchner funnel and washed 3 times in a solution of basic salts, i.e. KH_2PO_4 (1.5 mg/ml), $MgSO_4$ (0.5 mg/ml) and $CaCl_2$ (0.01 mg/ml), to prevent transfer of nutrients to the media to be tested. The washed

(2) USDA-ARS Collection of Entomopathogenic Fungal Cultures, USDA-ARS Plant Protection Research Unit, Boyce Thompson Institute for Plant Research, Ithaca, New York. Cultures of isolate ARSEF 714 are also available from the Entomology Department, International Rice Research Institute, P.O. Box 933, Manila, the Philippines.

mycelium was suspended in 500 ml sterile basic salts solution to which antibiotics (300 units/ml of penicillin-streptomycin) were added. The suspension was blended in a sterile Waring blender for about 20 s at medium speed (about 50 rpm) to break up mycelium pellets. The final inoculate was a smooth, homogenized suspension. The density of the mycelium in the inoculum suspension was adjusted to 2 mg/ml.

THE MEDIA

— *Preparation of the media*

All media were prepared with a basic salt solution and trace elements ; components of the media were reagent quality. The basic salts were (in g/l) : KH_2PO_4, 1.5 ; $MgSO_4.7H_20$, 0.5 ; $CaCl_2$ 0.01 ; the trace elements (in mg/l) : H_3BO_3, 0.03 ; $MnSO_4.4H_2O$, 0.04 ; $Na_2MoO_4.2H_2O$, 0.025 ; $CuSO_4.5H_2O$, 0.08 ; $ZnSO_4.7H_2O$, 0.4 ; $FeCl_3.6H_2O$, 0.5 ; $CoCl_2.6H_2O$, 0.4. Of each medium 8 flasks were prepared and sterilized (at 110 °C for 20 mn.). After cooling the pH was adjusted with a sterile 2 N NaOH solution to pH 6.25-6.50. Each flask contained 27.5 ml of medium to which 2.5 ml of inoculate was added, an inoculation rate equivalent to 5mg/replicate. To counteract the dilution effects of the inoculate the media were prepared at increased (10 %) concentrations of nutrients.

— *The media*

The media are grouped in 2 main groups, i.e. the « carbohydrate concentration groups » (CS and CM groups) and « yeast extract concentration groups » (YS and YM groups).

— *Carbohydrate concentration group*

Preliminary experiments showed that mycelium grown in sucrose and maltose broth produced more conidia compared to mycelium grown in similar media with starch, and dextrose as carbohydrate sources (**M. C. Rombach,** unpubl.). Moreover, starch often precipitates during fermentation, and dextrose can caramelize during heat sterilization, which complicates experimentation and mass production. Therefore, sucrose and maltose were tested in these experiments. In the concentration groups the influence of different concentrations (0, 0.5, 1, 2, 2.5, 3, 3.5, 4, 4.5, 5 and 6 %) of the same ratio (1 : 1) of sucrose (the CS group) and maltose (the CM group) and yeast extract were tested. Preliminary experiments showed maximum conidiation of dried mycelium when grown at 3 to 4 % of these media — therefore treatments were grouped around these values in this experiment.

— *Yeast extract concentration group*

In this group the influence of different concentrations (0, 0.1, 0.25, 0.50, 0.75, 1, 1.5, 2, 2.5, 3, 4 and 6 %) of yeast extract on growth and subsequent conidiation was tested. These quantities were added to a sucrose (2 %, in the YS group) and maltose (2 %, in the YM group) broth. Preliminary experiments showed that *B. bassiana* mycelium produced most conidia per mg dry mycelium when grown in a broth containing carbohydrates and yeast extract in about a 3 : 1 ratio. Therefore, the treatments were grouped around this ratio.

INOCULATION, INCUBATION AND EVALUATION

— *Inoculation*

The growing culture vessels used for the experiment were cylindrical, glass bottles (length 9 cm, diameter 3 cm), closed with autoclavable plastic screw caps.

After filling with the media (27.5 ml) the flasks were closed with the plastic caps. After inoculation (2.5 ml) the cap was loosened, to ensure oxygen exchange. For each media 8 flasks were inoculated — i.e. 3 replicates for dry weight measurements, 5 replicates for determination of sporulation. Immediately after inoculation the 348 flasks were placed on a rotary action flask shaker (150 rpm).

— Incubation and dry weight measurements

The shaker was kept in a constant temperature room at 23-25 °C under weak fluorescent illumination for 8-10 h per day. After 72 h on the shaker 3 replicates of each medium were removed, the mycelium collected by vacuum suction on pre-weighed filter paper, washed, dried (4 h at 75 °C), and weighted. At the same time the other replicates (5 for each medium) were removed and placed in a refrigerator (3 °C).

— Sporulation

After weighting the mycelium of the 3 replicates the contents of the 5 remaining replicates were pooled, and blended in a Waring blender for 10 s at medium speed (50 rpm). The blending procedure was standardized, as mycelium can be killed by excessive chopping. Using the data on dry weight obtained above, aliquots of liquid containing the equivalent of 160 mg mycelium were taken from the bottles with a pipet. These were placed on sterile filter papers (Whatman n° 1, diameter 5 cm) and the broth removed by vacuum suction in a sterile Buchner funnel. The mycelium mat was washed 3 times with sterile water to remove nutrients. After the mycelium mat was dry, the filter papers with mat were placed on filter papers in a Petri dish (9 cm diameter). One ml of sterile water was added and the dish closed with Parafilm to prevent desiccation.

For each treatment 5 to 8 replicates were prepared ; this number depended on mycelium growth and thus volume which carried the equivalent of 160 mg dry weight mycelium.

The Petri dishes were incubated on a laboratory bench under subdued fluorescent illumination (8 h/day) and room temperature (25-28 °C). During the conidiation period of 8 days the Petri dishes were randomized several times as to equalize experimental conditions. After this conidiation period the lids were inverted and the dishes placed for 24 h in a stove (75 °C) to dry. To evaluate conidial production conidia were sampled from the surface of the dry mycelium mat by transfer of the filter paper carrying the mycelium and conidia to a test tube with 10 ml Tween80®. The contents were vigorously mixed for about one minute. The filter paper was removed and the remaining conidia removed by washing with a further 10 ml Tween80 solution. Close examination of the remaining mycelium mass on the filter paper showed that virtually no conidia were left. The conidium suspension was adjusted to 39 ml and 1 ml formaldehyde (15 %) was added.

The concentrations of conidia were determined with an Improved Neubauer haemocytometer counting 5 squares of 16 cells in 2 drops of suspension for each replicate. The actual number of conidia produced by the 160 mg mycelium was calculated from these numbers.

— Analysis

Data on growth rate (dry weight of mycelium/30 ml media), and conidiation (conidia/160 mg dry weight mycelium) were analyzed, and data on conidial production/ml broth calculated. Differences in mycelium growth, production of conidia per mg mycelium, and conidial production per ml broth were tested on significance by Student's t-test. Differences were accepted as significant at the $P = 5$ % level.

RESULTS AND DISCUSSION

MYCELIUM YIELD (mg/ml, fig. 1)

Optimal yields were harvested from the CS(3.5 %) media (12.31 mg/ml), the CM(4 %) media, the Ys(1.5 %) media, and the YM(2.5 %) media. In the CS and the CM groups the yields decline with increasing concentration, after a peak has been obtained, but the yields are not significantly different from the peak value, except for media CM(6 %), which yielded significantly less compared to CM(4 %). These trends of decreasing mycelium yields after the peak value agree with findings of **Samsinakova** (1966) ; she found that increasing concentrations of dextrose and starch above 2.5 %, in combination with 1 and 2 % corn steep liquor resulted in decreasing yields of mycelium. In the same studies generally higher yields were obtained with maltose compared to sucrose media. This is in contrast with our findings, and might be caused by nutritional preferences of the different *B. bassiana* strains.

CONIDIAL YIELD (CONIDIA/mg, fig. 2)

Optimal conidiation was detected in mycelium from YM(0.75 %), YS(0.75 %), CS(3.5 %), and CM(3 %) media — and all these peaks differed significantly from the other media in the same groups, except for CM(1 %), which did not differ from CM(3 %). Significant differences were present between groups, with the YM(0.75 %) producing more conidia (4.62×10^6 conidia/mg) compared to YS(0.75 %), followed by CS(3.5 %) and CM(1 %).

CONIDIAL YIELD (CONIDIA/ml broth, fig. 3)

While the YM(0.75 %) medium produces maximum numbers of conidia/mg dry mycelium, the CS(3.5 %) medium produces a larger equivalent of conidia/ml broth — because of superposition of the significantly higher mycelium yields (mg/ml) on conidial production (conidia/mg). The conidial production is 3.72×10^7 conidia/ml. This yield is significantly higher compared to all other media, although the difference with YM(0.75 %) is of no practical value.

PRODUCTION ESTIMATES

Production of conidia on the mycelium particles in the field can be maximized in different ways, i.e. by maximizing *mycelium* production (**McCoy** *et al.*, 1972, 1975, 1978), or maximizing *subsequent conidial* production by the mycelium on the plant. We found that most conidia were produced by mycelium grown in a yeast extract (0.75 %)/maltose (0.75 %) broth. It should be noted, however, that virulence of conidia produced on different media can differ (**Kmitowa,** 1979) — this aspect was not tested in these experiments.

B. bassiana grows in cheap liquid media — and, therefore, cost of fermentor space is probably the production limitation rather than costs of media. Thus, for large scale fermentation, yields should be optimized for volume fermenter space (ml) necessary to produce the mycelium with maximum conidium production rather than for mg dry weight. The yield expressed per ml fermentor space can be calculated by superimposing mycelium yield (mg/ml) on subsequent conidium production (conidia/mg). We found that the largest equivalent of conidia/ml broth was produced by the sucrose (3.5 %)/ yeast extract (3.5 %) broth.

It should be noted that the data on absolute mycelium yield, from shaker flasks can not be extrapolated directly to large scale fermentors — aeration and slurry turnover in the bottles on the shaker is poor compared to laboratory and industrial fermentors. However, relative differences between media are likely to remain the same.

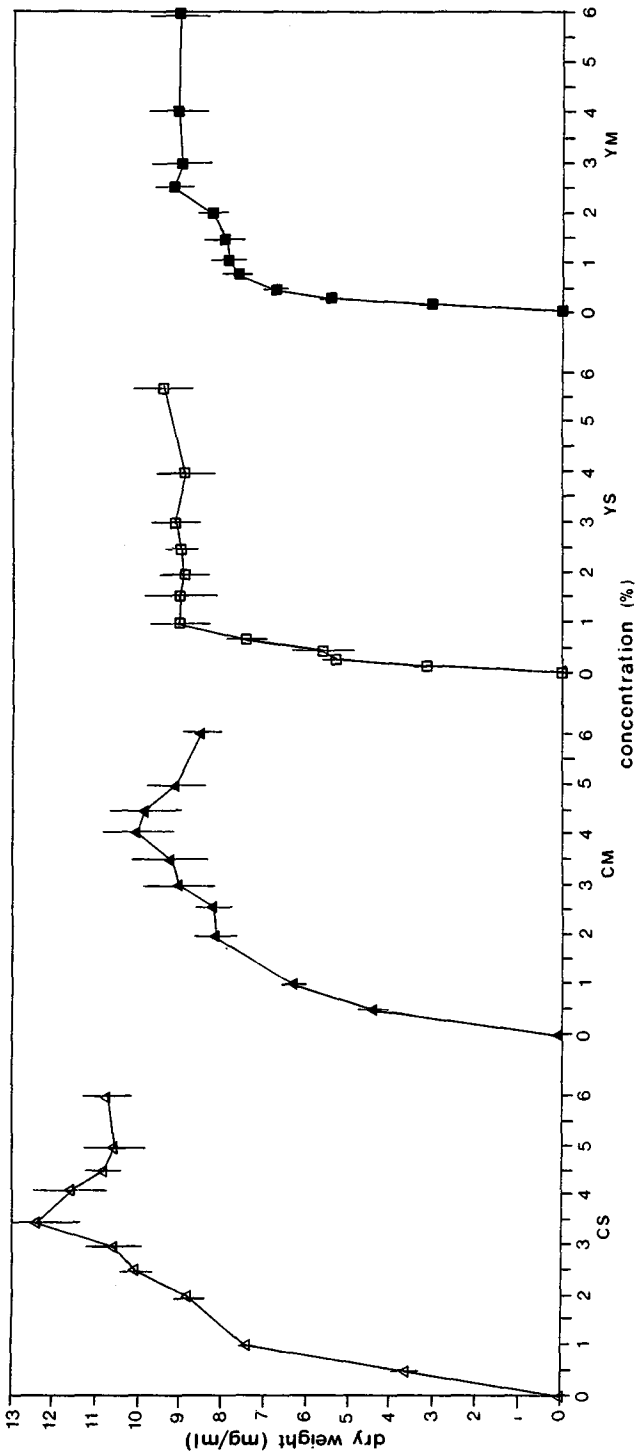

Fig. 1. Dry weight (mg/ml) of *B. bassiana* mycelium harvested from media of different composition. Bars represent standard deviation.

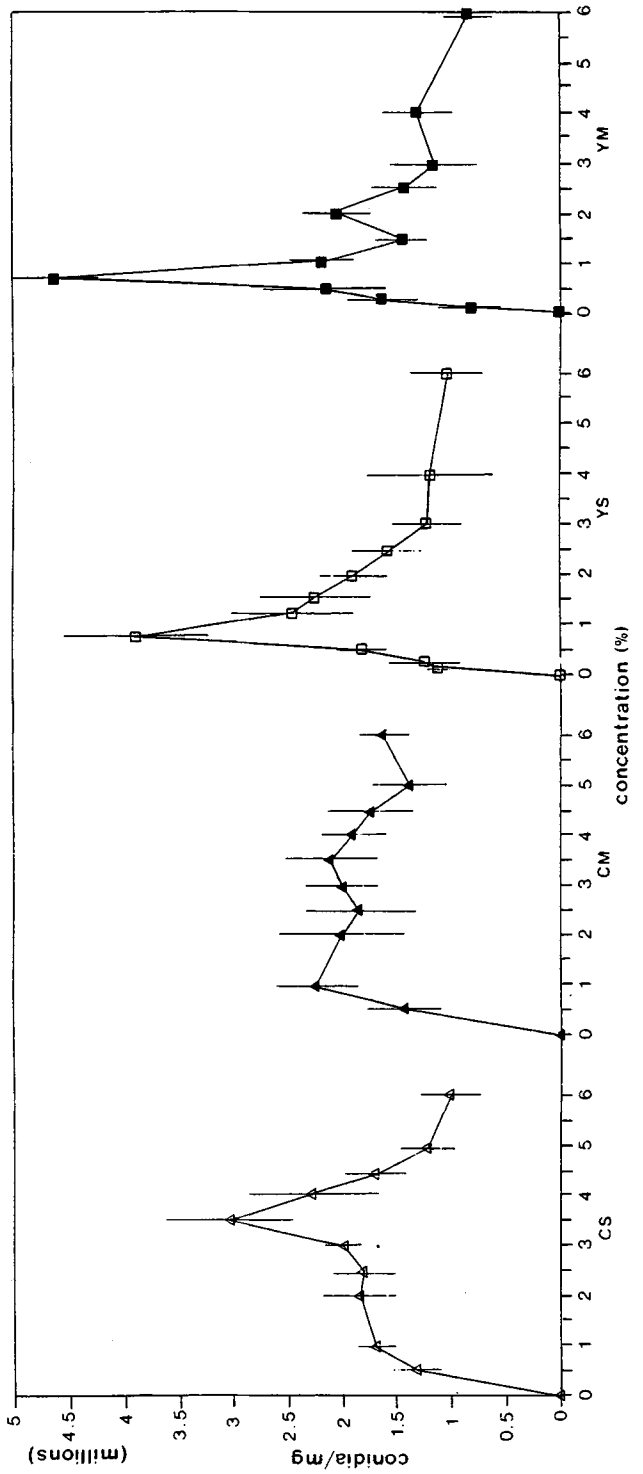

Fig. 2. Numbers of *B. bassiana* conidia produced per mg dry and washed mycelium harvested from media of different composition. Bars represent standard deviation.

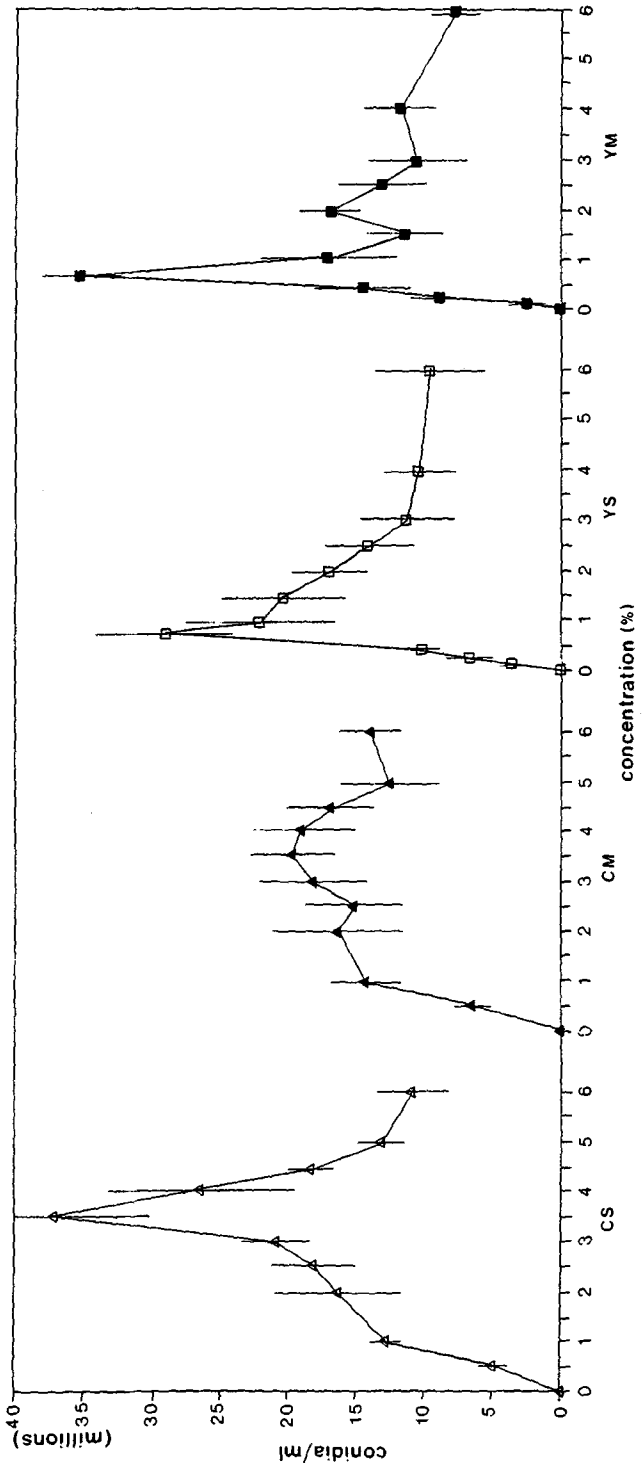

Fig. 3. Calculated numbers of *B. bassiana* conidia produced per ml media of different composition. For details see text. Bars represent standard deviation.

In fermentors *B. bassiana* can probably be grown up to 25 mg/ml dry weight. In rice, brown planthopper and black bug can be successfully controlled with doses of conidia of $2.5\text{-}5 \times 10^{12}$ conidia/ha (**Rombach** *et al.*, 1986a, 1986b, 1987 ; **Aguda** *et al.*, 1987). These data, combined with our findings of a maximum conidium production of about 5×10^6 conidia/mg suggest 20-40 l fermentor space to produce the equivalent conidia for treatment of 1 ha or rice for these insects. These findings suggest that the production of dry mycelium might be a practical solution for *B. bassiana* mass production for use on rice.

ACKNOWLEDGEMENTS

We thank **Joop C. van Lenteren** for his valuable suggestions on the manuscript, and **Maureen Rombach** for the statistical assistance and preparation of the figures.

This study was partly funded by a grant from the Office of the Science Advisor, United States Agency for International Development.

RÉSUMÉ

Production de *Beauveria bassiana* [*Deuteromycotina* : *Hyphomycètes*] dans différents milieux liquides et production consécutive de conidies du mycélium sec

Le mycélium de *Beauveria bassiana* peut être développé en milieu liquide, filtré et le mycélium séché. Après réhydratation, le mycélium sporule.

Deux sources d'hydrates de carbone (sucrose et maltose) et une source azote/vitamine (extrait de levure) furent essayées pour la croissance du mycélium et la production consécutive de conidies. La croissance maximale du mycélium (12,31 mg/ml) en culture liquide, intervenait dans un milieu : sucrose (3,5 %)/extrait de levure (3,5 %), mais le mycélium issu d'un milieu maltose (2 %)/extrait de levure (0.75 %) produisait le maximum de $4{,}62 \times 10^6$ conidies/mg de mycélium sec après incubation en boîtes humides de Pétri.

En utilisant les données sur la production de mycélium (en culture liquide) et sur celle de conidies (par le mycélium sec), on a calculé que les milieux sucrose (3,5 %)/extrait de levure (3,5 %) et maltose (2 %)/extrait de levure (0,75 %) produisaient la plus grande quantité de conidies par volume de milieu (l'équivalent de $3{,}52\text{-}3{,}72 \times 10^7$ conidies/ml).

MOTS CLÉS : *Beauveria bassiana,* production de masse, fermenteur, mycélium sec, production de conidies.

Received : 16 April 1987 ; Accepted : 22 June 1987.

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