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Development of the entomopathogenic fungus *Beauveria bassiana* in liquid cultures

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Abstract

Growth and development of *B. bassiana* was followed in four liquid media: peptone, peptone-glucose, glucose and glucose-peptone-yeast extract. Six developmental stages were defined: (I) the unswollen conidium, (II) the swollen conidium, (III) emergence of the germ tube, (IV) elongation of the germ tube and formation of the first septum, (V) polar and bipolar elongation (growth) of the resulting mycelium and initiation of a blastospore and, (VI) seccession of that blastospore. Conidia of *B. bassiana* produced germ tubes in all liquid media. Blastospores were produced in all liquid media except glucose. In peptone-glucose, the yield of blastospores was four-fold higher than in glucose-peptone-yeast extract. However, biomass production was highest in peptone-glucose-yeast extract.

Introduction

Beauveria bassiana (Balsamo) Vuill. (Deuteromycotina), the cause of muscardine disease in insects has been used to control pests of a wide variety of crops [3, 6]. MacLeod [12] gave a detailed description of its growth on solid media and in insects: on solid media globose conidia are produced in succession for air-borne dispersal; in infected insects ellipsoid conidia are produced. Samsinakova [14] observed that in liquid cultures, *B. bassiana* produced only ellipsoid shaped conidia. These were initially named blastospores [14] a term which has been defined in several ways [8]. Because this term has been used to describe the ellipsoid conidia of *Beauveria bassiana* [3, 14, 16, 18], it is used in the sense throughout this paper.

Research on the production of extracellular enzymes [11], toxins [17], biomass yield in various media [4, 18], and commercial production of *B. bassiana* for use as a bioinsecticide [10] is undertaken with *B. bassiana* growing in liquid media. Stages of differentiation in *B. bassiana* may be correlated to product formation for commercial use. In spite of this, very little is known about fungal development and growth in liquid culture, and in particular, ontogeny of blastospores has not been investigated.

We have undertaken studies to define the time sequence differentiation and production of blastospores by *B. bassiana* in liquid media. We believe this to be the first report which describes morphological differentiation and development of *B. bassiana* in various liquid media.

Materials and methods

Preparation of inoculum

Beauveria bassiana, our laboratory strain designat-

ed GK2016, was inoculated on yeast extract-peptone-dextrose agar and allowed to grow for 7 days at 27 °C. Conidia were harvested by flooding the plate with 10 ml of 0.02% (v/v) Tween 80 in sterile distilled water. A bent glass rod was used to disrupt the conidia from the mycelial mat. The suspension was filtered through glass wool and adjusted to 10^8 conidia/ml using a haemocytometer for counting. Light microscopy was used to ensure that the stock conidial suspension was free of mycelia. When describing developmental stages the general protocol defined in Ainsworth and Bisby's Dictionary of the Fungi was followed [8].

Liquid media and fungal growth

Four liquid media were used to grow *B. bassiana*: 0.2% yeast extract-1% peptone-2% glucose medium (YPG), 1% peptone-2% glucose medium (PG), 1% peptone medium (P), and 2% glucose medium (G) (BDH Chemicals Canada Ltd) and concentrations were on a w/v basis. Ten ml of each medium was placed in 50 ml erlenmeyer flasks. Each flask was inoculated with 1% (v/v) of the stock conidial suspension. The fungal cultures were grown in a rotary shaker (New Brunswick Scientific Co., Inc. Edison, N.J.) at 180 rpm and 27 °C. A 0.1 ml sample from each flask was taken every 6 h for 48 h. Each sample was assessed by light microscopy (450 ×) for the number of individuals in class I–V as described in Table 1.

All counts were done in duplicate from two replicate flasks by non-overlapping scans of glass slides and scoring individuals in each developmental class. The class percentage was calculated by dividing the number of individuals counted for each class by the sum of individuals (N = 600) counted per time point × 100. Daily counts of blastospores (class VI) for each medium were done by light microscopy with a haemocytometer.

Dry weights were estimated daily by filtering 10 ml of culture fluid through preweighed membrane filters (.22μm, GS-type) and drying for 2 h at 90 °C.

Table 1. Classification scheme of *Beauveria bassiana* developmental stages.

Class	Description
I	Phase bright conidiospores, small, compact, 2–3 μm diameter.
II	Phase bright swollen conidiospore, spherical, 3–5 μm diameter.
III	Conidiospore with emerging germ tube, less than 10 μm length.
IV	Condiospore with elongated germ tube 10–30 μm length, 2–3 μm width.
V	Germ tube elongates to 30 μm (mycelia). Bipolar emergence, in some cases, of another germ tube. Blastospores appear laterally of terminally from mycelia.
VI	Phase bright detached blastospores, ellipsoidal, approximately 6.5 μm length, 2.5 μm width.

Blastospore differentiation

Blastospores were isolated from the PG medium (grown for 4 days) by filtering the culture through glass wool. The filtrate was centrifuged at 17000 × *g* for 30 min, washed with distilled water, and the pellet resuspended in distilled water. Light microscopy revealed a suspension which was 99% blastospores. The suspension was placed into P, G, PG or YPG. Samples from each medium were taken daily and the course of production and development of blastospores recorded.

Results

Table 1 describes the morphological changes leading to blastospore production in *B. bassiana.* Figure 1 shows phase contrast photographs of *B. bassiana* progressing through the six developmental classes during growth in YPG. The first stage of development is the conidia (Fig. 1a). They are 2–3 μm in diameter and mostly globose in shape. Under phase contrast, conidia inoculated into culture media showed little or no tendency to aggregate.

The onset of stage II is marked by the gradual swelling of the conidia (Fig. 1b and c). This stage is

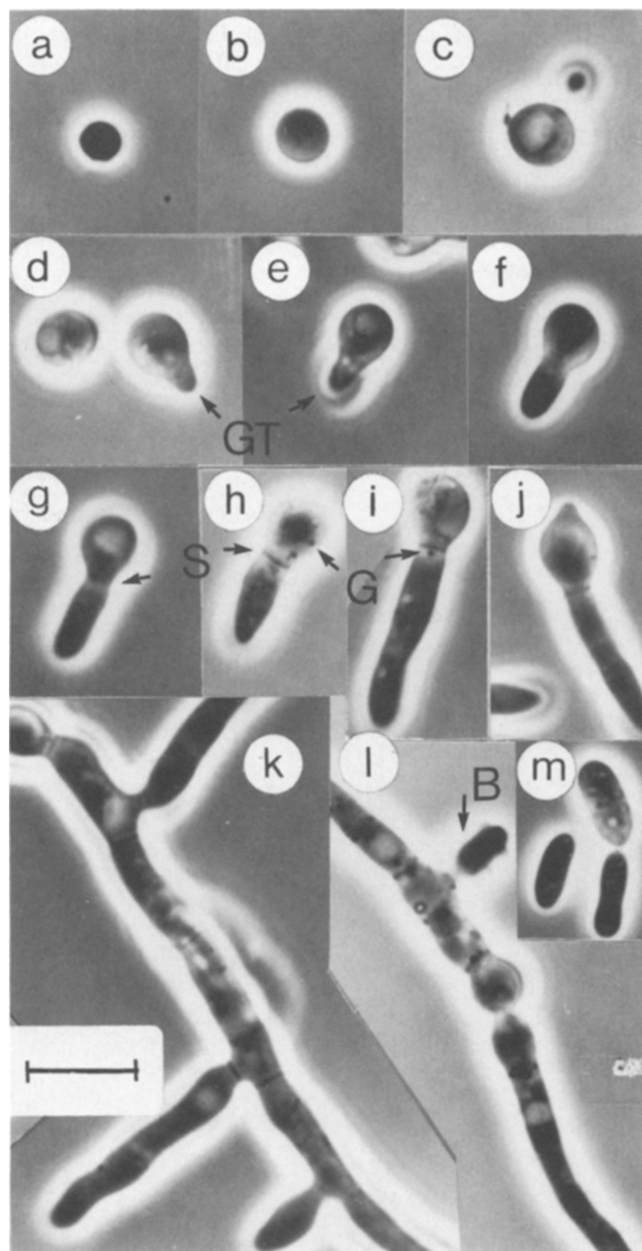

Fig. 1. Photomicroscopy of *B. bassiana* in various stages of development, described in Table 1, are shown: Stage I (a); Stage II (b, c); Stage III (d – f), arrow shows emergences of germ tubes (GT); Stage IV (g – i), arrow shows septation (S) and granule (G); Stage V (j – l B = blastospore); and Stage VI (m). Bar = 10 μm.

asynchronous throughout the population of spores in the media. In general, a 30 – 100% increase in the diameter of the conidia was observed. The swollen conidia do not lose their phase brightness, indicating no change in the refractile properties of the spores and their walls.

The emergence of a germ tube (Fig. 1d and e) from the conidium marks the start of stage III. In the early stages of germ tube emergence the conidium appears ampulliform (Fig. 1d). The germ tube is unipolar, physiologically attached to the conidium and septation is absent (Fig. 1f). The maximum length of the germ tube in this stage is less than 10 μm.

In stage IV, further elongation of the germ tube and septation are apparent (Fig. 1g–i). Mycelia can be 10–30 μm long and will contain 2–5 septa. Usually 1–3 dark cellular granules are observed near the septa which remain visible throughout stage V. The function of these granules remains unknown.

During the onset of stage V, bipolar growth of mycelia from the conidium can occur (Fig. 1j). Second polar growth begins much like that in stage III. After elongation of mycelia to 30 μm or more (Fig. 1k), the first blastospores appear laterally from mycelia (Fig. 1l). Mycelial septation occurs at approximately 6 μm divisions.

Stage VI is marked by the release of blastospores (Fig. 1m). The process of blastospore development as viewed by phase contrast microscope includes initiation, growth, physiological compartmentalization of the blastospore by a septum and finally, the physical separation of the blastospores from the mycelial base and release of a free blastospore. This process could be mediated by cell wall lytic enzymes or as result of outgrowth of the blastospore from the mycelial base. Detached blastospores are ellipsoid and phase refractile. With phase contrast microscopy, granules and organelles are visible in the blastospore.

The effect of different media on the time course of development was assessed (Fig. 2). The duration of stage I is medium dependent, lasting on average,

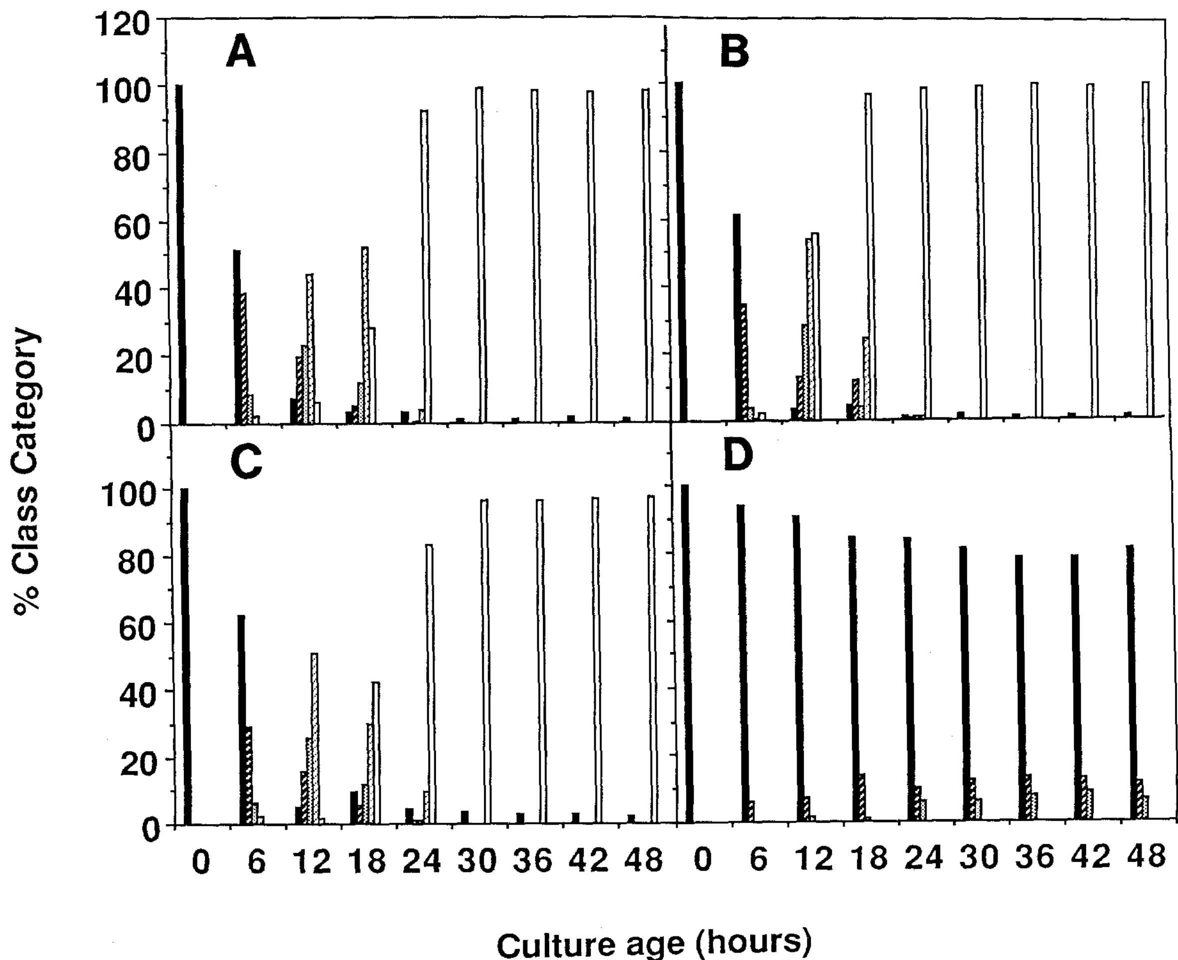

Fig. 2. Percentage frequency of *B. bassiana* developmental class types during a time course of growth in liquid culture containing (a) YPG, (b) PG, (c) P or (d) G. Stage I ■ ; Stage II ▨ ; Stage III ▩ ; Stage IV □ ; Stage V ▧ .

6 h in P, PG and YPG, and throughout the time course of the experiment for most conidia in G (Fig. 2). From 6–12 h, germ tubes emerged from conidia in P, PG and YPG media. The transition from one class to the next was expedited if glucose or glucose and yeast extract were present with peptone in the liquid media. For example, of the observed class types, 90% are class V by 30 h in P (Fig. 2). However, in PG and YPG, the 90% figure for class V is achieved by 24 h postinoculation. Conidia inoculated into G never progressed past class III as shown in Fig. 1f (Fig. 2).

At day one post-inoculation blastospores arise, without a pedicel, laterally and terminally directly from mycelia (Fig. 1k) and are observed in all cultures, except G. Blastospores are non-motile, hyaline, smooth, thin-walled, single-celled, ellipsoid bodies, varying from 5.0–7.4 μm in length ($\bar{X}$ = 6.45 μm) and from 2.0–2.6 μm in width ($\bar{X}$ = 2.33 μm).

Blastospore production is optimal between 4–6 days post-inoculation for all cultures, and is 2.5- and 4-fold higher in PG than in YPG and P respectively (Fig. 3). A decline in blastospore yield was observed after 4–5 days. However, in PG and YPG a corresponding decrease in dry weight was not observed (Fig. 4). Lower overall blastospore counts in YPG may be due to the rapid formation of mycelia from blastospores in the presence of yeast extract. The fate of blastospores in liquid culture was determined by inoculating isolated blastospores into P, PG and YPG. At day one post-inoculation of blastospores into YPG, more than 90% had mycelia. Mycelia had formed in 60%, 10% and 0% of the blastospores inoculated into PG, P or G respectively. This indicated that yeast extract is not necessary for blastospore production but expedited mycelial formation.

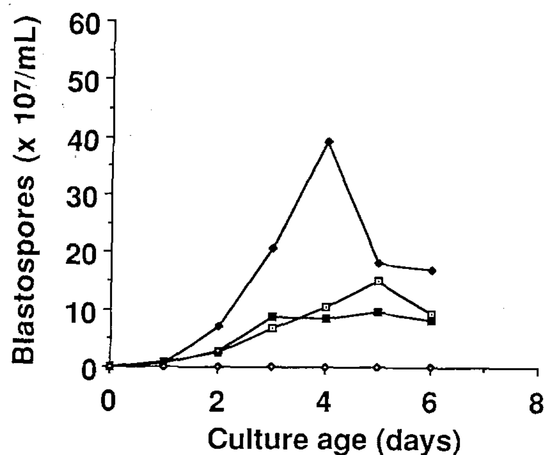

Fig. 3. Production of blastospores from *B. bassiana* during a time course of growth in YPG (■), PG (◆), P (□), or G (◇) liquid media.

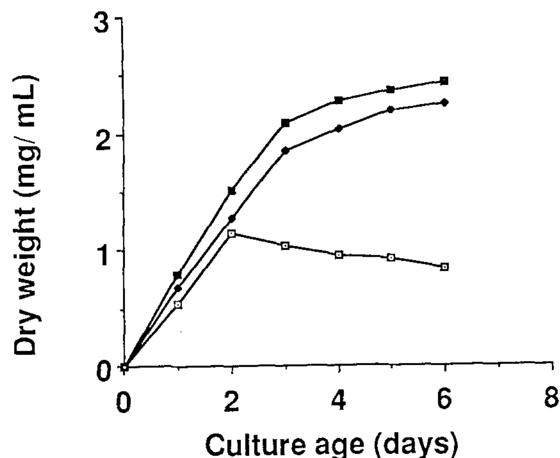

Fig. 4. Biomass production of *B. bassiana* grown in YPG (■), PG (◆), or P (□), liquid media.

Discussion

The development of *B. bassiana* in P, PG, YPG and G, has been described. A consistent pattern of differentiation in each media, although with differing time schedules, was observed. Initially, the conidia swell approximately twofold. Spores of the fungi *Penicillium griseofulvum* [7], *Mucor rouxii* [2] and *Aspergillus niger* [1] also increase approximately twofold to threefold in diameter during the first stage of germination.

Smith & Grula [15] observed that a carbon source could initiate germination in *B. bassiana*. However, a nitrogen source was necessary for mycelial growth. Our experiments confirm that a carbon source, such as glucose, can initiate germination. In the absence of an exogenous nitrogen source there are insufficient nitrogen reserves within the conidia for further mycelial growth. Many fungal spores germinate when incubated in a medium containing carbon and nitrogen sources [13] and yet

others are auto-activated by substances produced or associated with spores [5]. Kao & Leu [9] showed that more than 90% of sporangiospores of *Peronophythora litchii* germinated within 6 h in distilled water. The fact that less than 17% and 10% of *B. bassiana* conidia attain stage II and stage III, respectively, in G, indicates that these otherwise dormant conidia have received the stimulus which induces germination. The reason why most conidia do not germinate in this situation is unknown.

Smith & Grula [15] noted that vitamin B or other compounds which may be difficult to obtain under natural conditions were not essential for growth of *B. bassiana*. Our experiments show that yeast extract is not essential for blastospore production. In fact, the addition of yeast extract to PG lowered blastospore yield because of the formation of mycelia from blastospores. However, the addition of glucose to P increased blastospore yield. Blastospores are the only spore type observed in liquid media. This finding was previously reported by Samsinakova [14] for *B. bassiana* grown in corn steep liquor-minimal salts media, however, under different nutrient conditions, globose conidia formation can also occur [18].

The classification of morphological stages during a time course of germination and growth in *B. bassiana* may be applied to a variety of investigations. The isolation of morphological mutants which are arrested in a certain class of differentiation may be useful for genetic analysis in this fungus which has no known sexual cycle. The different morphological stages may be correlated to the production of toxins such as bassianolide [17], or extracellular enzyme production [11]. The time course of insect infection by *B. bassiana* may also be followed using a developmental stage classification.

Acknowledgements

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