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The Production of Conidiophores and Conidia by Newly Germinated Conidia of *Aspergillus niger* (Microcycle Conidiation)

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SUMMARY

The ability of *Aspergillus niger* conidia to produce conidiophores after germination in shaken culture at 30° was stimulated by the inclusion of glutamate in the medium. Incubation of the conidia at 35° to 41° increased swelling of the conidia and also the proportion which produced conidiophores. Although conidiophore initiation was stimulated at temperatures between 35° and 41°, maturation was poor and optimum conidiation was obtained by incubation at these temperatures followed by 30°. Conidiophore formation from conidia required a prior period of spore metabolism and at temperatures between 30° and 41° did not occur until several hours after germination. Direct conidiophore production from conidia in the complete absence of vegetative growth was achieved by incubation of the conidia at 44° (which allows only swelling) for a prolonged period (48 h.) followed by 30°. Although vegetative growth was absent the conidiophores were similar to, but smaller than, normal subaerial conidiophores and viable conidia were produced. These conidia differed from subaerial spores in lacking the dark pigmented spore coat.

INTRODUCTION

The normal germination of *Aspergillus niger* van Tieghem (IMI41873) conidia in submerged shaken culture has been described (Anderson & Smith, 1971*a*). Dormant conidia (3·5 μm. mean diameter) increase in diameter to 4·0 μm. in deionized water by imbibitional swelling and in the presence of nutrients swell to 6·5 to 7·0 μm. before germ-tube outgrowth. The majority of conidia produce one or occasionally two germ tubes between 6 and 9 h. after incubation at 30°, and following a period of vegetative growth, the extent and duration of which will depend on environmental conditions, will produce conidiophores (the asexual reproductive apparatus).

During studies on the effect of temperature on the morphology of germination of *Aspergillus niger* conidia (Anderson & Smith, 1971*a*) it was observed that at temperatures from 38° to 43° the proportion of conidia which produced germ tubes gradually decreased and at 44° germ-tube formation was completely inhibited although swelling of the conidia continued to occur over a prolonged period to produce large spherical cells (20 μm. mean diameter). It was further observed that some conidia were able to directly produce conidiophores without prior mycelial formation and that this occurrence was stimulated at higher temperatures and in the presence of glutamate. The stimulatory effect of amino acids on conidiation of this fungus in submerged culture has been described (Galbraith & Smith, 1969). Conidiophore formation from the conidium could be detected at an early stage and differed from germ-tube formation since it involved a much larger bulging of the conidial

wall and the subsequent outgrowth was broader with thicker walls than vegetative mycelium. This ability of conidia directly to produce conidiophores has been examined in more detail and the results of this study are reported here.

METHODS

Organism and culture conditions. The fungus used was *Aspergillus niger* and the maintenance of stock cultures and the production of conidia were as previously described (Anderson & Smith, 1971*b*).

The culture medium contained in 1 l. deionized water: glucose, 10 g.; glutamic acid (monosodium salt), 5 g.; KH_2PO_4, 1·0 g.; $MgSO_4.7H_2O$, 0·25 g.; $CuSO_4.5H_2O$, 0·234 mg.; $FeSO_4.7H_2O$, 6·32 mg.; $ZnSO_4.7H_2O$, 1·1 mg.; $MnCl_2.4H_2O$, 3·5 mg.; $CaCl_2$, 46·7 mg.; $(NH_4)_2SO_4$, 1·98 g.; adjusted with HCl to pH 4·5. The medium constituents were autoclaved together at 121° for 15 min. except for glucose which was autoclaved separately and added aseptically. The medium was dispensed in 5 ml. quantities into glass boiling tubes (150 × 24 mm.). Except in experiments on inoculum size all cultures were inoculated to give 1×10^6 conidia per ml. of medium. The tubes were sealed with rubber stoppers and the cultures agitated by clamping on to a Griffin flask-shaker. Temperature control (± 0·3°) was obtained by immersing two-thirds of the tube length into a water bath. This culture system gave good temperature control and minimized spore clumping which readily occurred in this culture medium when the conventional shake flask system was used. Sealed tubes prevented loss due to evaporation at the higher temperatures and in prolonged experiments the tubes were flushed with sterile air every 12 h. to prevent oxygen limitation and accumulation of volatile metabolites.

Microscopy. Conidiophore production from conidia was assessed by direct counting of 100 conidia per sample. Conidiophore counts included both immature (conidiophore stalk) and mature (conidiophore stalk with vesicle, phialides and conidia) structures. A conidiophore was scored as present if it was as long as it was broad. Each experiment was carried out in triplicate. Measurements of conidiophore dimensions were made on 20 conidiophores per sample, with a microscope fitted with an ocular micrometer. The above measurements were made on freshly prepared samples or on samples stored in 4% formaldehyde at 4°. All photographs were taken on freshly prepared samples using phase-contrast microscopy.

RESULTS

Effect of glutamate on conidiophore initiation

The presence of glutamate approximately doubled the number of conidia with conidiophores although the time of production (12 to 18 h.) remained the same (Table 1). The conidiophores produced at 30° were usually not the fully formed asexual reproductive structures of *Aspergillus niger* but only shortened conidiophore stalks without vesicle, phialides or conidia.

Effect of temperature

High temperature has marked effects on conidium swelling and germ-tube morphology (Anderson & Smith, 1971*a*), swelling being increased and formation of germ tubes decreased. The production of a conidiophore occurred more often from these enlarged conidia than from the normally germinating conidia when both were at 30°. At temperatures which allowed both swelling and at least partial germ-tube formation (30° to 41°) higher temperatures resulted in greater conidiophore production from conidia (Table 2). At 44°

conidia swelled but no germ tubes or conidiophores were produced. The formation of vesicles and phialides occurred better at 35° and 38° than at 41°. At 41° although conidiophores reached normal lengths most were unable to differentiate the final stages of the reproductive structure. This resulted in aberrant conidiophore tips (Fig. 1, 2).

Table 1. *The production of conidiophores from conidia of Aspergillus niger at* 30° *in culture media with and without* 30 mM-*glutamate*

	Percentage of spores producing conidiophores					
Time (h.)	6	12	18	24	30	36
Complete medium	0	0	29	32	35	32
Complete medium minus glutamate	0	0	13	12	16	14

Table 2. *Effect of temperature on the production of conidiophores from conidia of Aspergillus niger*

	Percentage of conidia producing conidiophores				
Time at initial* temperature (h.)	5	10	15	20	40†
Initial temperature					
30	33	30	37	32	35
35	36	51	58	48	52
38	44	66	59	63	61
41	38	88	95	90	78
44	31	59	61	74	0
47	36	34	29	31	0

* Following incubation at the initial temperature for the time stated (5 to 20 h.) conidia were incubated at 30° for a further 24 h.

† Conidia exposed to the initial temperature for 40 h., however, were not incubated further.

Since conidiophore initiation was stimulated in the enlarged spores at higher temperatures but maturation was better at lower temperatures, experiments were conducted to find the best combination of temperatures (Table 2). An initial incubation at temperatures between 35° to 44°, especially if for 10 h. or more, increased the number of conidiophores which eventually developed at 30°. At 47° no swelling occurred (the conidia remaining dormant) and no increase over the control (30°) value was obtained when these conidia subsequently germinated and developed at 30°. The optimum temperature combination was found to be a 15 to 20 h. incubation at 41° followed by 30°. With this combination 90% or more of the conidia produced fully mature conidiophores (Fig. 3), with good synchrony. Conidiophore production began about 16 h. after inoculation (Table 4) and complete maturation of almost all of the conidiophores had occurred by 26 h. after inoculation. Although the ability of a conidium to form a conidiophore was increased by incubation at 41° and 44° the actual formation of these structures occurred more readily at 30° (Table 2).

Effect of medium

The effect of varying the constituents of the culture medium on conidiophore production from conidia was assessed after incubation of the conidia for 15 h. at 41° followed by 24 h. at 30° (Table 3). The stimulation of conidiophore formation by glutamate observed at 30° (Table 1) was confirmed. Ammonium sulphate could be removed from the medium without effect, glutamate being an adequate nitrogen source for both germination and conidiation. Omission of glucose, which is required for maximal swelling of the conidia (Anderson & Smith, 1971*a*), decreased conidiophore production.

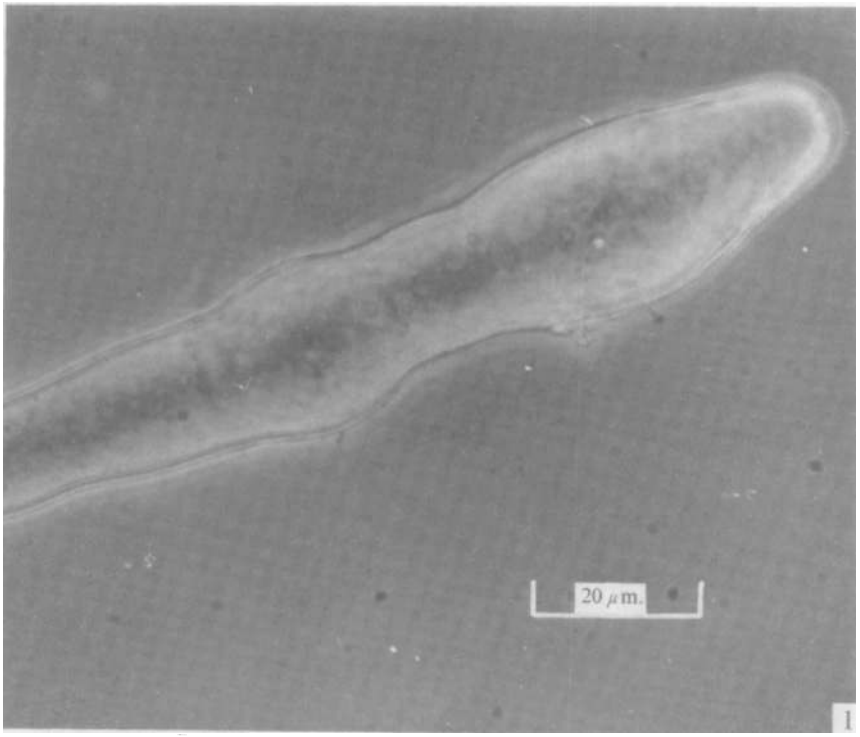

Fig. 1. Conidiophore stalk produced from a conidium of *Aspergillus niger* in submerged culture after incubation at 41° for 30 h. Normal vesicle formation is inhibited.

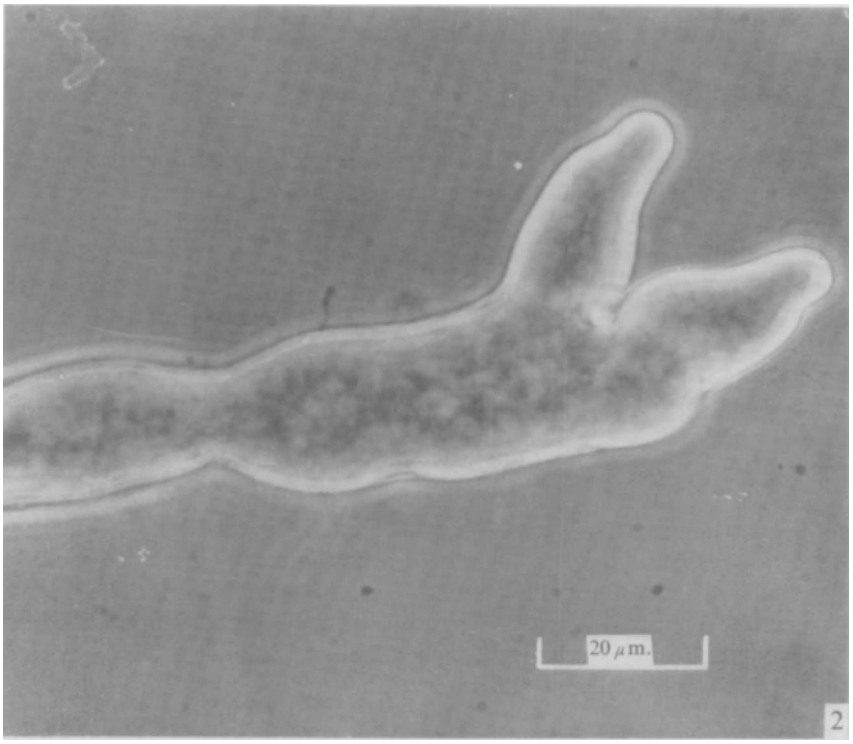

Fig. 2. Branching of the conidiophore tip after inhibition of vesicle formation.

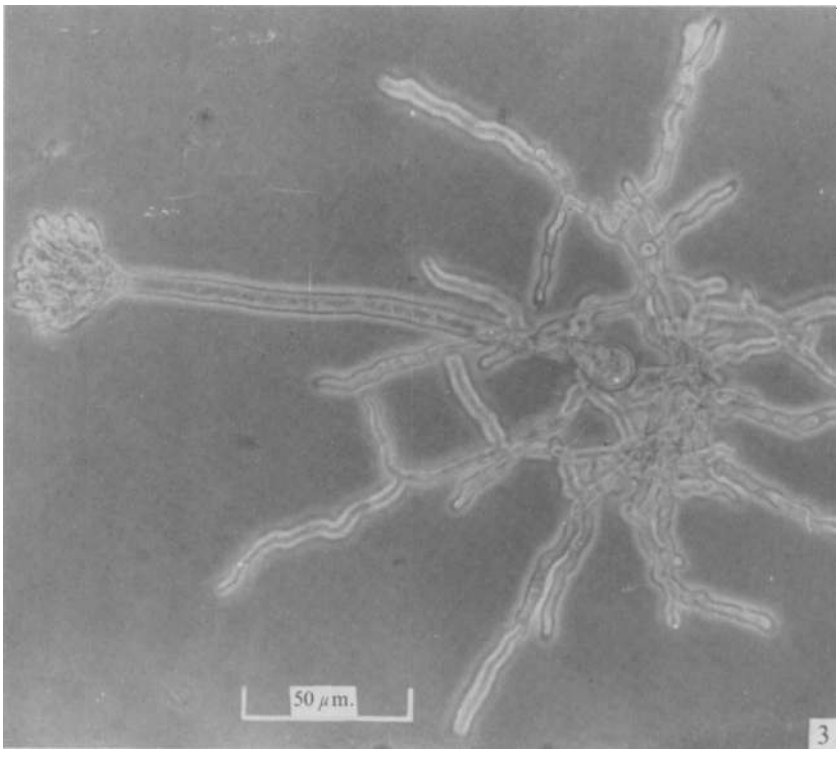

Fig. 3. Production of a branched mycelium and a mature conidiophore from a conidium. Incubation at 41° for 15 h. followed by 30° for 10 h.

Table 3. *Effect of medium composition on the production of conidiophores* from conidia of Aspergillus niger*

Medium	Percentage of conidia producing conidiophores
Complete	96
Complete–glutamate	41
Complete–ammonium sulphate	94
Complete–glucose	62

* 15 h. incubation at 41° followed by 24 h. at 30°.

Table 4. *Effect of an initial increase in temperature* on the time of production of conidiophores from conidia of Aspergillus niger*

	Initial temperature							
	30°	35°	38°	40°	41°	42°	43°	44°
Time of appearance of conidiophores (h.)	16	14	14	16	16	18	20	22

* 15 h. incubation at initial temperature followed by incubation at 30°.
(In 35° and 38° cultures conidiophores had been produced by 14 h. at the initial temperature.)

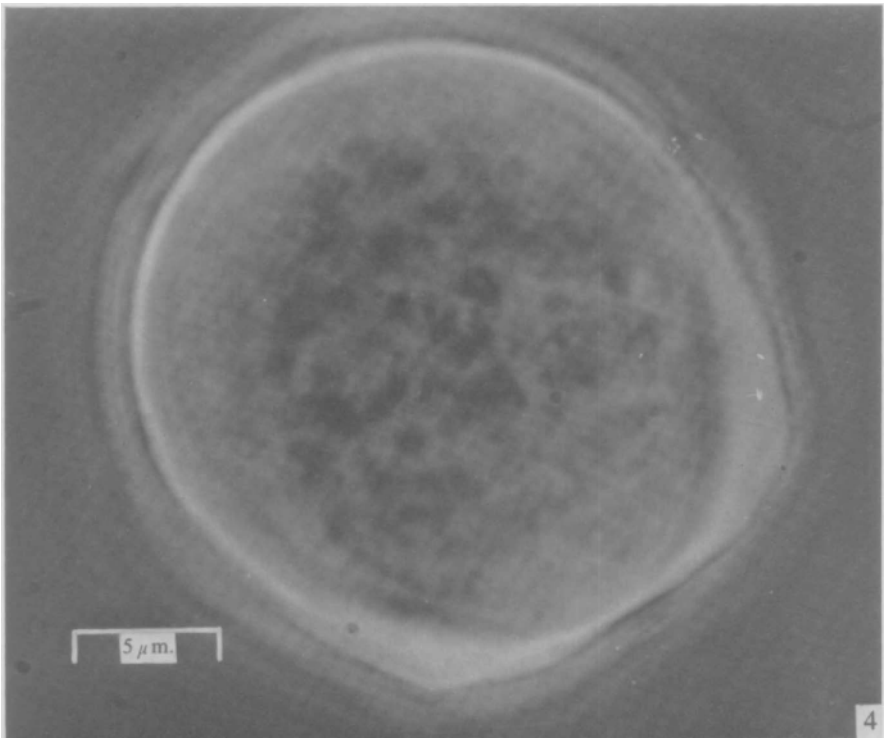

Fig. 4. Beginning of polarized wall development in an enlarged conidium prior to production of the conidiophore stalk. Incubation at 44° for 48 h. followed by 30° for 3 h.

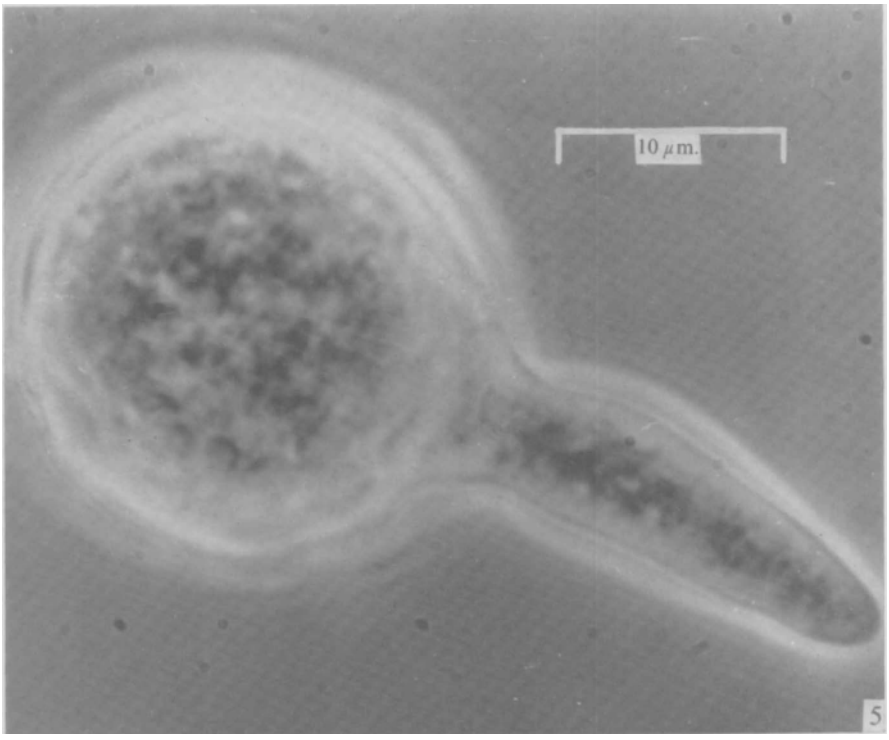

Fig. 5. Production of a conidiophore stalk from an enlarged conidium, 6 h. after transfer to 30°.

Time of conidiophore formation

The effect of temperature on the time at which conidiophores were produced from the conidia was investigated; 15 h. incubation at temperatures from 30° to 44° being followed by 30°. Cultures were examined every 2 h. and the time when 20% of the conidia had produced conidiophores noted. Conidiophores were produced first from conidia initially incubated at 35° or 38° (Table 4) and above 41° the time taken for conidiophore production increased markedly.

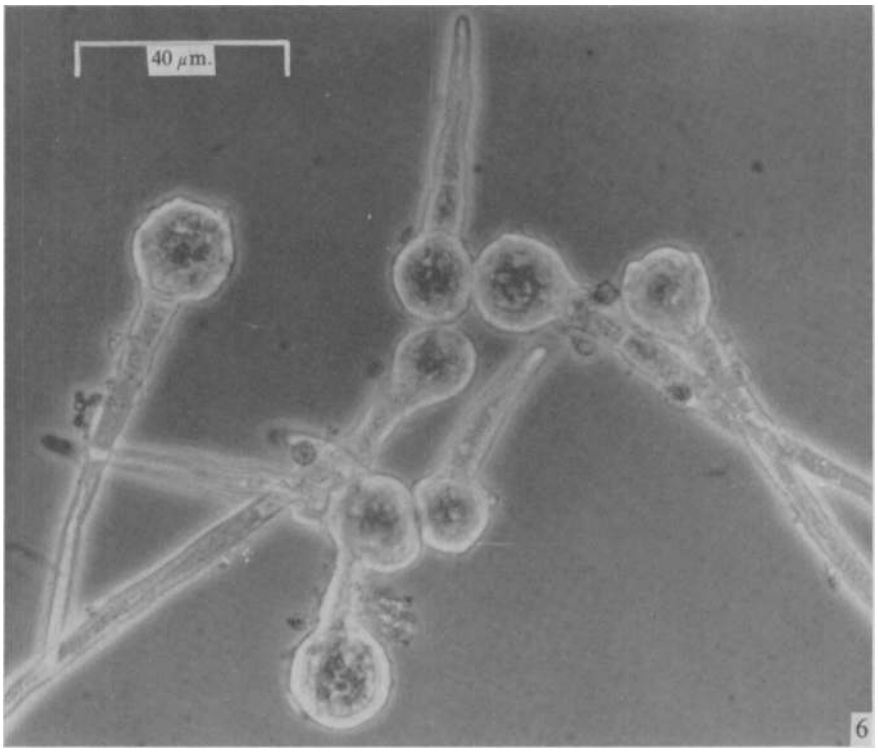

Fig. 6. Elongated conidiophore stalks from enlarged conidia, 9 h. after transfer to 30°.

Prolonged incubation of spores at 44°

Conidia were incubated for varying periods (12, 24, 36 and 48 h.) at 44° and transferred to 30°. After 12 h. and 24 h. incubation at 44° all conidia were able to produce germ-tubes at 30° and a number were also able to produce conidiophores. After 36 h. at 44° most conidia were still able to produce germ tubes as well as conidiophores at 30° and a few were able to produce conidiophores only. After 48 h. at 44° almost all of the conidia did not produce germ tubes at 30° but did produce conidiophores. The proportion (about 5 to 10%) which were still able to form germ tubes were the smallest conidia in the population. Conidiophore production from the conidia did not occur with complete synchrony indicating that some conidia were more advanced towards conidiation than others. This lack of synchrony and the ability of some of the smaller conidia to germinate and grow vegetatively (leading ultimately to complete vegetative overgrowth of the culture) most likely reflected a failure of the shaken-tube culture system to provide completely homogeneous conditions.

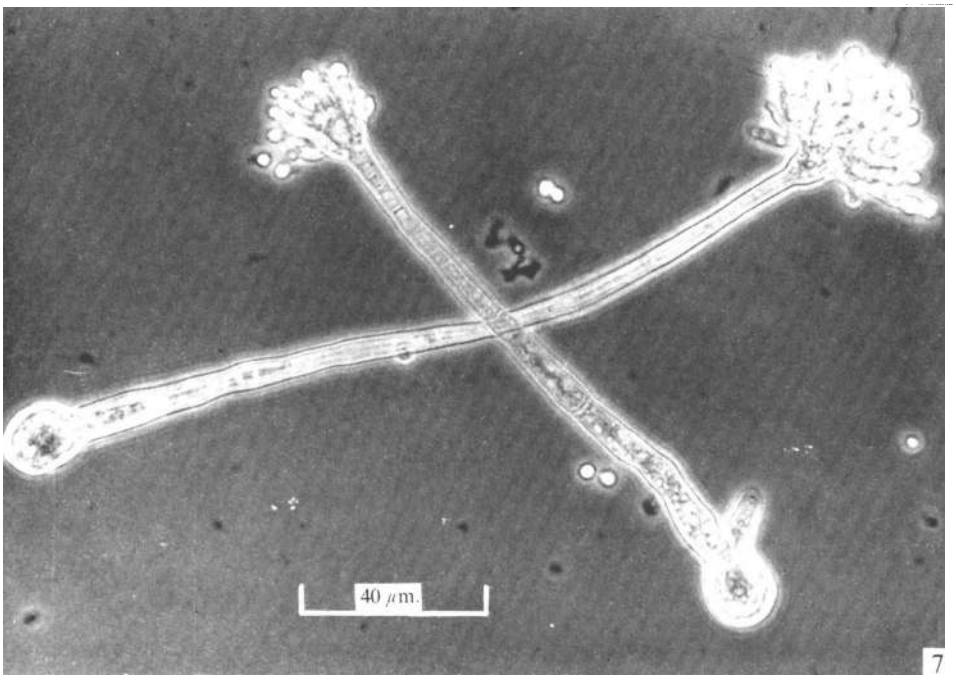

Fig. 7. Single mature conidiophores have been produced from two enlarged conidia, and a secondary conidiophore is developing from one conidium at the base of the primary conidiophore, 18 h. after transfer to 30°.

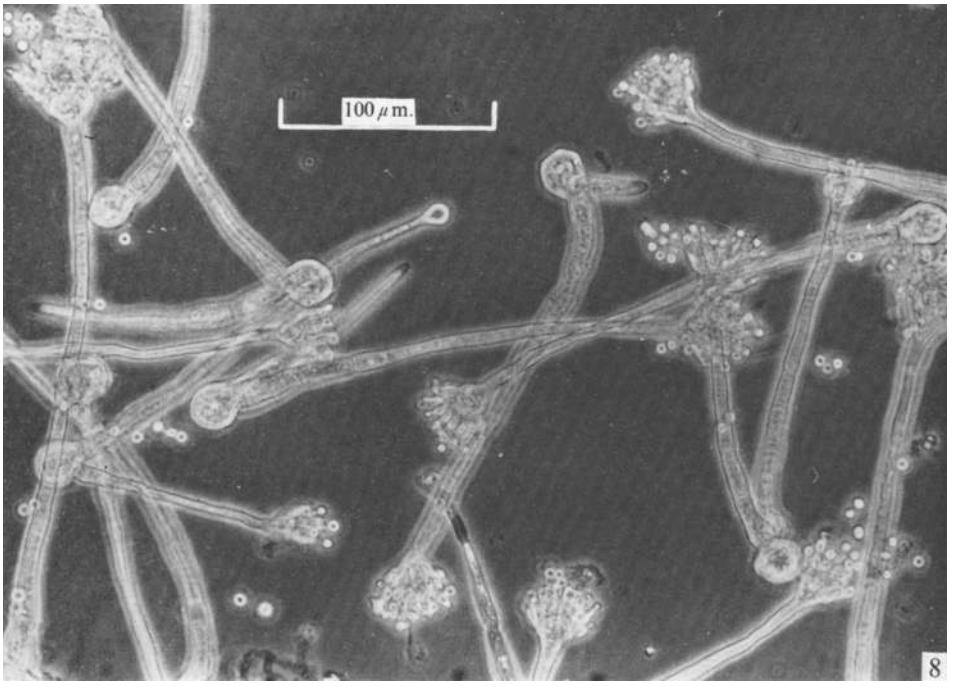

Fig. 8. Numerous enlarged conidia with mature conidiophores, 18 h. after transfer to 30°.

Early stages of conidiophore production from a conidium are shown in Fig. 4 and 5 and elongating conidiophores from a group of conidia are shown in Fig. 6. Whereas germ-tube emergence appeared as the emergence of a thin-walled protrusion from a small area of the conidium wall, conidiophore emergence was seen as a direct bulging and outgrowth of a large area of the conidium wall. Conidiophore elongation was followed by the formation of a vesicle, phialides and conidia which were released into the medium (Fig. 7, 8). Initially, a single conidiophore developed and this was then frequently followed by a second and occasionally more (Fig. 9). These usually arose directly from the conidium but occasionally occurred as branches from the first formed conidiophore (Fig. 10). Hyphal growth did not occur from these conidia or their conidiophore stalks even after prolonged incubation.

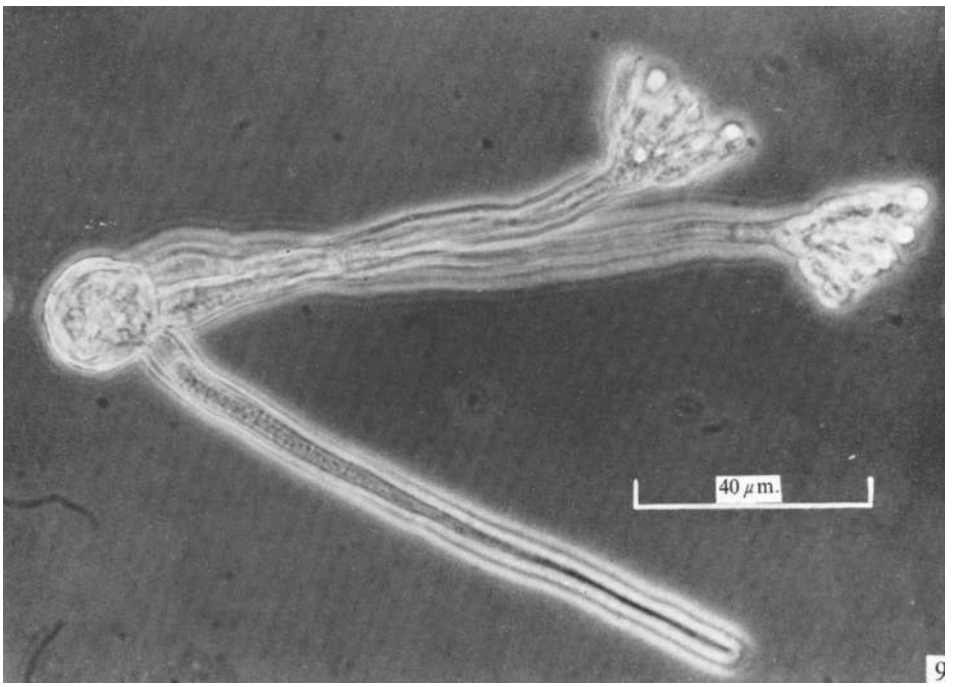

Fig. 9. Multiple conidiophore production (two mature and one immature) from an enlarged conidium, 18 h. after transfer to 30°.

The conidiophores produced from conidia in submerged cultural conditions were compared with the normal conidiophores of *Aspergillus niger* produced in subaerial culture from conidiating mycelium (Fig. 11). Conidiophores produced from conidia were smaller than the normal conidiophores of *A. niger* and the relative proportions of the structures differed. The most marked difference was in stalk length, the subaerial being more than five times longer than the submerged. The large vesicles of the subaerial structures had both phialides and sterigmata or metulae whereas those produced from conidia had only phialides which were occasionally two-celled. The conidiophores produced from conidia frequently formed cross walls on ageing (Fig. 10) whereas this rarely occurred with subaerial conidiophores. Conidia produced from the smaller submerged conidiophores were the same size as normal subaerial conidia. However, submerged conidia (Fig. 12) differed from subaerial conidia (Fig. 13) in lacking the dark pigmented spore coat. The submerged conidia germinated only if transferred to a fresh medium.

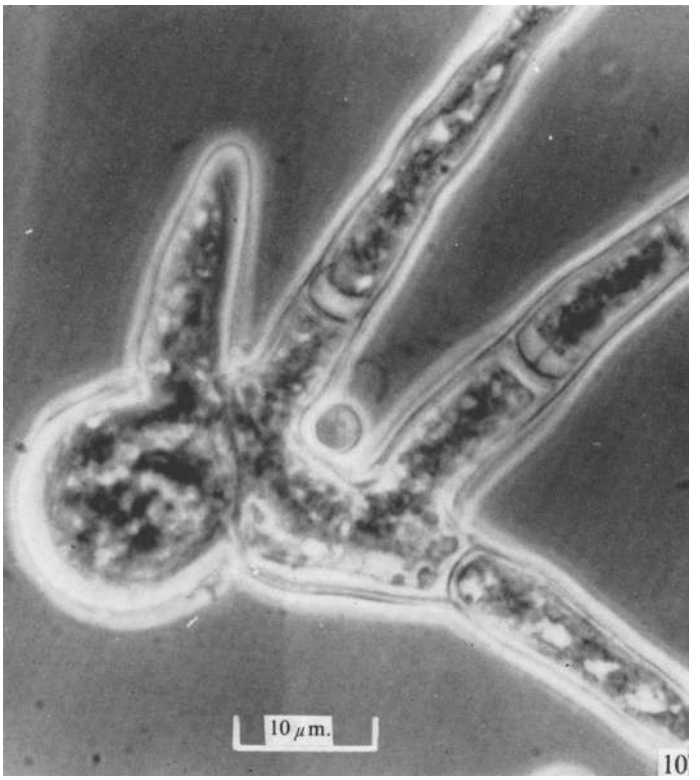

Fig. 10. Multiple conidiophore formation from an enlarged conidium, 30 h. after transfer to 30°. Three conidiophore stalks arise directly from the conidium and a fourth arises as a branch from a previously formed conidiophore. Three conidiophore stalks have developed septa.

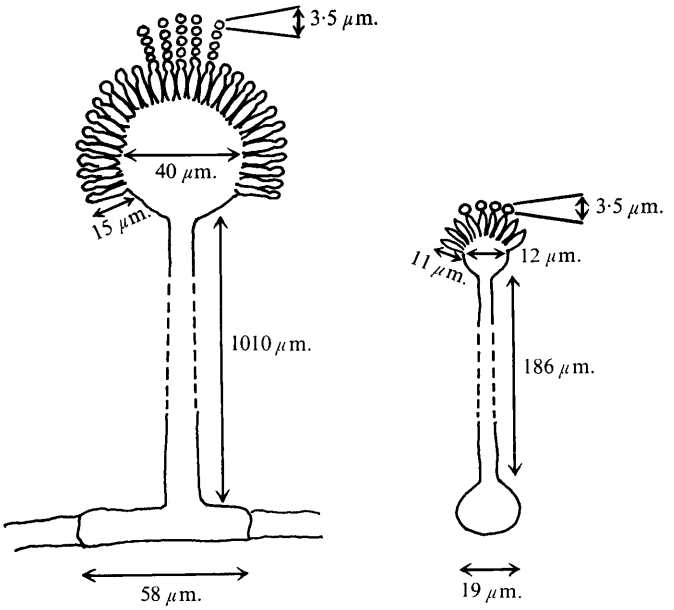

Fig. 11. Comparison of the typical subaerial conidiophore of *Aspergillus niger* produced from conidiating mycelium with the conidiophore produced from microcycle conidiation.

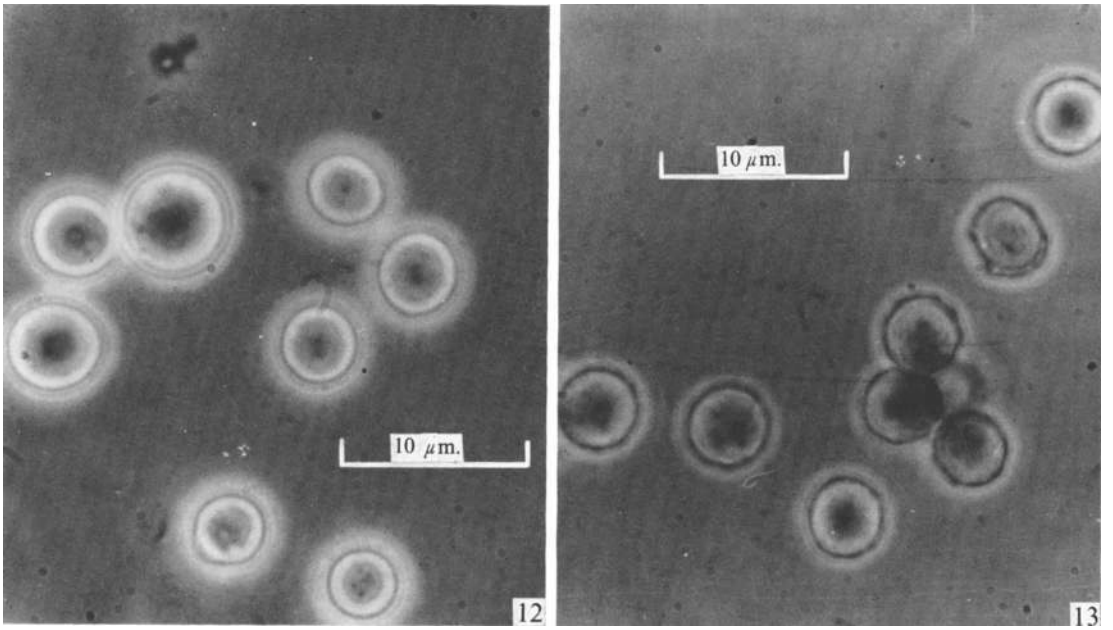

Fig. 12. Conidia from submerged culture. Conidia lack the dark pigmented spore coat.
Fig. 13. Conidia from subaerial culture. Conidia possess a dark pigmented spore coat.

DISCUSSION

Although germ-tube formation is the usual outcome of fungal spore germination alternative developments are known. In some fungi germination can occur by budding, either by direct budding of the spore or by the formation of a tube and its subsequent budding (Wolf & Wolf, 1947; Bartnicki-Garcia, 1969; Lingappa & Lingappa, 1969).

The formation of secondary spores is well documented in some groups of fungi notably the Phycomycetes and in particular the Entomophthorales (Wolf & Wolf, 1947; Lilly & Barnett, 1951; Cochrane, 1958). This occurs either by the endogenous formation of secondary spores in the mother spore as in *Phytophthora* (Bessey, 1950) or by the formation of secondary spores on a single erect germination hypha as in *Basidiobolus* (Eidam, 1887; Callaghan, 1969). Isolated reports of limited secondary spore formation by other fungi indicate that this process may be widespread in fungi. Secondary spore formation occurs in *Cephalosporium asteri* (Dowson, 1923), *Penicillium notatum* (Hadley & Harrold, 1958), *Alternaria* spp. (Rotem & Bashi, 1969), *Glomerella cingulata* (Lingappa & Lingappa, 1969) and in *Geotrichum candidum* (Park & Robinson, 1969). This immediate sporulation with absence of or very limited vegetative development is not limited to fungal spores since a similar phenomenon termed 'microcycle sporogenesis' has also been demonstrated in bacterial spores (Vintner & Slepecky, 1965; Slepecky, 1969).

The most general hypothesis regarding the induction of conidiation in filamentous fungi is that reproduction is initiated by factors which limit the growth of the fungus (Cochrane, 1958). Rotem & Bashi (1969) concluded that the direct formation of secondary conidia in *Alternaria* spp. and *Stemphylium botryosum* was induced by various factors which inhibited or partially inhibited the vegetative development of the mother conidium. Auto-inhibitors associated with the conidia were considered by Lingappa & Lingappa

(1969) to be responsible for the inhibition of mycelial growth and the preferential development of secondary conidia in *Glomerella cingulata*. An effect of germination inhibitors is also indicated in an observation by Hadley & Harrold (1958) in which secondary conidium formation occurred from *Penicillium notatum* conidia when these developed in the medium in which they were produced. Induction of conidiation in sporelings of *Geotrichum candidum* was considered by Park & Robinson (1969) to result from an interference with the metabolic condition characteristic of the somatic phase. In this study on *Aspergillus niger* although direct conidiation from conidia could be obtained without vegetative development this conidiation was not the direct result of the complete inhibition of vegetative growth since under most conditions vegetative growth and conidiation occurred together. However, higher temperatures which increased the degree of conidiophore production from conidia has previously been shown to cause increased swelling of the conidia and a greater degree of mycelial branching (Anderson & Smith, 1971*a*). These effects were considered to result from a complete or partial inhibition of apical growth. Therefore, in this fungus also, conidiation appears to be stimulated by factors which interfere with rapid apical growth. A similar explanation may apply to an observation of the thermostimulation of conidiation in *Neurospora crassa* (Ojha & Turian, 1968) since higher temperatures not only stimulated the total number of conidia produced but also induced an early conidiation.

The complete loss of ability of conidia to produce vegetative growth in a complete growth medium at 30° after a prolonged period of swelling at 44° while retaining the ability to form a complex reproductive structure and viable conidia, appears remarkable. This may represent a differential stability of those nuclear and/or cytoplasmic elements which are responsible for these developments. In fact, temperatures near those used in this study have been found to have marked effects on the conidial ribosomes of *Aspergillus oryzae* (Horikoshi & Ikeda, 1969). Ribosomes autolysed, possibly by ribonuclease action, at 43° while thermal denaturation occurred at 46°.

At present the mechanism involved in germ-tube emergence from fungal spores is not clearly understood. In spores in which emergence involves a passage through an outer wall or an outer layer of the same wall it is not known whether this involves only expansion forces or enzymic degradation (Bartnicki-Garcia, Nelson & Cota-Robles, 1968). If germ-tube emergence from *Aspergillus niger* conidia occurs by emergence of the germ tube wall through an outer layer of the conidium wall then possibly failure to develop germ tubes may be the result of an altered or thickened wall. Conidiophore development, which occurs as an outgrowth of the complete conidium wall, is however not affected. It is interesting that conidiophore development from the conidium appears morphologically identical with the large bulging of the wall of a hyphal cell during the formation of a conidiophore initial (Anderson & Smith, 1971*b*). Clearly the mechanism which determines this form of wall extension represents one of the earliest events in conidiation of this fungus.

Although the conidia were able to produce a germ tube at an early stage, conidiophores did not form till some time later. In addition, at high temperatures which gave a slow rate of growth of the fungus the time to conidiophore production was considerably delayed. This indicates that a certain degree of growth must precede this development. Vinter & Slepecky (1965) also observed the necessity of partial development of the outgrowing bacterial spore before sporogenesis could occur. Whether this early development of germ-tubes and the later development of conidiophores reflects the timing of RNA synthesis for these events is at present under investigation.

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