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Effect of liquid culture media on morphology, growth, propagule production, and pathogenic activity of the Hyphomycete, *Metarhizium flavoviride*

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Abstract

Two isolates of *Metarhizium* spp. were studied for propagule production, because of their pathogenic activity towards locusts and grasshoppers (*Mf189 = M. flavoviride* (or *M. anisopliae* var. *acridum*) strain *IMI 330189*, and *Mf324 = M. flavoviride* strain *ARSEF324*). Both isolates were grown in seven different liquid media, which have been developed for mass production of various Hyphomycetes, considered as candidates for microbial control of noxious insects. Shake-flask experiments were carried out at 28 °C in the dark. Production was quantified for 72 h and the effects of the tested media were evaluated on propagule concentration, morphology and pathogenicity. Based on preliminary experiments, all tested media were supplemented with 0.4% Tween 80 to avoid the formation of pellets and to produce unicellular propagules. Submerged propagule yields were higher with *Mf189* than with *Mf324* in all seven media. While high concentrations of propagules (1.4 to 2.4 $\times$ 10^8 propagules ml^{-1} for *MF189* and 1.4 to 8.3 $\times$ 10^7 propagules ml^{-1} for *Mf324*) were produced in four media (Adamek, Catroux, Jackson, and Jenkins–Prior media), production of propagules was lower in the three other media (Goral, Kondryatiev, and Paris media). Both isolates produced oblong blastospore-like propagules, except in Kondryatiev medium in which they provided ovoid propagules. In this case, *Mf189* submerged propagules looked like aerial conidia, but scanning observations did not demonstrate a typical conidiogenesis via phialides. In Kondryatiev medium, *Mf324* submerged propagules were significantly smaller than aerial conidia. Infection potential of submerged propagules was assayed on *Schistocerca gregaria*. Second-instar larvae fed for 48 h on fresh wheat previously contaminated by a spraying suspension of each inoculum titrated at 10^7 propagules ml^{-1}. All seven media produced submerged propagules that were highly infectious for *S. gregaria* larvae. Shake flask culture assays permitted us to select three low-cost media, Adamek, Jenkins–Prior, and Catroux for improving scale-up of liquid fermentation focused on mass-production of *Metarhizium* propagules for mycoinsecticides devoted to locust control.

Key words: *Metarhizium flavoviride*, *Metarhizium anisopliae* var. *acridum*, liquid media, mass-production, propagule morphology, pathogenic activity, microbial control, *Schistocerca gregaria*

Introduction

The entomopathogenic hyphomycete *Metarhizium flavoviride* Gams and Rozsypal (*Metarhizium anisopliae* (Metschnikoff) Sorokin var. *acridum*, pro parte) is currently being investigated for biocontrol of locusts, especially *Schistocerca gregaria* Forskål and *Locusta migratoria* L (Orthoptera: Acrididae) [1–3]. For practical use of these biopesticides, it must be possible to produce high yields of propagules which are pathogenic towards target insects [4]. Because they can

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affect propagule quantity and quality, both medium composition and culture conditions require study.

When grown on solid media, *M. anisopliae* and *M. flavoviride* produce asexual spores by specialized sporogenous cells called phialides [5]. In submerged culture, these hyphomycetous fungi usually form either mycelial pellets or single cells or both [6–7].

This paper describes the influence of nutrition in liquid cultures on two isolates of *M. flavoviride* pathogenic to locusts and grasshoppers [2, 8–10]. This comparative study was carried out with seven different media originally improved for mass production of various isolates of *Beauveria* spp. [11–14), *Paecilomyces* spp. [15], and *Metarhizium* spp. [6, 7, 16, 17]. The effect of each medium was evaluated on concentration of propagules and on both morphological and pathogenic characteristics. Five of the seven media tested had not been studied for production of propagules of *M. flavoviride*. Thus this paper reports new elements about the production of this fungal species in liquid culture.

Materials and methods

Inoculum preparation and cultural conditions

Fungal isolates. Two isolates (*Mf189* and *Mf324*) of *M. flavoviride* were studied because of their infectivity towards locusts [8–10]. *Mf189* (1M1330189) was isolated from *Ornithacris cravoisi* Finot (Orthoptera: Acrididae) in Niger in 1988, and *Mf324* (ARSEF324) (= *M. anisopliae* var. *acridum*) was isolated from *Austracris guttulosa* Walker (Orthoptera: Acrididae) in Australia in 1979 [2, 10].

Culture media. Seven different media were tested (Table I). The Adamek medium [16] was originally described for producing submerged spores of *Metarhizium anisopliae* (Metschnikof) Sorokin and recently it was tested successfully using *M. flavoviride* [17]. The Jenkins–Prior medium was improved for liquid production of submerged conidia of *M. flavoviride* [7]. The Catroux medium, investigated for production of *Beauveria brongniartii* (Saccardo) Petch (= *tenella*), supported blastospore production [11]. The Paris medium (also called semi-synthetic medium) has also been studied for *B. brongniartii* [14]. The Goral medium was originally developed to obtain submerged conidiospores of *Beauveria bassiana* (Balsamo) Vuillemin [12]. The Kondryatiev medium was originally intended for production of conidia in submerged culture of *B. bassiana*, although the authors also observed some production of blastospores [13]. The recently developed Jackson medium was tested for production of desiccation-tolerant blastospores of *Paecilomyces fumosoroseus* (Wize) Brown and Smith [15]. The original composition of all these media was slightly modified by supplementing them with 0.4% Tween 80, because in a first series of cultures supplemented with 0.2% Tween 80 and 0.05% chloramphenicol we observed that the isolate *Mf324* produced mainly mycelial pellets. Moreover, in Catroux and Kondryatiev media, glucose was used as a carbohydrate source in place of sucrose and maltose, respectively.

Culture and sampling procedures. Shake-flask cultures were prepared by inoculating the medium with the suspension of aerial conidia to yield a final concentration of 10^6 conidia ml^{-1} culture. Flasks were then incubated on a reciprocal shaker (Biolafitte AT 640, Gourdon, Maisons-Lafitte, France) (90 ± 2 travels min^{-1}) at 28 ± 0.5 °C in the dark. Each series of experiments consisted of two 200-ml shake-flasks containing 70 ml of each specific medium and it was replicated at least twice over time. Thus samplings were taken on four shake-flasks for each tested culture medium.

Flasks were sampled after 72 h incubation for determination of concentration, morphology, and infectivity of propagules.

Production estimates. The culture flasks were sampled at 72-h culture for propagule concentration. Four 0.5-ml aliquots were removed from each flask. The aliquots were examined at 320 magnification and the propagules were counted using a hemacytometer. If propagule number was large, the aliquot was diluted before counting.

Morphological characteristics of propagules

Scanning observations. For scanning electron microscopy (SEM), propagule samples were centrifuged and washed twice. Precipitates were fixed at 4 °C for 12 h in 2% glutaraldehyde in a 0.2 M cacodylate buffer, pH 7.4, rinsed three times in the cacodylate buffer for 10 mn, and then postfixed in 2% OSO_4 for 1 h. Specimens were dehydrated through a graded series of ethanol, dried by the critical-point method, coated with a film

Table 1. Composition of seven media (g l^{-1} distilled water) of shake-flask cultures *M. flavoviride* isolates

Ingredients	Ademek	Catroux	Goral	Jackson	Jenkins–Prior	Kondryatiev	Paris
KH_2PO_4	–	6.8	2	2	–	2	0.36
$MgSO_47H_2O$	–	0.1	2	0.3	–	2	0.60
$Na_2HPO_412H_2O$	–	–	–	–	–	–	1.42
$FeSO_47H_2O$	–	–	–	0.05	–	–	–
$MnSO_4H_2O$	–	–	–	0.016	–	–	–
$ZnSO_47H_2O$	–	–	–	0.014	–	–	–
$CoCl_26H_2$	–	–	–	0.037	–	–	–
KCl	–	–	–	–	–	–	1.00
$CaCO_3$	–	2	–	–	–	–	–
$CaCl_2$	–	–	–	0.4	–	10	–
KNO_3	–	5	–	–	–	–	–
$NaNO_3$	–	–	5	–	–	5	–
NH_4NO_3	–	–	–	–	–	–	0.70
Casamino acids	–	–	–	13.2	–	–	–
Sucrose	–	–	–	–	30	–	–
Glucose	20	20	20	80	–	20	20
Maltose	–	–	–	–	–	–	–
Corn steep liquor	15	20	9	–	–	9	–
Yeast extract	20	–	–	–	20	–	5
Vitamin mixture*	–	–	–	3.15	–	–	–
Tween 80°	4	4	4	4	4	4	4

Vitamin mixture: thiamine, riboflavin, pantothenate, niacin, pyridoxamine, thiotic acid, 0.5 mg l^{-1} each; folic acid, biotin, vitamin B12, 0.05 mg l^{-1} each.

of gold palladium, and examined with a Zeiss DSM 950 operated at 15 kV.

Propagule size and shape. The length and width of 20 propagules per aliquot (four 0.5-ml aliquots per flask), was measured using an ocular micrometer at 720 × magnification.

An electronic particle counter (Coulter Counter ZB, Coultronics France, Margency, France) was used to determine the number and size of fungal propagules suspended in an electrolyte solution [18]. The size of propagules was estimated as the diameter of an equivalent sphere. The apparatus was initially adjusted to 1.69 μm as the minimal size of *M. flavoviride* particles. The successive thresholds, corresponding to each size class, were automatically determined by the apparatus. After dilution and agitation, the particles of 4 replicated 0.5-ml samples of the suspension were counted, first to know the total number of propagules (size from 1.69 μm to 18 μm), then to know the number in size each class. Although these classes were not equal, the polygon of frequencies was established by transforming the numbers given by the Coulter Counter as percentages of the total number of counted propagules. This polygon represents the morphological distribution of the particles in the tested suspension.

Infectivity test method

Insect rearing. A laboratory colony of *S. gregaria* originated from insect eggs provided by the International Institute for Tropical Agriculture (IITA) laboratory in Cotonou (Benin) was maintained at 28/35 °C (12/12 h thermoperiod) in cages. Nymphs and adults were reared on a diet of wheat leaves and bran.

Preparation of the fungal inocula. Propagules were harvested after 72 h of submerged cultivation in the most productive media by centrifugation at 3,500 rpm for 15 mn (Jouan CR 1000, Jouan, St Herblain, France), and then resuspended in distilled sterile water. These suspensions were filtered on a piece of polyamid tissue because of the presence of excess of mycelium (individual hyphae or pellets). Ten ml of each suspension was placed in a 45-ml glass vial with approximately 50 glass beads (3 mm diam.) and agitated for 5 mn at 700 oscillations per mn on a

mechanical shaker (10 cm vertical travel). Propagule concentrations were estimated using a hemocytometer and suspensions were then adjusted to 10^7 propagules ml^{-1}.

Locust inoculation. Two lots of 10 second-instar larvae of *S. gregaria* were exposed for 48 h to fresh wheat leaves (4.0 g of 10–15 cm long leaf blades per group of ten larvae) previously sprayed with a suspension of each fungal inoculum titrated at 10^7 propagules ml^{-1} (i.e., providing a deposit of 3×10^4 propagules cm^{-2}) [9]. Controls consisted of larvae treated with water only. These tests were replicated four times.

Locust larvae were placed in cylindrical cages (8-cm high and 10.5-cm diameter). Fresh wheat was added daily. Dead larvae were removed daily and were transferred to dishes kept at saturated humidity at 25 °C. Cadavers were examined for presence of a sporulating layer of *M. flavoviride* 4–8 days post mortem. All experiments were terminated 10 days after inoculation.

Analysis of data

Log-10 transformed propagule counts, lengths and widths of propagules, and angular transformed mortalities (arcsine square-root on rates) were analyzed by analysis of variance. The Student–Newman–Keuls (SNK) multiple-range test ($\alpha = 0.05$) was used to compare means [19].

Results

Production of propagules. Culture components affected the growth of both *M. flavoviride* isolates (Table 2). In most tested media, supplemented with 0.4% Tween 80, *Mf189* produced more submerged propagules than *Mf324*, and in both cases it depended on the culture components (medium effect after 72-h incubation: $F = 41.11$; df = 6,21; $P < 0.001$ for *Mf189*; and $F = 8.87$; df = 6,21; $P < 0.001$ for *Mf324*) (Table 2).

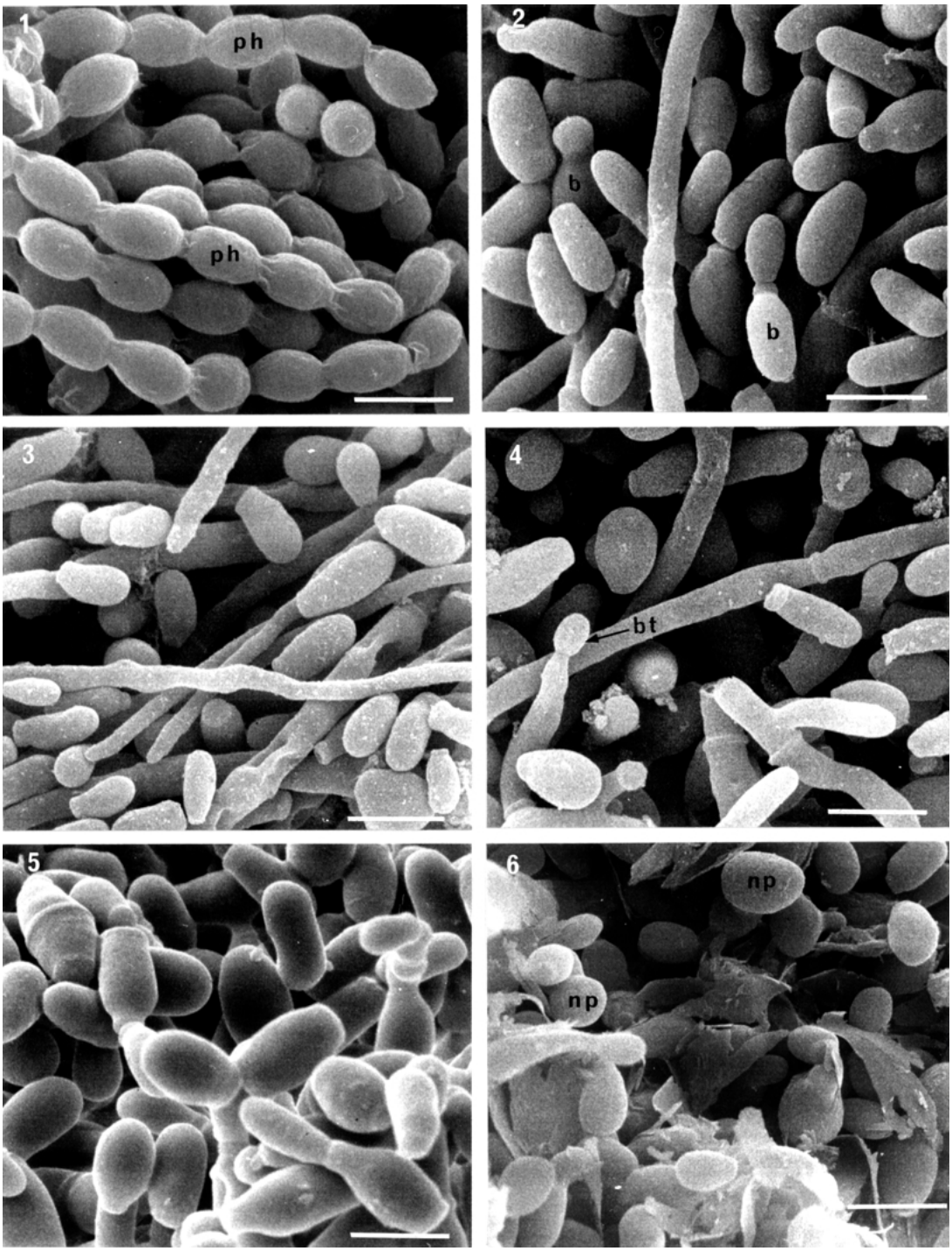
Mf189 produced many submerged propagules, except in Kondryatiev and Paris media. *Mf324* produced submerged propagules in all media, except in Paris medium, in which blastospore production was erratic and occurred in some culture flasks, while other flasks provided large quantities of pellets.

Highest concentrations of *M flavoviride* propagules were harvested after 72 h incubation from Adamek, followed by Catroux, Jackson, and Jenkins–Prior media (Table 2). The maximal yield, observed after 72 h culture of *Mf189* in these media, was ca. 300 times greater than the initial concentration. When the concentration of propagules at the end of the incubation period (72 h) did not differ significantly from that of the culture just after inoculation (10^6 propagules ml^{-1}) (e.g. *Mf324* culture in the Goral medium), investigations on the morphology of propagules (see below) showed that they consisted of newly formed propagules.

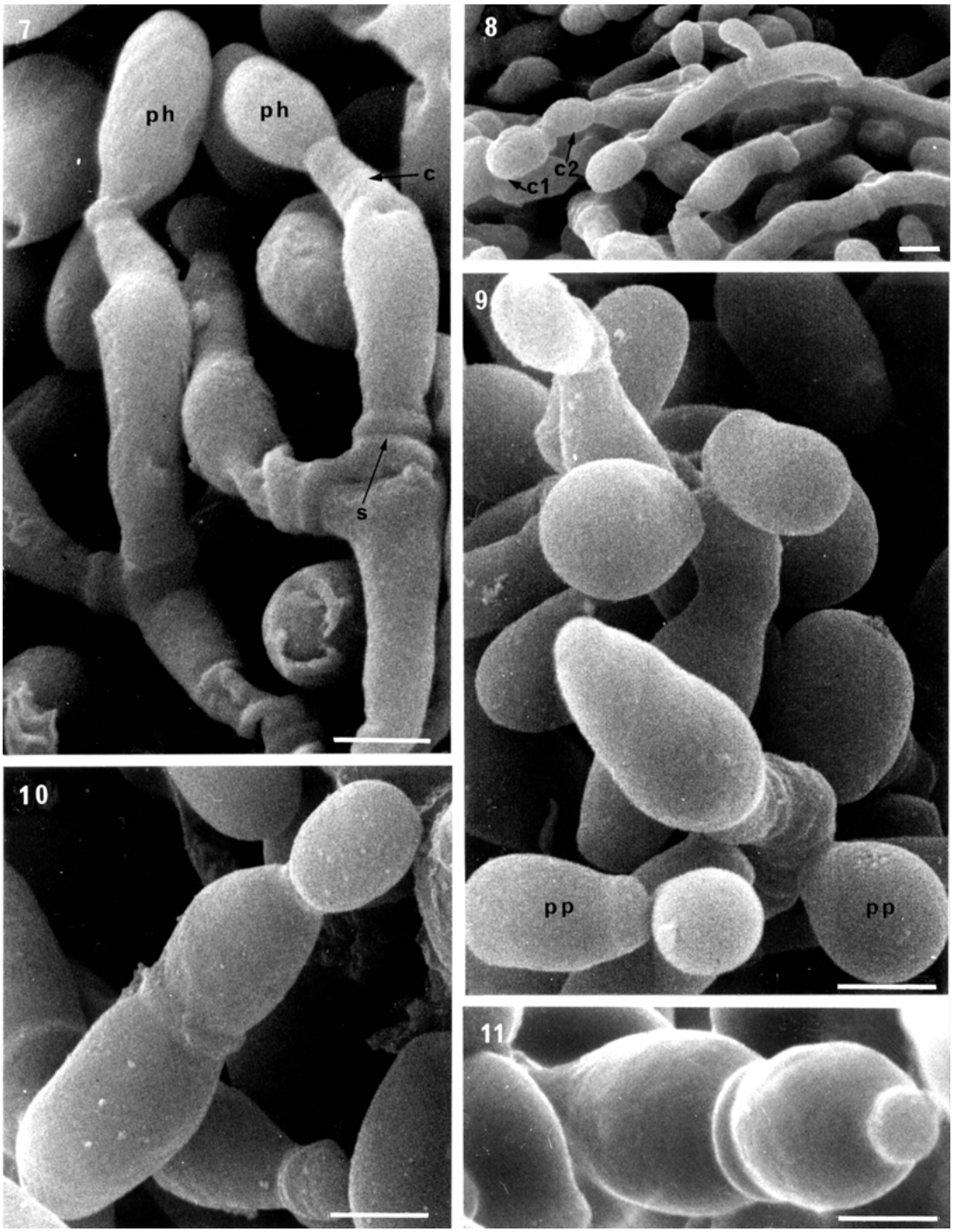
Propagule formation. Both isolates of *M. flavoviride*, *Mf189* (Figures 1–11) and *Mf324* (Figures 12–17) produced blastospores by hyphal constriction, separation at the septa, or yeast-like budding from hyphae in submerged culture (Figures 2–5, 8–11, 14–17). Under the liquid culture conditions, the isolates did not produce conidiospore-like propagules according to typical conidiogenesis, with chains of phialoconidia emerging terminally on elongated phialides as was observed in solid culture on semisynthetic medium (Figures 1, 7–8, 12–13).

In submerged culture, *Mf189* showed a greater variation in both growth form and propagule morphology than did *Mf324*. Among the four most productive media for the isolate *Mf189* (Adamek, Catroux, Jackson, and Jenkins–Prior), Adamek and Jenkins–Prior induced mainly yeast-like budding (Figures 2, 5, 10–11). In Catroux and Jackson media, this isolate also produced blastospores arising either terminally or pleurally branched hyphae or yeast-like propagules (Figures 3–4, 9). In contrast, in most tested media, the isolate *Mf324* formed mainly elongated cylindrical blastospores (Figures 13–17). In Kondriatiev medium, both isolates produced ovoid to spherical propagules (Figure 6) mainly, but not exclusively, at the tip of hyphae.

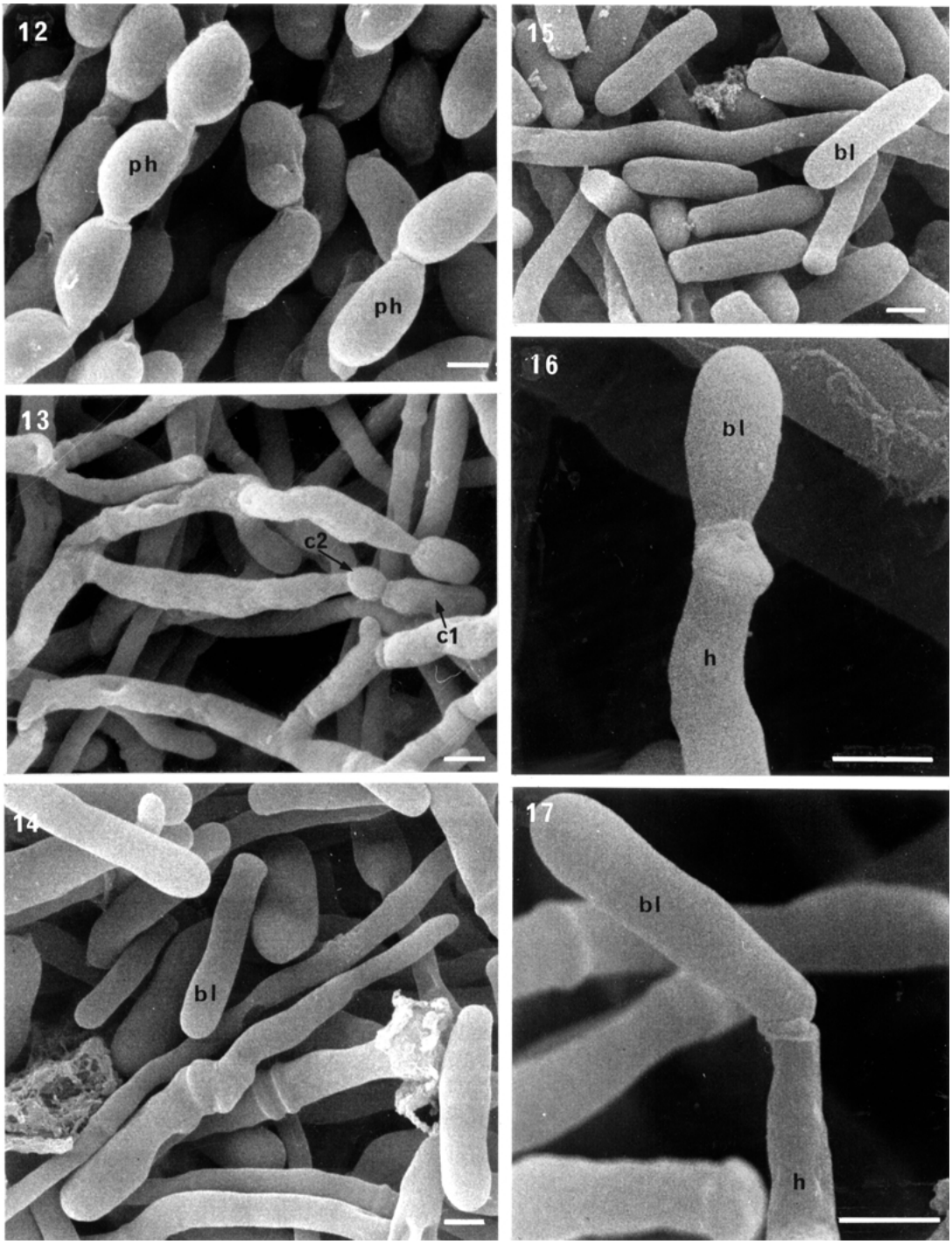
Propagule size and shape. Nutrition and culture conditions significantly affected propagule length ($F = 34.37$; df = 7,24; $P < 0.001$ for *Mf189* and $F = 39.62$; df = 7,24; $P < 0.001$ for *Mf324*) and width ($F = 16.40$; df = 7,24; $P < 0.001$ for *Mf189* and F = 10.64; df = 7,24; $P < 0.001$ for *Mf324*) (Table 2). Aerial conidia of *Mf189* and *Mf324*, harvested from Paris nutrient agar after incubation for 14 d at 25 °C, and used for initial inoculation, were 4.66 ± 0.27 μm long and 3.57 ± 0.26 μm wide, and 6.49 ± 0.25 μm long and 3.61 ± 0.32 μm wide (mean of two series of 4 samples of 30 propagules per sample ± SD), respect-



Figures 1–6. Propagule production of *Metarhizium flavoviride* (*Mf189* = IM1330189 = *M. anisopliae* var. *acridum*). Figure 1. Chains of aerial phialoconidia (ph) aggregated in columns, produced on agar culture in Paris medium. Figure 2. Blastospores in Adamek medium. Note budding propagules (b). Figure 3. Blastospores in Catroux medium. Figure 4. Blastospores in Jackson medium. Note a blastospore (bt) arising terminally on one hypha. Figure 5. Propagules in Jenkins–Prior medium. Note that budding produced discrete cells. Figure 6. Propagules in Kondriatiev medium. Note that the newly formed propagules (np) were ovoid to subspherical instead of ovoid to cylindrical as in the other liquid media. Figures 1–6 bar = 5 μm.



Figures 7–11. Propagule production of *Metarhizium flavoviride* (*Mfl89* = IM1330189 = *M. anisopliae* var. *acridum*). Figure 7. Branched filament with phialides producing phialoconidia (ph) on agar culture in Paris medium. Note the collarette (c) at the tip of the conodiophore and septum constriction at the bottom of the phialide (s). Figure 8 Formation of the first (CI) and the second (C2) conidia at the tip of a phialide in the same cultivation condition. Figure 9. Propagules arising terminally and pleurally (pp) on a short branched filament in Jackson medium. Figures 10–11. Yeast-like structures without immediate separation after the daughter cell has fully developed in Adamek medium (Figure 10), and in Jenkins-Prior medium (Figure 11). Figures 7–11 bar = 2 μm.



Figures 12–17. Propagule production of *Metarhizium flavoviride* (*Mf324* = ARSEF324): Figure 12. Chains of aerial phialidoconidia (ph) produced on agar culture in Paris medium. Figure 13. Basipetal succession of phialoconidia 1 (C1) and 2 (C2) at the distal tip of a phialide. Figures 14–15. Cylindrical blastospores (bI) in Adamek medium (Figure 14) and in Catroux medium (Figure 15) Figures 16–17. Apical emission of blastospores (bl) at the tip of hyphae (h) in Adamek medium (Figure 16) and in Jackson medium (Figure 17). Figures 12-17 bar= 2 μm.

Table 2. Effect of seven submerged culture media containing 0.4% Tween 80 on both propagule production and propagule size (×) of two *Metarhizium flavoviride* (*MF189* and *MF324*) at 72-h incubation

Culture media	Fungal isolate	Mycelial pellets	Propagule production CFU ml^{-1} (log)	Size of propagules*	
				Length(μm)	Width(μm)
Adamek	*Mf189*	No	2.4×10^8 (8.38 ± 0.12) a1	6.91 ± 0.10 a1	3.60 ± 0.29 b1,c1
	Mf324	No	2.4×10^7 (7.38 ± 0.44) a2,b2	9.66 ± 1.38 a2,b2	3.01 ± 0.20 d2
Catroux	*Mf189*	No	2.0×10^8 (8.30 ± 0.26) a1	5.93 ± 0.62 b1	3.76 ± 0.32 b1
	Mf324	No	3.3×10^7 (7.52 ± 0.32) a2,b2	9.32 ± 0.52 b2,c2	4.02 ± 0.83 b2,c2
Goral	*Mf189*	No	6.0×10^7 (7.88 ± 0.47) a1	5.53 ± 0.28 b1	3.60 ± 0.21 b1,c1
	Mf324	No	5.1×10 (5.71 ± 0.41) c2	8.39 ± 0.62 c2	3.35 ± 0.17 c2,d2
Jackson	*Mf189*	No	1.4×10^8 (8.17 ± 0.06) a1	6.61 ± 0.41 a1	3.24 ± 0.13 c1
	Mf324	No	1.4×10^7 (7.16 ± 0.15) a2,b2	10.8 ± 0.66 a2	4.74 ± 0.41 a2
Jenkins–Prior	*Mf189*	No	1.6×10^8 (8.21 ± 0.02) a1	6.93 ± 0.34 a1	4.03 ± 0.20 b1
	Mf324	No	8.3×10^7 (7.92 ± 0.05) a2	9.67 ± 0.20 a2,b2	3.61 ± 0.32 b2,c2
Kondryatiev	*Mf189*	No	1.1×10^6 (6.06 ± 0.09) b1	4.46 ± 0.19 c1	3.31 ± 0.18 b1,c1
	Mf324	No	1.2×10^7 (7.08 ± 0.05) b2	4.73 ± 0.43 e2	2.93 ± 0.31 d2
Paris	*Mf189*	No	1.2×10^6 (6.07 ± 0.66) bl	7.03 ± 0.36 a1	4.69 ± 0.18 a1
	Mf324	Yes/No	6.6×10^7 (6.82 ± 0.03) b2	10.11 ± 0.32 a2,b2	4.18 ± 0.05 a2,b2

(×) Means (± SD) in the column followed by the same letter are not significantly different (one-way ANOVA; Student-Newman-Keuls test, $P < 0.05$).
*Size of aerial conidia as a reference:
length = 4.66 ± 0.27 μm (c1), width = 3.57 ± 0.26 μm (b1,c1), for *Mf189*;
length = 6.49 ± 0.25 μm (c2), width = 3.61 ± 0.32 μm (b2,c2,d2), for *Mf324*.

ively. After 72 h incubation in most tested liquid media (Adamek, Catroux, Goral, Jackson, Jenkins–Prior, and Paris), both fungal isolates produced significantly longer propagules (blastospores) than aerial conidia used as reference. For these media, length and width of *Mf189* propagules varied from 5.53 to 7.03 μm × 3.24 to 4.69 μm and that of *Mf324* propagules from 8.39 to 10.80 μm × 3.01 to 4.74 μm. In contrast, the shortest submerged propagules (conidiospore-like propagules) were observed in the Kondryatiev medium, with 4.46 × 3.31 μm for *Mf189* and 4.73 × 2.93 μm for *Mf324*. The longest blastospores came from the Catroux medium and the largest ones from the Jackson medium.

Size characteristics of propagules, expressed as the diameter of the volume of the equivalent sphere, were determined after 72 h incubation. The mean diameter values of the equivalent sphere ranged from 3.40 μm (aerial conidia as Reference, Goral and Kondryatiev media) to 4.28 um (Adamek and Jenkins–Prior media) for *Mf189* (Figure 18), and from 3.80 μm (Reference and Paris medium) to 4.80 μm (Jenkins-Prior and Catroux media) for *Mf324* (Figure 19).

The size distribution, based on modal diameters, of the submerged propagules from all the liquid media was larger than that obtained with the air-borne conidia (Figures 18–19).

Pathogenic activity of propagules. Comparisons of pathogenic activities of propagules produced in the five most productive media for each isolate are shown in Figures 20 and 21. For both isolates, the propagules produced in any medium caused significantly higher cumulative mortality of all larvae compared to controls from day 5 for *Mf324* ($F = 6.45$; df = 5,26; $P < 0.001$), and from day 6 for *Mf189* ($F = 20.12$; df = 5,34; $P < 0.001$).

Because of the high pathogenic activity of propagules produced in any liquid medium, there was no significant effect of the composition of the liquid culture media on insect mortality ($F = 2.53$; df = 4,27; $P = 0.063$ for *Mf189*, and $F = 0.37$; df= 4,27; $P = 0.827$ for *Mf324*, at day 8).

Discussion

Propagule production in tested media

With both *M. flavoviride* isolates, nutritional conditions strongly influenced growth and morphology of newly-formed propagules. The result of our study corroborates similar research on the effect of nutrition and propagule production in *Metarhizium* spp. and other entomopathogenic hyphomycetes [6, 15, 18, 20, 21].

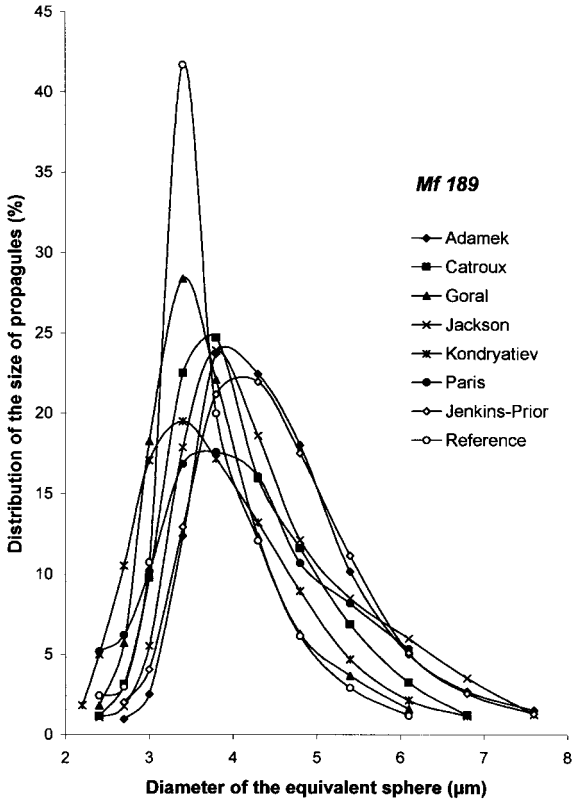


Figure 18. Effect of seven submerged culture media on size distribution of propagules of *Metarhizium flavoviride Mf189* based on the diameter of their equivalent sphere volume: Scanning of samples recorded after 72 h shake-flask culture at 28 °C. Aerial conidia issued from nutrient agar used as Reference.

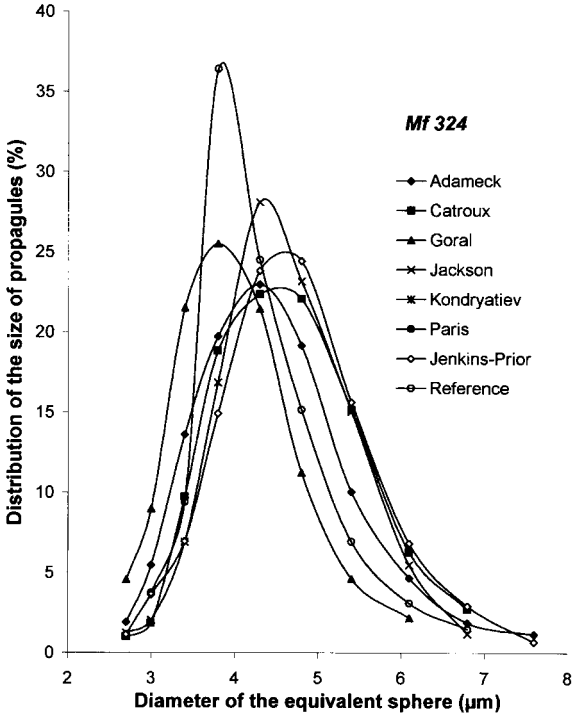


Figure 19. Effect of seven submerged culture media on size distribution of propagules of *Metarhizium flavoviride Mf324* based on the diameter of their equivalent sphere volume: Scanning of samples recorded after 72 h shake-flask culture at 28 °C. Aerial conidia issued from nutrient agar used as Reference.

As reported by Adamek [16] for *M. anisopliae*, we noted in preliminary experiments that the addition of Tween 80 to the liquid media changed the culture morphology of the isolate *Mf324* by suppressing the growth of pellets and enhancing propagule production. As the Tween 80 concentration increased, the propagule production of the isolate *Mf189* also increased. Changes in water activity based on glucose or polyethylene glycol concentration may also affect both fungal morphology and propagule yield [6, 22].

Four media (Adamek, Catroux, Jackson, and Jenkins–Prior) supported high propagule production of *Mf189*, with yields over 100 times after 72 h incubation. In contrast, *Mf189* produced few propagules in the Kondryatiev medium. With *Mf324*, all tested media induced relatively low propagule production. There was no significant production in the Paris medium for *Mf189* and in the Goral medium for *Mf324*.

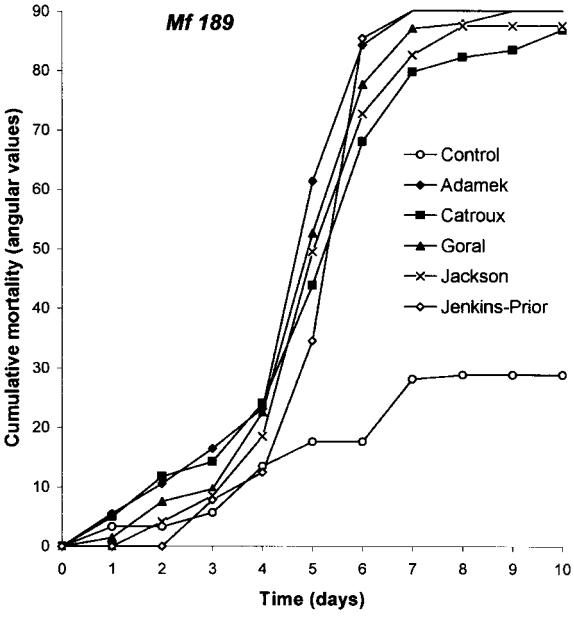


Figure 20. Infectivity of *Metarhizium flavoviride Mf189* propagules, produced in five liquid media, towards second-instar larvae of *Schistocerca gregaria*: Cumulative mortality recorded 8 days after 48 h exposure to contaminated fresh wheat leaves.

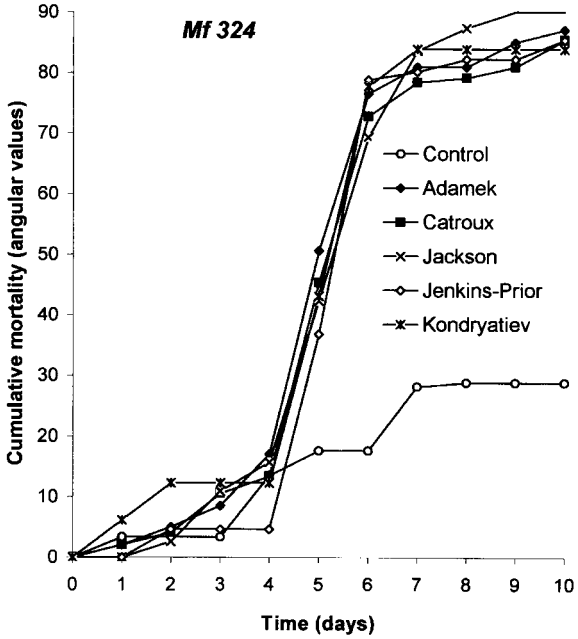


Figure 21. Infectivity of *Metarhizium flavoviride Mf324* propagules, produced in five liquid media, towards second-instar larvae of *Schistocerca gregaria*: Cumulative mortality recorded 8 days after 48 h exposure to contaminated fresh wheat leaves.

Jenkins and Prior [7] reported that the isolate IMI 330189 (= *Mf189*), incubated at 24 °C on a rotary shaker at 150 rpm produced 1.5×10^9 submerged conidia ml^{-1} after 7 days. Under these conditions, the intensive production of conidia occurred between days 3 and 6. The authors underlined that this high yield in submerged conidia was approximately one order of magnitude greater than figures quoted for blastospore productions in *M. anisopliae* [6, 22–23].

Blastospore yield with *Mf189* in the Jackson medium was similar to that reported by Jackson et al. [15] and Vidal et al. [18] with *P. fumosoroseus*. In the Catroux medium, it was also close to that observed with *B. brongniartii* [11], and *P. fumosoroseus* [18]. Depending on fungal isolates and both medium composition and culture conditions, the authors reported lower and higher propagule productions than observed in our tests [12, 13, 21, 24, 25].

Formation and morphology of submerged propagules

On nutrient agar, *Metarhizium* spp. phialoconidiogenesis was typical [26–27], producing enteroblastic conidia in basipetal succession from the tip of phialides [5, 28–29]. Aerial conidia of *Mf189* were subspherical to ovoid and *Mf324* conidia were mainly ovoid. In contrast with the typical asexual conidial ontogeny on solid media, there was in submerged culture a great variation in growth form depending on the composition of the culture medium [6, 30–31]. That was more demonstrative with the isolate *Mf189* than with *Mf324*. Our observations of *Mf189* revealed intermediate forms between yeast-like budding and emergence of blastospores from hyphal tips. In insect mycology the term blastospore has been commonly applied to any hyphal body generally produced in either insect blood or submerged culture via a process of hyphal budding, excluding the formation of phialoconidia [24, 32]. In lungal taxonomy, however, the term blastoconidia describes all types of propagules issued of a blastic development and thus does not exclude enteroblastic phialospores [26–27].

In all submerged media, except Kondryatiev, *Mf189* produced oblong to cylindrical blastospores. Similarly *Mf324* produced cylindrical blastospores, except in Kondryatiev medium. In Kondriatiev medium, propagules of both isolates appeared at the end of mycelial branches and detached very rapidly (because of culture shaking), making it impossible to observe possible formation of chains characterizing conidiation in Phialosporae on solid media [28–29]. Propagules produced in Jenkins–Prior medium under our culture conditions undoubtedly consisted of blastospores. In contrast to this study, Jenkins and Prior [7] observed phialide formation and submerged conidiation with the isolate IMI 330189 (= *Mf189*). This contradiction may be related to culture conditions, such as aeration (reciprocal shaker instead of rotary shaker) and temperature (28 instead of 24 °C). However, Jenkins (personal communication) observed similar changes in the type of propagules produced depending on the nature of the yeast extracts, and even on the batch for one manufactured yeast extract.

Influence of different components of media

We cannot be certain why the media differed in effects on both *Metarhizium* isolates, but some speculation is useful. Among the most favorable media for high propagule production of *Mf189*, there were no significant effects of the concentrations of glucose (80 g l^{-1} in the Jackson medium and 20 g l^{-1} in both Adamek and Catroux media). The unfavorable effect of yeast extract on production of biomass [33] could be involved in the low growth of *Mf189* in the Paris medium. However, the brewers' yeast extract of the Jenkins–Prior medium stimulated its blastospore production. The

Catroux medium, with 20 g of corn extract l^{-1}, produced more propagules than the Goral medium (with only 9 g l^{-1}), which seems to confirm the beneficial effect of this nutrient for production of blastospores [11, 30].

The composition of the tested liquid media did not determine the type of propagules produced by *M. flavoviride*. In contrast, the presence of nitrates, KNO_3 and $NaNO_3$, in the Goral and Kondryatiev media may favor formation of conidiospore-like propagules in *B. bassiana* [13, 25, 34], and in *P. fumosoroseus* [18].

Although the effect was statistically insignificant, propagules of *Mf189* (and to a lesser extent of *Mf324*) produced in Adamek medium tended to be more virulent than those produced in the other media. The influence of medium composition on pathogenic activity is poorly documented. However, in using several media varying in the carbon sources, Kmitowa [35–36] observed differences in the pathogenic activity of *P. fumosoroseus* blastospores towards *Galleria mellonella* L. (Lepidoptera: Pyralidae), but not to *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae). In contrast, when the nitrogen sources varied, differences in susceptibility appeared in both insects. Trinci et al. [37], comparing virulence and longevity of blastospores of *B. bassiana* produced in carbon- and nitrogen-limited batch-cultures, found similar median lethal doses (LC_{50}), but obtained significantly lower median lethal times (LT_{50}) in nitrogen-limited blastospores than in carbon-limited blastospores. Fargues and Robert [38] showed that soybean peptone induced a greater virulence of *Metarhizium* conidia to larvae of the scarabeid *Cetonia aurata* L. (Coleoptera: Scarabeidae) than did other nitrogen sources.

Mass-production technology is clearly one important way for improving biopesticides based on propagules of Hyphomycetes. The diphasic liquid-solid fermentation system seems promising for mass producing mycoinsecticides based on aerial conidia of *M. fiavoviride* for locust control [4, 39]. Nevertheless, short-term liquid fermentation could potentially provide economically blastospores in deep tank fermentors as an alternative for producing mycoinsecticides [4, 6]. Among the four most productive media for submerged culture of *Mf189*, three of them, Adamek, Jenkins-Prior and Catroux are simple in composition and cheap in price. They could be improved with minor changes for developing a low-cost medium for mass-production. Thus, shake flask culture assays permitted us to select appropriate media, which will be now submitted for scale-up evaluation for commercial development.

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