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The Physiology of Mould–Yeast Dimorphism in the Genus *Mycotypha* (Mucorales)

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SUMMARY

Mycotypha africana and four strains of *Mycotypha microspora* were studied. The former displayed total conversion from mycelium (M) to yeast (Y) when grown under N_2 or in the presence of some electron-transport inhibitors with either glucose, fructose or mannose as carbon source. In air, mixed filamentous and yeast-like forms were frequently observed. Acid pH, high temperature, dense inoculum and high hexose level increased the proportion of the Y-form. Yeast and mycelial phases were interconvertible. All but one strains of *M. microspora* tested displayed similar dimorphism, but strain to strain variations were observed.

INTRODUCTION

Mould-yeast (MY) dimorphism has been investigated in a number of pathogenic and non-pathogenic fungi from a wide range of taxonomic groups (see reviews of Romano, 1966, and Bartnicki-García, 1968*a*). Physiological control of this phenomenon varies between organisms; temperature, medium composition and nature of the gas-phase have all been implicated.

In the Mucorales, only the genus *Mucor* has been reported to display MY dimorphism (Bartnicki-García & Nickerson, 1962*a*). The major determining factors were the gas-phase and hexose sugar concentration (Bartnicki-García, 1968*b*). Yeast-like growth occurred under CO_2 and a mycelium under N_2 or air. These growth patterns were constant for *Mucor rouxii* over a wide range of physical and nutritional conditions but induction of the Y-phase by CO_2 could be reversed by EDTA or other N-acetic acid type chelating agents (Bartnicki-García & Nickerson, 1962*b*). *Mucor subtilissimus* grew as a yeast under both N_2 and CO_2. Hence, the existence of an EDTA-sensitive inducer of the Y-phase was postulated, the synthesis of which required CO_2 in *M. rouxii* but not in *M. subtilissimus*, and which was thought to act on the wall polymers responsible for maintaining cell shape. A greatly increased mannose content in the Y-phase wall (probably present as a mannose-polysaccharide) supported this suggestion (Bartnicki-García & Nickerson, 1962*c*).

More recently Terenzi & Storck (1968) showed that 0·2 % (v/v) of β-phenylethyl alcohol (PEA) also induced the Y-phase, inhibited the expression of cytochrome oxidase activity and stimulated alcoholic fermentation in *Mucor rouxii*, provided that 2 % or more of a hexose was provided in the medium.

In the present communication the factors controlling dimorphism in another genus of the Mucorales, namely *Mycotypha*, which was recently placed in the family Thamnidiaceae (Young, 1969), are described.

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METHODS

Organisms. Mycotypha africana (CBS122.64) and four strains of *Mycotypha microspora*, namely CBS186.68, IMI108621, IMI31239 and an isolate from Madras soil, designated here as *M. microspora*, Subramanian strain, were studied. Slopes grown on Sabouraud-maltose agar at 30 °C and stored at 4 °C were used up to 6 weeks old, after which spore viability markedly decreased. In some experiments, *Mucor rouxii* was used for comparison.

Media and chemicals. The medium (YPG) of Bartnicki-García & Nickerson (1962*a*) was used. The composition (g/l) was: yeast extract (Difco), 3·0; peptone (Fisher Scientific), 10·0; glucose, 20·0. Solid medium contained, in addition, 15 g agar (Fisher)/l. Variations in glucose concentration are indicated by a number following the letters YPG, e.g. YPG-50 means basal medium with 50 g glucose/l. Unless otherwise stated, all media were adjusted to pH 5 with H_2SO_4, dispensed in 50 ml portions in 250 ml Erlenmeyer flasks and autoclaved at 121 °C for 15 min. In experiments in which pH and nature or concentration of carbon source were varied, the carbohydrates were autoclaved separately at 115 °C for 10 min. Aqueous solutions of KCN, cysteine, tryptophol (3β-hydroxyethyl-indole) and the disodium salt of EDTA were sterilized by membrane filtration (Millipore Ltd, Type HA, 0·45 μm). B-phenylethylalcohol (Fluka Chemical Co., Burchs, Switzerland) was added directly without sterilization. Antimycin A (Sigma), oligomycin (Sigma) and 2-thienoyltrifluoroacetone (TTFA, Fluka Chemical Co.) were added as ethanolic solutions. Rotenone (Sigma) was first dissolved in benzene and then appropriately diluted into ethanol. The solvents alone were without effect. All chemicals were of analytical grade and distilled water was employed throughout.

Submerged cultivation. Spores were used either directly or after pre-germination. For pre-germination, a 10 ml suspension was transferred to 50 ml YPG broth in static culture at 30 °C for 8 h, during which time 60 to 70 % of the spores swelled to spheres. The medium was centrifuged, the pellet resuspended in 10 ml of YPG broth and the spores counted in a haemocytometer. The inoculum was adjusted to give 500000 spores/ml of culture broth. Incubation, unless otherwise stated, was at 35 °C and 175 rev./min in a Metabolite water bath shaker (New Brunswick) with a circular throw of 1·27 cm diam. To separate the growth forms the mixture of M and Y was filtered through cheese cloth. The mycelial pad was then resuspended in water and refiltered. This procedure was repeated three times. The two phases were then separately filtered through pre-dried, pre-weighed filter-paper discs and dried to constant weight at 50 °C.

Control of the gas phase. Oxygen-free N_2 and CO_2 were used. Gasses were mixed to the desired percentage composition by means of pre-calibrated microflowmeters. Accuracy of composition was verified by testing samples with an Orsat Gas Analyser (Elsworth, 1970). Gas was passed from flask to flask and flow rate adjusted to 2 to 3 bubbles/s by using a gas bleed on the supply line (Bartnicki-García & Nickerson, 1962*a*). To purge air from the system, media were incubated for 2 h at a high gas-flow rate before inoculation.

Growth on solid media. Solid YPG medium in 9 cm Petri dishes was transferred immediately after inoculation to a desiccator which was evacuated and refilled with N_2 or CO_2. This procedure was repeated five times to ensure removal of all oxygen. When N_2 was used the desiccator also contained a beaker of 20 % KOH to absorb metabolic CO_2.

Observations on individual spores. YPG medium containing spores was introduced into a sterile haemocytometer slide (density about 100 spores/grid) and the edges of the cover glass sealed with wax. The squares of the grid were numerically coded and the position and shape

of the spores noted. The haemocytometer was incubated in a N_2-filled moist chamber. The spores were subsequently located and observed for swelling and budding.

RESULTS

The words 'yeast phase' and 'yeast' are used to describe the yeast-like morphology only when multipolar budding cells were observed. Swollen spores, which often deposited wall material and produced gas but failed to bud, are referred to as 'swollen spores'.

Microscopic examination failed to detect arthrospore formation in any experiment, the Y-phase cells arising directly from spores without an intermediate mycelial phase.

Studies with Mycotypha africana

Effect of pH and incubation temperature. YPG medium at initial pH values of from 2 to 9 was incubated at temperatures from 25 to 37 °C. The organism grew between pH 3 and 7·5, and at all temperatures tested. Stimulation of Y-phase formation was evident at higher temperatures and at lower pH values. The effects were slightly additive and maximum Y-phase (about 15 %) was produced at pH 4·5 and 37 °C.

Effect of variation in culture medium and inoculum level. Varying the glucose concentration in YPG medium from 0·5 to 100 g/l (Table 1) indicated that total growth was depressed at levels above 40 g/l and that the proportion of Y-phase rose with increased carbohydrate up to 50 g/l, after which it declined. At higher carbohydrate levels an inoculum effect became apparent. Thus at 20 g/l, inocula of 9×10^5, 10^5 and 2×10^4 spores/ml culture broth resulted in 19·1, 14·8 and 3·4 % Y-phase respectively. As the sugar level rose the inoculum effect became more marked and at 50 g/l the Y-phase reached 50 to 60 % of the total.

The nature of the carbon source had no influence on MY dimorphism of *Mycotypha africana* growing *in air* (Table 2). Growth of both Y- and M-phases was most abundant on hexose sugars while some pentoses failed to support growth. Morphology was unaffected by variations in the type or concentration of peptone employed.

Effect of the gas phase. Mucor rouxii behaved in the manner described by Bartnicki-Garcia & Nickerson (1962*b*) and produced a sparse mycelium under N_2 and spherical budding cells under CO_2. By contrast, *Mycotypha africana* grew as the Y-phase under N_2 and produced thin-walled swollen spores under CO_2 (Fig. 1; Table 3). Extensive examination of the latter failed to detect budding and no increase in cell number was detectable by haemocytometer counting, indicating that proliferation was absent. These morphological forms were unaffected by changes in pH or temperature but were influenced by the nature of the carbon source as shown in Table 2. Similar forms were observed in solid YPG medium. It was noteworthy that upon removal from the CO_2 or N_2 into air, the Mucor plates failed to produce sporangiophores (Bartnicki-García & Nickerson, 1962*b*) in contrast with Mycotypha which developed small though otherwise normal sporangiophores in 24 to 36 h.

The data above suggested that low oxygen tension induced the Y-phase. This was further supported by experiments in which *Mycotypha africana* was grown in N_2/O_2 mixtures at different oxygen partial pressures (Fig. 2). With agitation at 150 rev./min the Y-phase predominated up to an oxygen partial pressure of 0·10 to 0·11. Increased agitation (175 rev./min) decreased the oxygen partial pressure at which the transition from Y to M occurred.

Effect of inhibitors of the cytochrome system. The addition of KCN (10^{-3} M) to cultures of *Mycotypha africana* growing in YPG-10 medium in air induced almost 100 % Y-phase. At lower concentrations of KCN, Y formation was enhanced but to a lesser degree (Table 4). Similarly, 0·4 μg oligomycin/ml and 0·2 μg antimycin A/ml induced 100 % Y-phase.

Table 1. *Effect of glucose concentration and β-phenylethyl alcohol (PEA) on growth and morphology of Mycotypha africana under air*

Glucose concentration (g/l)	No. PEA		0·2 % (v/v) PEA	
	Total growth* (mg dry wt/50 ml)	Yeast (%)	Total growth‡ (mg dry wt/50 ml)	Yeast (%)
0	70 (62–92)	0	40 (33–46)	0
0·5	140 (129–147)†	0	N.T.	N.T.
5·0	173 (164–181)	0	142 (131–151)	1 (0–3)
10·0	256 (231–289)	2 (0–9)	145 (136–151)	90 (87–96)
20·0	304 (292–320)	7 (3–25)	135 (130–138)	82 (76–90)
30·0	272 (256–288)	25 (16–30)	130 (122–136)	96 (94–100)
40·0	291 (260–316)	50 (28–61)	105 (96–114)	76 (69–80)
50·0	263 (239–281)	62 (27–65)	75 (66–87)	60 (54–69)
100·0	204 (184–215)†	31·5 (13–56)	N.T.	N.T.

N.T. = not tested.

* Figures in this column represent average data from four experiments each with three replicates.
† Only two experiments were conducted.
‡ Average data from two experiments each with three replicates.

Table 2. *Effect of carbon source and gas phase on the growth and morphology of Mycotypha africana and three strains of Mycotypha microspora*

Figures represent mg dry wt/50 ml.

Carbon* source	*Mycotypha africana* under:			Strains of *Mycotypha microspora* under N_2		
	Air	CO_2	N_2	108621	186.68	31239
Glucose	255 (M)	85 (SS)	127 (Y)	90 (Y)	50 (Y)	10 (M)
Fructose	305 (M)	85 (SS)	129 (Y)	99 (Y)	44 (Y)	10 (M)
Galactose	245 (M)	35 (SS)	18 (SS)	37 (Y/M)	30 (Y)	8 (M)
Mannose	270 (M)	40 (Y)	138 (Y)	76 (Y)	52 (Y)	34 (M/Y)
Sucrose	205 (M)	15 (SS)	87 (SS)	23 (Y)	9 (Y)	6 (M/SS)
Maltose	205 (M)	11 (SS)	21 (SS)	N.T.	N.T.	N.T.
None	145 (M)	N.G.	N.G.	N.G.	N.G.	N.G.

Lactose, D-xylose, D-arabinose, mannitol, citrate and starch failed to support growth of *M. africana* or *M. microspora* under CO_2 or N_2. Under air, growth of *M. africana* was similar to that with no added C source.
N.G. = no growth; N.T. = not tested.
M = mycelial phase; Y = yeast-phase; SS = swollen spores.
* All tested at a concentration of 20 g/l.

Experiments with TTFA were more variable and at the highest non-toxic concentration tested (10^{-4} M) conversion varied from 34 to 88 %. PEA differed in exhibiting its Y-inducing effect only in the presence of 1 % or more glucose (Table 1). Amytal and rotenone were inactive up to 5×10^{-3} M, although distorted filamentous forms were often produced.

Effect of EDTA, cysteine and tryptophol. The effect of EDTA on *Mycotypha africana* was tested by using either spores or pre-germinated spores as inoculum, with essentially the same results. Low concentrations (5×10^{-6} M) stimulated growth of both phases by 10 to 14 %. Above this, growth was decreased to a point of complete inhibition at 5×10^{-4} M. Under a N_2 gas phase the cells at 5×10^{-5} M were enlarged (mean diameter 46·1 μm, compared with 30·6 μm normally) but still exhibited multipolar budding, while at 10^{-4} M budding was completely inhibited and most cells had rudimentary hyphae 100 to 150 μm long.

Tryptophol slightly stimulated production of the Y-phase but cysteine was without effect on MY dimorphism.

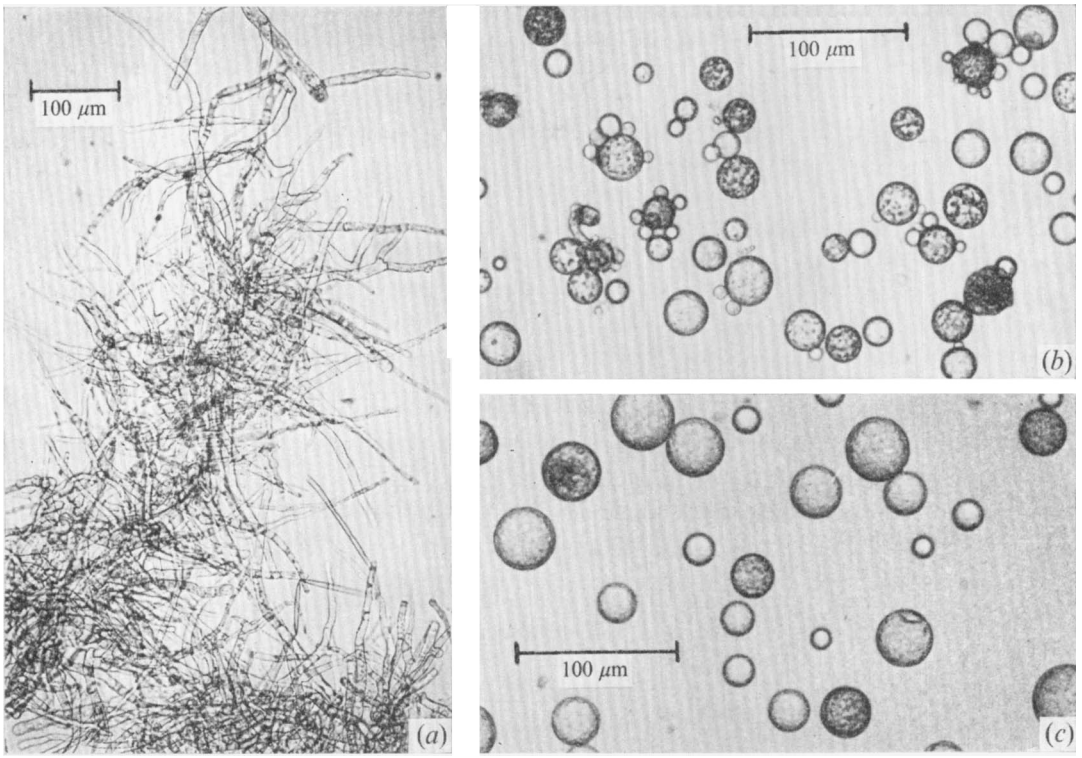

Fig. 1. Morphological forms of *M. africana* grown under different atmospheres in YPG medium for 48 h. (*a*) Typical mycelial development in air; (*b*) Y-phase cells under N_2 exhibiting multipolar budding; (*c*) swollen spores under CO_2.

Table 3. *Comparison of the growth and dimorphism of M. africana and Mucor rouxii in YPG medium at* 30 °*C under* N_2, CO_2 *and air*

	M. africana			*Mucor rouxii*		
Gas phase	Dominant morphology	% as mycelial phase	Total dry weight (mg/50 ml)	Dominant morphology	% as mycelial phase	Total dry weight (mg/50 ml)
Air	M	87	269	M	99	384
N_2	Y	0	112	M	96	35
CO_2	SS	0	48	Y	0	77

M = mycelium; Y = yeast-phase; SS = swollen spores (no budding).

The relationship of spore and vegetative dimorphism. A total of 730 individual spores were observed for 120 h, comprising 216 more or less spherical (2·8 to 3·2 μm dia), 294 oval/elliptical (3·3 to 5·3 μm long) and 220 which were more or less rectangular in profile (5·4 to 6·2 μm long). After 24 to 36 h, 90 % of the oval and rectangular groups and 75 % of the spherical spores had swollen to spheres. Between 36 and 50 h, 7 rectangular, 6 oval/elliptical and 2 spherical spores developed small bud initials. No further development took place under these conditions. Attempts to separate the spore types by centrifugation and filtration were unsuccessful. However, it seemed likely that all spore types could develop into the Y-phase, and that spore dimorphism (Young, 1969) was not related to vegetative development.

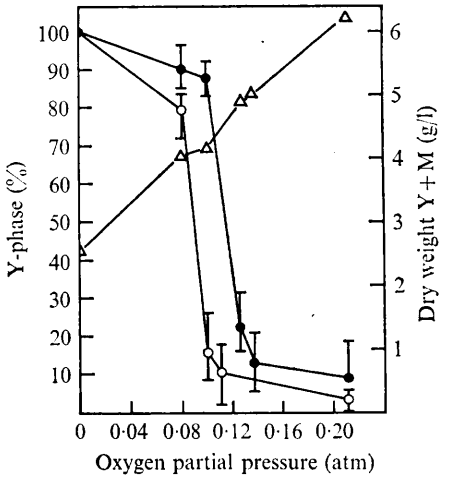

Fig. 2. Effect of oxygen partial pressure in N_2/O_2 gas mixtures on the total growth and development of the Y-phase of *M. africana* in YPG medium at 30 °C. △, total growth; ●, % Y at 150 rev./min; ○, % Y at 175 rev./min.

Table 4. *Effect of KCN on the growth and morphology of Mycotypha strains in YPG-10 medium at 25 °C*

	KCN concentration					
	Zero		10^{-4} M		10^{-3} M	
Organism	Total growth (mg/50 ml)	Yeast (%)	Total growth (mg/50 ml)	Yeast (%)	Total growth (mg/50 ml)	Yeast (%)
M. africana	215	8·6	135	66·6	100	90
M. microspora IMI108621	210	0	120	17·0	50	100
M. microspora CBS186.68	230	7·3	210	31·5	52	52·2
M. microspora (Subramanian)	150	0	110	9·0	75	55
M. microspora IMI31239	200	0	240	0	0	0

Interconvertibility of Y- and M-phases. By transferring a small (0·1 ml) inoculum to fresh YPG medium under N_2 every 48 h, *Mycotypha africana* was maintained growing as the Y-phase for 30 days, with no change in morphological form. Such Y-cells reverted to a mycelium within 6 to 8 h of transfer to air, indicating that the capacity for mycelial development was not lost. Conversely, when young mycelium (up to 8 h after germ-tube formation) was transferred to a N_2 atmosphere, the cells became swollen and began budding to form a Y phase (Fig. 3*a*). Older mycelium failed to develop and became darkly pigmented. The Y-phase of *Mycotypha microspora* did not grow for more than two or three passages, probably because of the formation of many thick-walled cells susceptible to osmotic lysis upon transfer (Fig. 4*b*).

Studies with Mycotypha microspora

Although some strain to strain variation was observed, MY dimorphism in IMI108621, CBS186.68 and the Subramanian strain was essentially similar to *Mycotypha africana*. However, triangular and stellate cells were produced in many experiments, preventing accurate quantification of the growth forms. Strain IMI31239 formed a few Y-cells in only one experiment and lacked the morphological flexibility of the other strains.

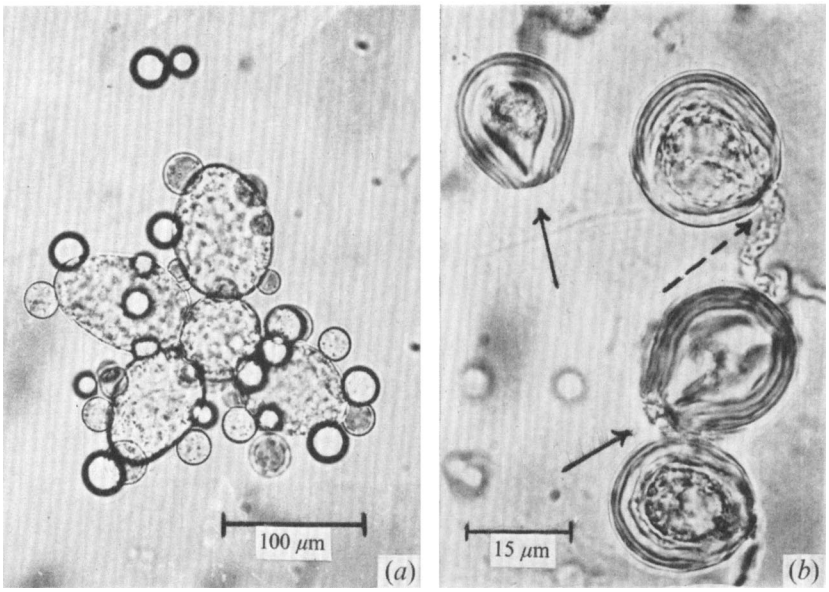

Fig. 3. (*a*) Reversion of a young mycelium of *Mycotypha africana* to budding Y-phase after transfer from air to N_2. The original central cell and four germ tubes have swollen and commenced budding. (*b*) Y-phase of *Mycotypha microspora* CBS186.68 grown in the presence of 5×10^{-4} M KCN. The cells display heavily thickened walls. One cell (broken arrow) is in the process of discharging its protoplast and two others (solid arrows) are empty.

Effect of medium composition and incubation conditions in air. The effects of temperature, pH and C source were generally similar to those described for *Mycotypha africana*. All strains of *Mycotypha microspora* resembled *Mycotypha africana* in displaying maximum dry weight at 20 g glucose/l. Strain IMI108621 produced mixed mycelium and budding yeast at high (100 g/l) glucose but mycelium alone at low (10 g/l) concentrations. With CBS186.68, the Subramanian strain and IMI31239, budding was not detected at any glucose level, though in the first two strains non-budding spherical cells were present at high concentrations of glucose. The spherical cells formed by these two organisms and IMI108621 had heavily thickened walls, the tendency being most marked in CBS186.68 and IMI108621. Such cells frequently burst, especially on transfer to fresh medium (Fig. 3*b*).

Growth under nitrogen and carbon dioxide. With the exception of IMI31239, which formed a stunted mycelium (Fig. 4), all strains grew as a Y-form in YPG medium under N_2 provided a hexose or sucrose was the C source (Table 2). Strain IMI31239 developed as a yeast only on mannose when a few clusters of budding cells were present among the sparse mycelium. Under CO_2, development of all strains was inhibited except for partial swelling of a few spores.

Effect of inhibitors of the cytochrome system. All strains of *Mycotypha microspora* differed in their behaviour. IMI108621 resembled *Mycotypha africana* in forming a pure Y-form at 10^{-3} M KCN (Table 4). The same trend was noted in CBS186.68 and the Subramanian strain but conversion was only 50 to 60 %. IMI31239 failed to grow. Lower KCN concentrations (10^{-4} and 5×10^{-4} M) had the same general effect, but mixtures of forms were produced depending on the strain employed.

Rotenone, antimycin A and amytal were inactive against all strains except for some growth inhibition. Oligomycin induced the Y-phase only in CBS186.68, the conversion reaching

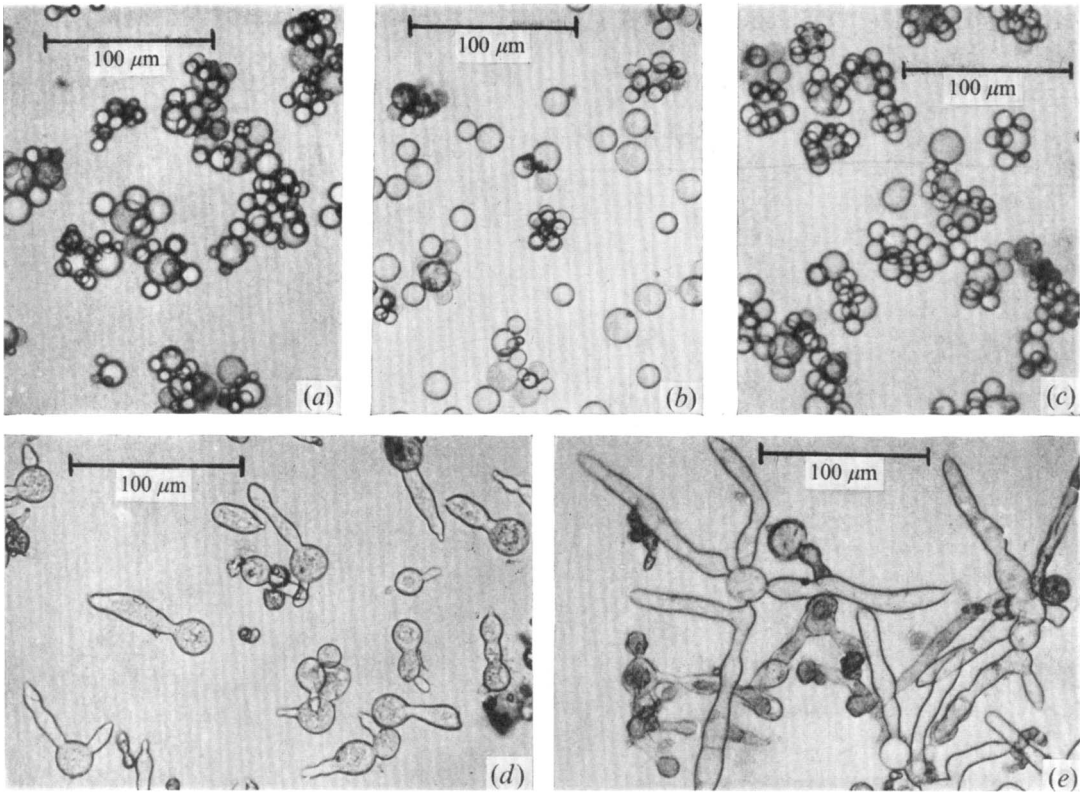

Fig. 4. Growth of four strains of *M. microspora* under N_2 in YPG medium. (*a*) The Subramanian strain 48 h.; (*b*) CBS186.68, 48 h; (*c*) IMI108621, 48 h; (*d*) IMI31239, after 24 h; (*e*) IMI31239, after 48 h.

100% at 4 μg/ml, a tenfold higher concentration than was effective with *Mycotypha africana*. With other strains, this compound significantly reduced the dry weight but did not affect the morphological form. TTFA was active only against the Subramanian strain, where maximum Y-phase (31%) formed at 5×10^{-5} M. Higher concentrations were toxic. PEA induced a mixture of stunted mycelium, yeast and stellate forms in IMI108621 and the Subramanian strain and showed the same glucose dependence as with *Mycotypha africana*.

DISCUSSION

Of eight genera of Mucorales tested by Bartnicki-García & Nickerson (1962*b*), only *Mucor* displayed MY dimorphism. The present communication establishes that the phenomenon is also a characteristic of the genus *Mycotypha*.

Control of dimorphism in Mucor rouxii and Mycotypha africana. Extensive studies of *Mucor rouxii* (Bartnicki-García & Nickerson, 1962*a*, *b*, *c*; Bartnicki-García, 1968*b*; Terenzi & Storck, 1968; Bartnicki-García & Lipmann, 1969, 1972) revealed that partial pressure of CO_2 and hexoses were the major factors controlling yeast-phase development. At low hexose or CO_2 levels, or both, development was filamentous. At high hexose (above 8%) it was in the Y form, even in air, while at lower levels supplementation of up to 30% CO_2 in the incubation atmosphere was necessary (Bartnicki-García, 1968*b*). Anaerobic conditions in themselves did not stimulate Y formation and growth under pure N_2 was filamentous. Other nutritional factors were largely without effect. The CO_2-induced Y-phase reverted to a filamentous form in the presence of low EDTA concentrations.

Mucor rouxii and *Mycotypha africana* responded differently to N_2 and CO_2 atmospheres (Table 3). Carbon dioxide was not a major factor in MY dimorphism in Mycotypha although enhancement of the Y-phase has been noted in a N_2 atmosphere enriched with 4·2 % CO_2 (G. Kraepelin and B. Schultz, private communication). Rather, the evidence suggested that anerobiosis, whether induced by control of the gas phase or by inhibitors of electron transport, was the key factor. This was supported by the observation that a faster agitation rate, which would increase the oxygen transfer rate into solution (Finn, 1954) decreased the oxygen partial pressure at which the M to Y transition occurred (Fig. 2).

Similarly, the increased proportion of Y-cells which accompanied increased hexose levels (Table 1) could be attributed to O_2 depletion of the broth by the more actively growing organism.

The cytochrome inhibitors fell into two categories. Those which blocked electron flow or acted as uncoupling agents (KCN, oligomycin, antimycin A) produced total conversion to the Y-phase, while those which acted on a specific branch of the electron transport chain (TTFA, rotenone, amytal) were inactive or less absolute in effect. PEA which was known to favour Y development in *Candida albicans* (Lingappa *et al.* 1969) showed a glucose-dependent effect on both *Mucor rouxii* and *Mycotypha africana*. In *M. rouxii*, it stimulated alcoholic fermentation, possibly through its action as a cytochrome-oxidase inhibitor (Terenzi & Storck, 1968). A similar explanation could apply to *M. africana*, although the effect of PEA on alcoholic fermentation was not determined in this case.

In *Mycotypha africana*, MY dimorphism was more sensitive to temperature, pH and inoculum change than in *Mucor rouxii* though none of the factors were of great individual significance. The effect of EDTA suggested that a common metal ion-dependent enzyme might be involved in yeast formation in both organisms.

Dimorphism in Mycotypha microspora. Strains IMI108621, CBS186.68 and the Subramanian strain resembled *Mycotypha africana* in their response to pH, temperature, gas phase and requirement for a hexose sugar for Y-cell formation. However, they differed in their general failure to form a Y-phase at high glucose levels (although other morphological effects such as changes in hyphal diameter and degree of branching were evident) and in their response to cytochrome inhibitors (Table 4). In the latter case the abnormally thick walls and restricted intercellular spaces suggested that an oversynthesis and deposition of wall material was occurring (Fig. 3).

The remaining strain, IMI31239, usually failed to display dimorphic behaviour, either producing a mycelium or failing to grow at all (Fig. 4, Table 4). Only under N_2 with mannose as carbon source were a few large clusters of Y-cells observed among the filaments.

Mannose was also the only sugar on which *Mycotypha africana* produced budding cells under CO_2 (Table 2). In view of the involvement of mannose in the formation of the yeast cell wall (Bartnicki-García & Nickerson, 1962*a*; Bartnicki-García, 1968*b*; Falcone & Nickerson, 1956; Marks, Keller & Guarino, 1971), preliminary analyses of the Y-phase of *M. africana* were made but failed to reveal the presence of this sugar. Clearly there is scope for further work to establish the biochemical basis of dimorphism in the genus *Mycotypha*.

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