Alternative Fruiting Pathways in *Phycomyces*[1]

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ABSTRACT

Developmental distinctions between giant and dwarf fruiting bodies of *Phycomyces blakesleeanus* (Burgeff) were studied by means of size measurements and growth analyses. Histograms of fruiting body lengths showed a bimodal distribution, with peaks around 0.3 millimeter (dwarfs) and 30 millimeters (giants). Individual cultures contain both giants and dwarfs. Differences between giants and dwarfs appear in the first phase of development; the apex of the giant is tapered, whereas the dwarf apex is dome-shaped. Probable cytological distinctions at this stage are cited in discussion. The dwarfs terminate enlargement upon expansion of the sporangium, thus lacking the subsequent phase of rapid elongation (stage IV) that contributes 90% of the length in the case of giants. It was concluded that *P. blakesleeanus* maintains two developmental patterns for asexual fruiting, with dwarfs and giants differing radically in growth regulation.

The giant asexual fruiting bodies of *Phycomyces blakesleeanus* have long been studied in the search for basic mechanisms of growth regulation (2). Experiments in this laboratory have led to the impression that *P. blakesleeanus* can produce two types of asexual fruiting bodies, one of which has previously been ignored in the literature. The second type may conveniently be termed the "dwarf" fruiting structure owing to its small stature compared to the better known "giant" type. Our interest has been drawn by the possibility of elucidating developmental mechanisms by comparing the physiology of the dwarf and giant fruiting pathways. A precedent for this anticipation may be found in the aquatic fungus *Blastocladiella emersonii*, where the ability to form two types of vegetative spore has been given intensive metabolic study (5). Relevant work on the giant and dwarf fruiting systems in *P. blakesleeanus* is in progress in this laboratory.

The first question must be whether the giant and dwarf fruiting bodies of *P. blakesleeanus* are truly distinct in development, or whether they are merely quantitative variants arising from a single pathway. At least two related species are known to have binary fruiting capabilities: *Choanephora cucurbitarum* produces both conidia and sporangia (1); and *Thamnidium elegans* produces sporangia as well as sporangioles (10). But the situation in *P. blakesleeanus* is less clear. The giant and dwarf fruiting bodies in this species are similar in general morphology. Further, the giant fruiting system can be made to produce dwarf-sized variants by germinating large numbers of spores in close proximity (2, 4, 8, 11, 14). Therefore if a real distinction can be drawn between "stunted giants" and "true dwarfs," then critical experiments are needed to show it. Such experiments are offered in this paper and form the essential basis for comparative studies of the dwarf and giant fruiting pathways.

MATERIALS AND METHODS

Cultures of *Phycomyces blakesleeanus* (Burgeff), American Type Culture Collection strain 8743a, were grown in 10-cm plastic Petri dishes on 20 ml of an artificial medium. Medium I was a 1:1 mixture of solutions A and B (see below). Medium II was a 1:1 mixture of solutions A and C. Solution A contained 15 g of Difco purified agar for nutritional studies plus 500 ml of distilled water, autoclaved for 15 min at 20 pounds pressure. Solution B was a similarly autoclaved solution of 50 g of dextrose, 15 g of KH_2PO_4, 5 g of $MgSO_4·7H_2O$, 2 mg of asparagine, 13.9 μg of thiamin HCl, and 500 ml of distilled water. Solution C was an autoclaved solution of 30 g of dextrose, 1 g of KH_2PO_4, 0.5 g of $MgSO_4·7H_2O$, 2 mg of asparagine, 13.9 μg of thiamin HCl, and 500 ml of distilled water. Inocula were provided as follows. The type strain was maintained in pure culture on potato dextrose agar medium. With a sterile 5-mm cork borer, plugs of PDA[2] medium containing the young growing tips of hyphae were cut and transferred to dishes of artificial medium, which served as intermediate stock cultures. Similar plugs, containing hyphal tips but not spores, were cut from the latter dishes for use as inocula in experimental cultures. Each culture received one centrally placed inoculum. All cultures were incubated in darkness. Temperature controls and variable details of incubation are described in the text.

An AO binocular dissecting microscope with variable magnification up to 60 diameters was used for microscopic work. An ocular micrometer calibrated at $\frac{1}{30}$ mm per scale division was used in measuring fruiting bodies below 3 mm in length; longer fruiting bodies were excised with watchmaker's forceps and were measured against a millimeter scale. Variable details of measurement and additional procedures for growth determination are described in the text.

RESULTS AND DISCUSSION

Grown in darkness at room temperature (15 to 26 C) on medium I, every culture of *P. blakesleeanus* produced both small and large fruiting bodies. After 3 or 4 weeks of incubation, all the fruiting bodies with mature sporangia were measured in a total of eight cultures. The results are presented in Figure 1 as a series of histograms on a logarithmic abscissa. Two of the cultures had been grown in tall cans to provide for free growth of large fruiting bodies (dashed curve). The

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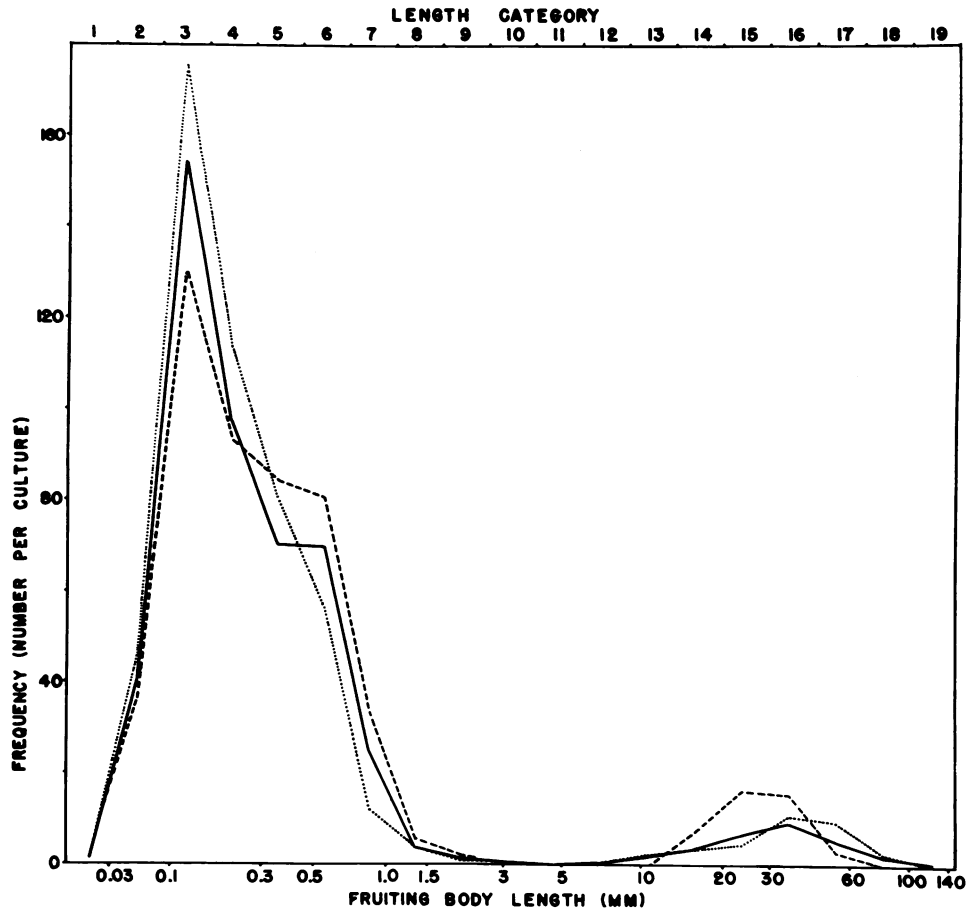[2] Abbreviation: PDA: potato dextrose agar.

Fig. 1. Lengths of fruiting bodies in mature cultures of *Phycomyces*. The upper and lower abscissae together show the limits of successive length categories; the length scale is logarithmic. Solid curve: average of eight cultures; dashed curve: average of two cultures grown in tall cans to allow free growth of large specimens; dotted curve: average of three cultures grown in closed Petri dishes.

rest, in two trials, were confined in closed Petri dishes. All the histograms in Figure 1 are bimodal. The larger peak, comprising over 90% of the fruiting bodies, represents dwarfs with an average length near 0.3 mm. The giants occupy a smaller peak with an average length near 30 mm. About 4,000 fruiting bodies contributed to Figure 1.

These data suggest that the dwarf and giant fruiting bodies fall into separate size ranges rather than forming the two ends of a continuum. However, the right-hand peak in Figure 1 could be an artifact of the logarithmic scale. The following test was conceived to discriminate between the above two interpretations. If the right-hand peak is a real clustering of fruiting bodies around a preferred (30 mm) size, then the specimens above 3 mm in length should yield a sigmoid cumulative distribution. If the population is unimodal with a long right-hand tail, then the cumulative distribution should be linear or concave downward.

For the test, additional cultures on medium I were incubated without lids in a sterile thermostat at 21.5 C, allowing free growth of large fruiting bodies. The fruiting bodies in sets of three or four cultures were measured after 2, 3, and 4 weeks of growth. The resulting cumulative distributions of specimens above 3 mm in length are shown in Figure 2. The numerous dwarf bodies, below 3 mm in length, are not shown in the figure. All the distributions are sigmoid. The observed deviations from linearity were examined by reference to the Kolmogorov-Smirnov test (3), which showed that the hypothesis of linearity can be rejected with better than 99.5% confidence in each of the curves of Figure 2. Further, the tend-

ency of the large fruiting bodies to cluster in the region from 20 to 65 mm and to avoid the 3 to 20 mm region was tested by reference to the binomial distribution. The first cultures to be harvested in Figures 1 and 2 were used to formulate test hypotheses; these cultures were omitted from the test data. The hypothesis that all sizes in the distribution are equally probable led to probability figures considerably below 0.001 for the data in each plot of Figures 1 and 2. Both statistical tests, therefore, support the conclusion that the giant and dwarf fruiting bodies in these cultures represent two distinct classes rather than the ends of a continuum.

To interpret the developmental relation between fruiting bodies in the dwarf and giant classes, it is necessary to know whether giant and dwarf specimens can be initiated in close proximity at the same time. A series of cultures were established on medium II in darkness at 21.5 C and were examined microscopically at 24-hr intervals, using dim green light for observation. The bottom of each dish was inscribed with concentric circles of radius 2, 3, 3.5, and 4 cm about the central inoculum. The locations of fruiting bodies were marked at daily intervals on matching maps.

Giant fruiting bodies first began to appear in a surge of initiations within 24 hr after the mycelium reached the radial limit of the nutrient medium. About 85% of the early giants stood beyond 3 cm from the inoculum. Occasional new giants continued to appear for several weeks, with the locus of initiation shifted from the perimeter to the central regions of the culture.

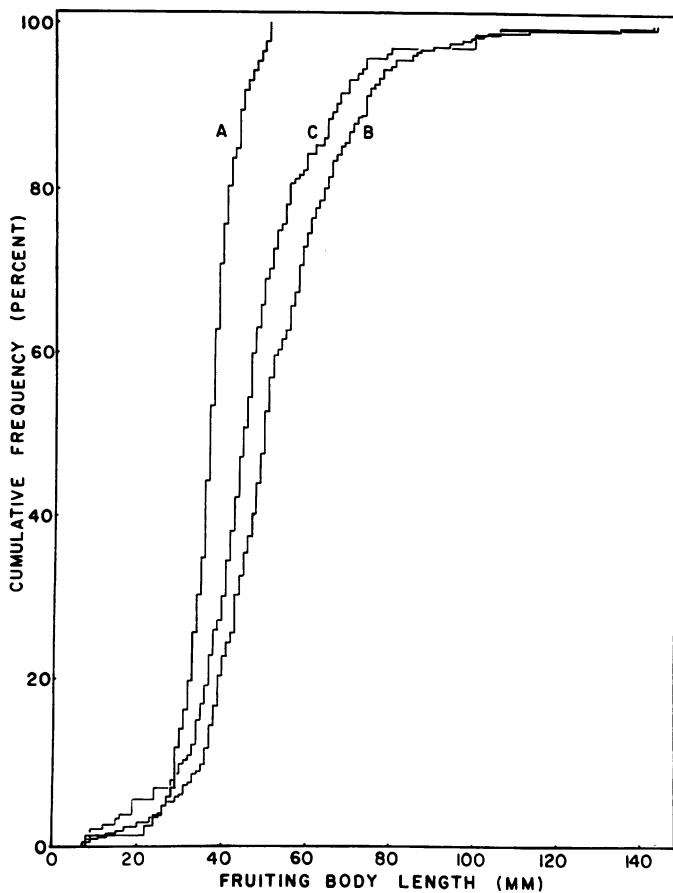In these cultures dwarfs appeared before giants, becoming

FIG. 2. Cumulative distribution of fruiting bodies in the size range above 3 mm. Data plotted as percentage of total. Curve A: total in three cultures, 2 weeks after inoculation; curve B: total in four cultures, 3 weeks after inoculation; curve C: total in three cultures, 4 weeks after inoculation. All cultures were grown in open dishes to permit free growth of large specimens.

abundant at first near the inoculum and steadily increasing in numbers throughout the culture during subsequent weeks.

About 4 days were required for the mycelium to reach the region beyond 3 cm from the inoculum. Between the 4th and 5th days 33 fruiting bodies were initiated between 3 and 3.5 cm from the inoculum in four cultures. Of these, 17 became giants and 13 became dwarfs. In the same time interval 151 fruiting bodies were initiated in the region between 3.5 and 4 cm from the inoculum. Of these, 34 became dwarfs and 111 became giants. In these regions giants and dwarfs often arose within a few millimeters of one another. Therefore, the initiation patterns of the giant and dwarf fruiting bodies differ radically but overlap sufficiently to negate the idea that giants and dwarfs are products of a single fruiting system as it acts under dissimilar environmental conditions.

Apart from size distinctions, the dwarf and giant fruiting bodies have also been found to differ in their mode of development. Giants display a well documented sequence of four developmental stages, involving two major shifts in the pattern of growth (7). In stage I, the giant is a tapered cylinder that elongates by tip growth. The first major shift in growth, inaugurating stage II, causes the extreme apex to balloon into a subspherical sporangium. Stage II merges into stage III, a period without growth during which the sporangium changes color from yellow to brown. The second major shift in growth inaugurates stage IV; the fruiting body begins a new phase of rapid elongation which continues for 24 hr or more.

Casual observation suggested that the dwarfs produced on medium I in darkness lack the second growth transition and consequently lack a developmental stage IV. Testing this impression, dwarf fruiting bodies were photographed at 30 diameters magnification at daily intervals after reaching stage III (specimens with brown sporangia). Cultures for this purpose were maintained on medium I at 10 C in darkness except during photography. The observations were terminated after 4 or 5 days, when the fruiting bodies showed signs of senescence (shrivelling of the sporangium). Prone specimens, identified at the conclusion of the experiment, were excluded from consideration. Pairs of successive photographs showing lateral movement of the fruiting bodies against the background of superficial hyphae were also deleted. With 11 specimens, 24 useful pairs of photographs were obtained, each pair representing the change in silhouette length of a specimen during a 24-hr interval. Four of these showed axial extension of the fruiting body. The most extreme instances (0.03 mm) represented about 2.5% of the length of the fruiting bodies in question. In three instances the micrographs showed a shortening of the silhouette length of the specimens. The mean change in length (0.002 mm) was less than the standard deviation (0.01 mm). Instances of apparent shortening suggested that the recorded silhouette changes were the consequence of changes in the leaning angle of the specimens. By this hypothesis, micrographs of horizontally-placed specimens would be expected to show occasional shortening but never extensions. Therefore, beside each of several new dwarf fruiting bodies, a pit was scooped from the nutrient medium. A sliver of microscope cover glass was pressed horizontally into the pit to preclude optical distortion, and the specimen was gently turned horizontally with its apex over the pit. A lump of agar was applied basally to hold the specimen in place. Photographic and incubation procedures were as before. As predicted, the 19 resulting pairs of micrographs showed no instances of axial extension during the period between stage III and senescence, whereas three instances of slight (0.03 mm) shortening were recorded. The mean change in length (−0.003 mm) was again less than the standard deviation (0.007 mm). Therefore it is highly probable that the dwarfs do not grow at all after the sporangium is formed.

Developmental stage IV can account for the size gap between giant and dwarf fruiting bodies. Under present conditions, the giants terminate stage I at lengths between 5 and 15 mm. Specimens occasionally reach 150 mm at maturity, so that 90% of the giant's growth derives from stage IV. A comparably proportioned stage IV in the dwarfs would have shifted the tallest dwarf specimens (3 mm in height) to 30 mm, well within the giant population. On the other hand, stage IV growth by the dwarfs would still have shifted the 0.3 mm population peak in Figure 1 only to about 3 mm. Thus the histogram would still have been highly skewed, if not bimodal, even if the dwarfs had a four-stage developmental pattern. Evidently the presence or absence of stage IV is but the last in a set of differences between giants and dwarfs that extend back to stage I.

In this connection, microscopic observations show that the apical growing zone of the stage I giant differs in structure from that of the dwarf. A well-defined pattern of axial and lateral wall expansion in the young giant gives the apex a complex taper (6). The dwarf, by contrast, is a simple cylinder with a hemispherically domed apex. These morphological differences must reflect dissimilar parameters governing the distribution of wall expansion (9), though attempts in this laboratory to define the pattern of expansion in the dwarf have been frustrated by a high tendency of applied markers to alter the growth of the domed apex.

Besides the difference in apical morphology, the stage I dwarf and giant differ in cytological structure. Phase optics show the giant to contain a mass of cytoplasm in the tapered apex, within which an ovoid lucent zone with a cluster of lipid droplets can be discerned. Electron microscopc surveys additionally show a cluster of nuclei just above the ovoid zone (13). By contrast, unpublished electron micrographs of dwarf specimens produced in this laboratory have shown neither an apical ovoid zone nor an apical cluster of nuclei.

The extensive differences between dwarf and giant fruiting systems suggested that the dwarfs might arise from a contaminant species rather than from *P. blakesleeanus.* Therefore individual dwarf and giant sporangia were plated in separate dishes of PDA medium. The two types of sporangia produced colonies that were morphologically indistinguishable. Strain G5 (+), used in previous physiological studies in this laboratory (13), and strain 8743b from the American Type Culture Collection, also produced dwarf fruiting bodies when plated on medium I. These results indicate that the dwarfs are products of *P. blakesleeanus* and are not contaminant fruiting structures.

In summary, the dwarf and giant fruiting bodies can be distinguished qualitatively by reference to the presence or absence of developmental stage IV, and by their dissimilar apical structure. Quantitative distinctions are also present in terms of differential apical morphology (which implies quantitative differences in the allocation of wall expansion), and in the discrete size ranges occupied by the two types of fruiting body. In the latter connection it must be recalled that conditions can be found under which the size distinction between dwarfs and giants vanishes. The giant fruiting pathway produces dwarf-like bodies when rich media are liberally seeded with spores. These stunted giants have been found by cinematic observations, however, to pass through a stage IV (8) and in that respect they are clearly distinguishable from true dwarfs.

It is concluded that *Phycomyces blakesleeanus* has two asexual fruiting capabilities, as in the pair of related species with binary fruiting patterns mentioned earlier. The dwarf pathway presumably escaped notice until this time through the customary provision of nutrient conditions under which the giant pathway is strongly preferred (*e.g.*, rich media). Appropriately, dwarf-like bodies appear in published figures of growth on deficient media (12).

Dwarfs and giants can be made to arise simultaneously in close proximity by suitable choice of growth conditions. However, the patterns of dwarf and giant initiation in aging cultures differ enough to suggest that the two fruiting pathways will achieve optimum expression under quite dissimilar conditions. This suggestion has formed the basis for a comparative study of factors that influence dwarf and giant initiation, the results of which will comprise the second paper in this series.

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