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Effects of Antibiotics on the Life Cycle of *Neurospora crassa*

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ABSTRACT. Some antibiotics and synthetic inhibitors affect, in several ways, the life cycle of *Neurospora crassa* (germination of conidia → myceliar growth → formation of conidia). Bikaverin, cyanein, scopathricin and phenethyl alcohol retard the germination of conidia, without inhibiting it completely. 5-Fluorouracil, ramihyphin A and zygosporin A (cytochalasin D) do not inhibit the germination. Bikaverin brings about a thickening of the hyphae of growing mycelium. Ramihyphin A, cyanein, scopathricin and zygosporin A stimulate the ramification of hyphae while 5-fluorouracil and phenethyl alcohol do not affect the myceliar morphology apart from their inhibitory effect on growth. Actinomycin D, 5-fluorouracil, cycloheximide, ramihyphin A and partially also sodium iodoacetate inhibit to a different degree the photoinduced formation of conidia. The inhibition by 5-fluorouracil is very conspicuous when the agent is present during the photoinduction but considerably weaker when it is applied 2 h after the photoinduction.

The morphological processes of filamentous fungi are characterized by two basic phases: initial phase of spore germination and the resulting phase of sporulation. These two stages delineate the whole life cycle of the fungus, defined as the development from a spore to a spore, which includes a shorter or longer period of myceliar growth (Turian, 1966).

The present work describes the effects of antibiotics and synthetic inhibitors on the germination of conidia, myceliar growth and photoinduced sporulation of *Neurospora crassa*.

MATERIALS AND METHODS

Microorganisms. Wild strain of *N. crassa* was maintained on potato-dextrose agar and the mutant *Abn. 1* on potato-dextrose agar with inositol (5 mg/litre).

Cultivation media. The wild strain of *N. crassa* was cultivated on potato-dextrose medium and Vogel's minimal medium (Vogel, 1956), mutant *Abn. 1* on the same media with the addition of inositol (5 mg/litre).

Inhibitors. The following antibiotics and synthetic inhibitors were used: Actinomycin D, bikaverin, cyanein, cycloheximide, 5-fluorouracil, phenethyl alcohol, ramihyphin A, scopathricin, sodium iodoacetate and zygosporin A.

Preparation of conidia. Sporulated cultures of *N. crassa* were incubated for 24 h at 4°C to facilitate the release of conidia (Combépine and Chervet, 1969). The resulting suspension of conidia in distilled water with the addition of 0.5% Tween 80 was filtered through sterile glass wool to remove mycelial fragments. The homogeneity of the filtered suspension was checked microscopically.

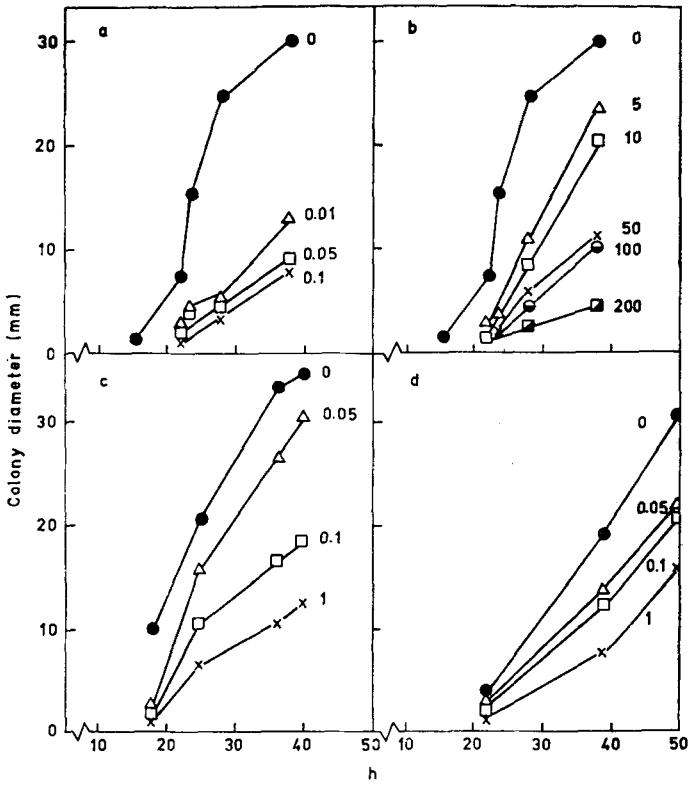

FIG. 1. Growth curves of *Neurospora crassa* (wild strain) and the mutant *Abn. 1* in the presence of inhibitors. a, Wild strain in the presence of ramihyphin A; b, wild strain in the presence of 5-fluorouracil; c, *Abn. 1* in the presence of ramihyphin A on Vogel's minimal agar with biotin and inositol; d, *Abn. 1* on PDA agar with yeast autolysate plus inositol. Medium with a and b: Vogel's minimal agar with biotin. Concentrations in μg/ml.

Effects of inhibitors on germination of conidia. 100-ml flasks were supplied each by 10 ml of Vogel's minimal medium and solutions of inhibitors in dimethylsulphoxide (DMSO) so that the final concentration of DMSO was 1%. The flasks were inoculated to a final density of 5×10^6 conidia/ml, and incubated on a reciprocating shaker (100 strokes/min, amplitude 8 cm) at 28°C. At intervals, samples were taken for the microscopic control of germination.

Effects of inhibitors on the growth of colonies. Petri dishes, 10 cm in diameter, were supplied with 0.15 ml solutions of inhibitors in DMSO and 15 ml medium with 2% agar. Each plate was inoculated at three points with the suspension of wild strain of *N. crassa* or myceliar suspension of the mutant *Abn. 1* and incubated at 28°C. The diameters of colonies were measured at intervals and the data obtained were used for drawing the growth curves and determining the ID_{50} values. At the same time the morphology of hyphae at the margin of the colonies was observed microscopically *in situ*.

Effects of inhibitors on hyphal morphology. The inhibitors were added in ID_{50} concentrations directly to agar or were applied on paper discs which were then placed at the margins of the growing colonies (Betina and Mičeková, 1973).

Photoinduction of conidiation and the effect of inhibitors. Discs of Whatman No. 1 paper, 0.5 cm in diameter, were placed around a growing colony of *N. crasa* on Vogel's minimal agar and, after being overgrown by the mycelium, were used to inoculate larger discs of Whatman No. 3 paper (diameter 2.5 cm) laid on plates with Vogel's minimal agar. These were incubated for 18 h in the dark at 28°C. Colonies growing on the larger discs were then transferred under red light on plates

TABLE I. ID_{50} values of inhibitors determined from growth curves of wild strain of *Neurospora crassa* and the mutant *Abn - 1.*

Inhibitor	ID_{50} (μg/ml)		
	Wild strain[a]	*Abn. 1.*	
		VMM[b]	PDA[c]
Bikaverin	10	—[d]	—[d]
Cyanein	5.6	15	5
5-Fluorouracil	160	—[d]	—[d]
Phenethyl alcohol	650	550	400
Ramihyphin A	0.1	0.1	0.1
Scopatricin	14	80	55
Zygosporin A	11	120	80

[a] On Vogel's minimal agar with biotin; [b] on Vogel's minimal agar with biotin and inositol, [c] on potato-dextrose agar with inositol; [d] not tested.

with aqueous agar (1%) and exposed to either daylight or UV light at 366 nm (UVIS, Desaga) from the distance of 10 cm and at various time intervals. At various times before or after the application of inhibitors to the "transplanted" colonies the plates were exposed for 15 min to the daylight and then incubated in the dark for 24 h.

Some experiments were carried out using a *N. crasa* mycelium grown in a submerged culture in the dark at 28°C for 24 h on a rotary shaker (240 r.p.m). Aliquot volumes of 3.5 ml were filtered under red light through membrane filters and washed with two 5-ml volumes of distilled water. Filters with mycelium were placed on aqueous agar (3 filters on a 10-cm diameter plate) with inhibitors added. At various intervals after or prior to the application of inhibitors the plates were illuminated for 15 min by the daylight and incubated for 25 h in the dark at 28°C. The myceliar matter was washed off individual membrane filters with 2.5 ml 0.5% Tween 80 in distilled water, the suspension was filtered through glass wool to remove mycelial fragments and the absorbance was measured at 650 nm in a SPEKOL spectrophotometer. The concentrations of conidia were read from a calibration curve which was set up for each separate experiment as the dependence of absorbance on conidia count determined by hemocytometer.

RESULTS

Bikaverin at a concentration of 50 μg/ml retarded the germination of *N. crasa* conidia but failed to stop it completely. At a concentration of 25 μg/ml it caused a thickening of primary hyphae. Cyanein up to 50 μg/ml, phenethyl alcohol up to 1000 μg/ml and scopathricin up to 200 μg/ml retarded conidial germination but the inhibition was not complete. 5-Fluorouracil (200 μg/ml), ramihyphin A (0.1 μg/ml) and zygosporin A (200 μg/ml) had no effect on the germination.

In various concentrations the inhibitors retarded the growth of the colonies of the wild strain and the *Abn. 1* mutant. Fig. 1 shows typical growth curves for the two strains in the presence of ramihyphin A and 5-fluorouracil. On Vogel's minimal medium both the wild strain and the mutant exhibit approximately the same sensitivity to ramihyphin A; on potatodextrose medium the *Abn.* 1 mutant is somewhat less sensitive. Ramihyphin A is the most potent inhibitor of growth among the inhibitors used; this becomes also apparent from Table I which presents the ID_{50} values.

TABLE II. Effects of inhibitors on photoinduced formation of conidia in *Neurospora crassa*.

Inhibitor	Concentration μg/ml	Number of spores per disc × 10^{-6}	% of illuminated control
Illuminated control without inhibitors	—	17.71	100
Unilluminated control without inhibitors	—	3.9	22
Actinomycin D	10	11.2	63
	50	9.85	56
	100	9.0	51
Cycloheximide	0.5	5.25	30
	1	2.75	14
	5	1.5	8
	10	1.25	7
5-Fluorouracil	100	8.75	49
	500	5.25	30
	1000	3.75	21
Sodium iodoacetate	100	12.75	75
	1000	9.25	52
Ramihyphin A	1	15.5	88
	5	7.5	42
	10	4.0	23
	50	2.8	16

In addition to the inhibition of colonial growth, bikaverin brings about a thickening of *N. crassa* hyphae. Cyanein, ramihyphin A, scopathricin and zygosporin A at subinhibitory concentrations stimulate the ramification of terminal hyphae (Plate 1) which becomes apparent already during the first hours of contact (Plate 2). 5-Fluorouracil and phenythyl alcohol in our experimental concentrations do not affect the character of myceliar growth. In contrast to the wild strain, the *Abn. 1* mutant does not exhibit any morphogenic effects of cyanein, ramihyphin A, scopathricin A and zygosporin A.

The colonies of *N. crassa* grown in the dark on paper discs were transferred on aqueous agar under red light and then illuminated by either daylight or near UV light at 366 nm. A partial conidiation proceeded even without illumination but it was appreciably stimulated by both daylight and longwave UV light. One-min illumination with daylight enhanced the formation of conidia by 40% on average. Illumination at 366 nm for 10—120 s stimulated the formation of conidia by about 30%, more intensive illumination had an inhibitory effect.

TABLE III. Effect of daylight (15 min) and 5-fluorouracil (1000 μg/ml) on the sporulation of submerged-growing mycelium of *Neurospora crassa* (details *cf.* Materials and Methods).

Treatment	Time of 5-fluorouracil action (h)[a]	Number of spores per disc × 10^{-6}	% of unilluminated control
No light	—	19.5	100
No light + 5-FU[b]	0 to +24	9.0	46
Light	—	27.6	142
Light + 5-FU	−4 to −1	12.1	62
Light + 5-FU	−1 to +2	10.0	51
Light + 5-FU	+2 to +5	19.0	98
Light + 5-FU	0 to +24	7.4	38

[a] Intervals relative to photoinduction; negative values — prior to photoinduction, positive values — after photoinduction;
[b] 5-FU — 5-fluorouracil.

In experiments with the inhibition of photoinduced conidial formation the colonies were subjected to a single illumination by daylight for 15 min with a subsequent incubation in the dark for 24 h. When the colonies of *N. crassa* on paper discs were transferred on aqueous agar with inhibitors 60 min prior to the photoinduction, actinomycin D, cycloheximide, 5-fluorouracil, sodium iodoacetate and ramihyphin A showed inhibitory effects presented in Table II. Analogous effects were also obtained during the photoinduced sporulation of a mycelium grown in the dark under submerged conditions. In these experiments the effect was also studied of a short action of 5-fluorouracil on a filtered mycelium before, during or after the photoinduction. The results are given in Table III. In comparison with the prolonged action of 5-fluorouracil added just before the photoinduction and present throughout the experiment, the most pronounced effect of a short application was observed when the agent was present in the interval of −1 to +2 h relative to photoinduction. Its action within another 3-h interval (−4 to −1 h) was less effective. Finally, when present in the interval from +2 to +5 h after the photoinduction its inhibitory effect was less pronounced than that found in the above experimental variants. Unilluminated mycelium in the presence of 5-fluorouracil produced about the same number of conidia as illuminated mycelium exposed to the effect of the inhibitor in the intervals of 0—24 h or −1 to +2 h.

DISCUSSION

Phenethyl alcohol inhibits the synthesis of DNA, RNA and proteins in Gram-negative bacteria (Treick and Konetzka, 1964) the inhibition of initiation of DNA replication being associated with its effect on the synthesis of membrane phospholipids (Nunn and Tropp, 1972). Phenethyl alcohol is an autoinhibitor of *Candida albicans* (Lingappa *et al.*, 1969) but the mechanism of this inhibition is not yet known. In our experiments this agent was found to retard the germination of conidia.

5-Fluorouracil, known to inhibit the RNA synthesis, did not affect the germination; this might be explained by the fact that the germination process includes the participation of fully synthesized mRNA which was detected in mature conidia of *N. crassa* (Bhagwat and Mahadevan, 1970).

Bikaverin and cyanein partially retarded the germination but their effect became manifest no sooner than during the myceliar growth. Bikaverin brings about an intensive vacuolization of fungi (Conrforth *et al.*, 1971); in our experiments it caused a thickening of *N. crassa* hyphae. Cyanein (Plate 1d and 2b) and zygosporin A (Plate 1f) induced a frequent ramification of hyphae similar to that found in *Botrytis cinerea* (Betina and Mičeková, 1973). Scopathricin is a derivative of scopamycine which is characterized by the stimulation of the ramification of *B. cinerea* hyphae (Hütter *et al.*, 1965). In our experiments with *N. crassa* scopathricin had similar effects (Plate 1e, 2e, f). Ramihyphin A acts on filamentous fungi, inducing an intensive ramification of hyphae even at subinhibitory concentrations (Baráth *et al.*, 1974; Baráthová *et al.*, 1975). It also induces chemical and morphological changes in cell wall of *Neurospora sitophila* (Betina *et al.*, in preparation) similar to the changes occurring during the genotypically or phenotypically induced "colonial growth" (Mahadevan and Tatum, 1965).

The increased frequency of ramification of *N. crassa* hyphae in our experiments was induced under the conditions of a partial elongation of the hyphae (Table I). Katz *et al.* (1972) suggest that the partial inhibition of the cell wall and cytoplasmic membrane synthesis at the site of growth should increase the frequency of ramification. They also attempt to explain single point mutations which alter the morphology of colonies and stimulate the frequency of ramification of *Aspergillus ni-*

dulans, in the following way: if these mutations suppress the rate of synthesis of some cell wall precursor, without affecting the mechanism of the initiation of ramification, they should result in multi-branched hyphae.

Our experiments showed that under given conditions light was not absolutely necessary for the conidiation of *N. crassa* but that it stimulated rather the differentiation of conidia. The conidiation of colonies kept permanently in the dark corresponded approximately to one fifth of conidiation after photoinduction (Table II). Apart from ramihyphin A and sodium iodoacetate, the photoinduced conidiation was also suppressed by inhibitors of RNA synthesis (or protein synthesis) such as actinomycin D, 5-fluorouracil and cycloheximide. When applied at 3-h intervals before, during or after the photoinduction, 5-fluorouracil permitted a different intensity of conidiation. 5-Fluorouracil present during the photoinduction inhibited the photoinduced conidiation in the dark (Table III). However, when applied 2 h after the photoinduction and removed after another 3 h, the inhibitor exerted a weaker effect. This may be explained by the fact that within 2 h after photoinduction a substantial part of photoinduced metabolic processes have already been finished. 5-Fluorouracil suppresses also the photoinduced conidiation of *Trichoderma viride* which is explained as being due to the inhibition of RNA synthesis necessary for conidiation (Galun and Gressel, 1966; Gressel and Galun, 1967).

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The plates will be found at the end of the issue.

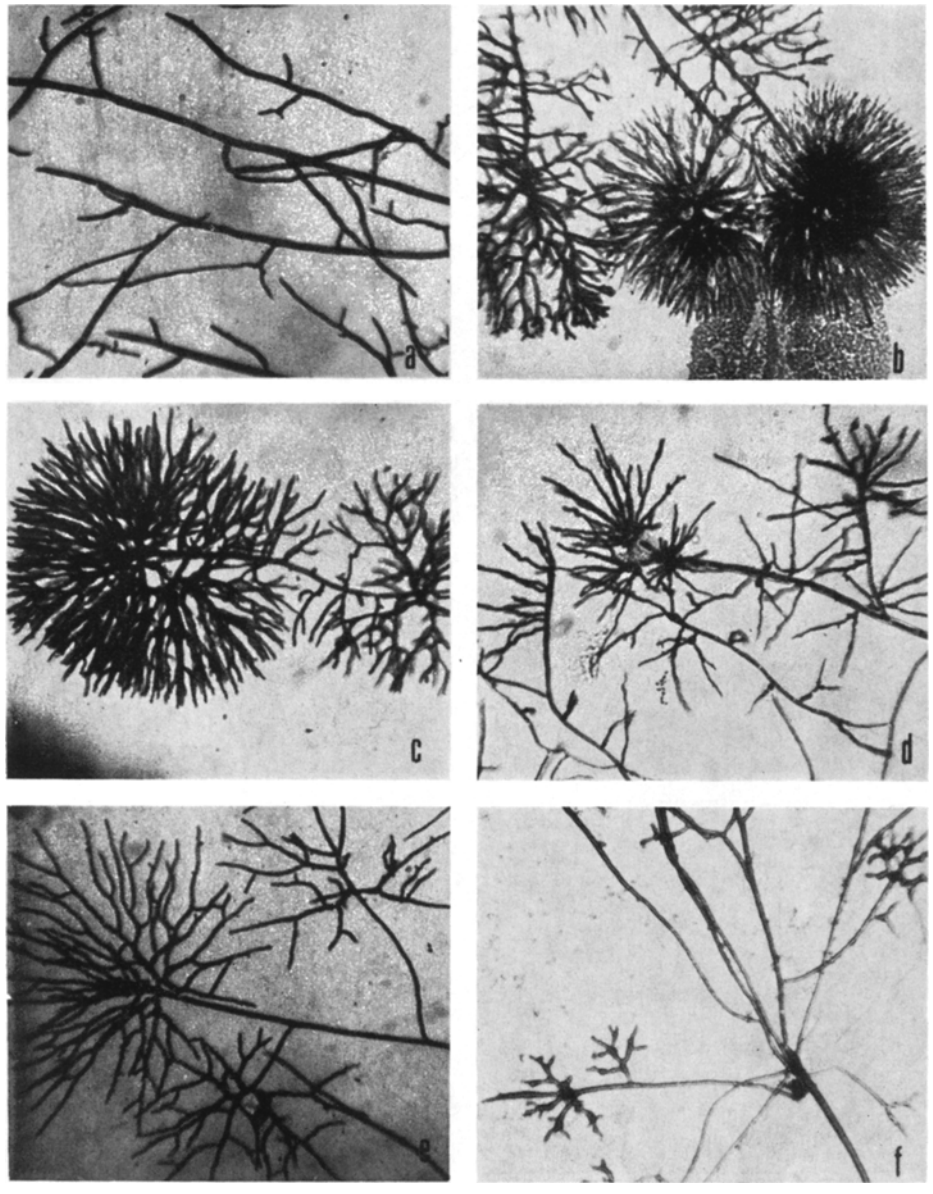

PLATE 1. Changes in myceliar growth of *Neurospora crassa* induced by antibiotics. a, Control without antibiotics; b, ramihyphin A 0.05 μg/ml; c, ramihyphin A 0.1 μg/ml; d, cyanein 25 μg/ml; e, scopathricin 200 μg/ml; f, zygosporin A 50 μg/ml. Magnification approximately 360×.

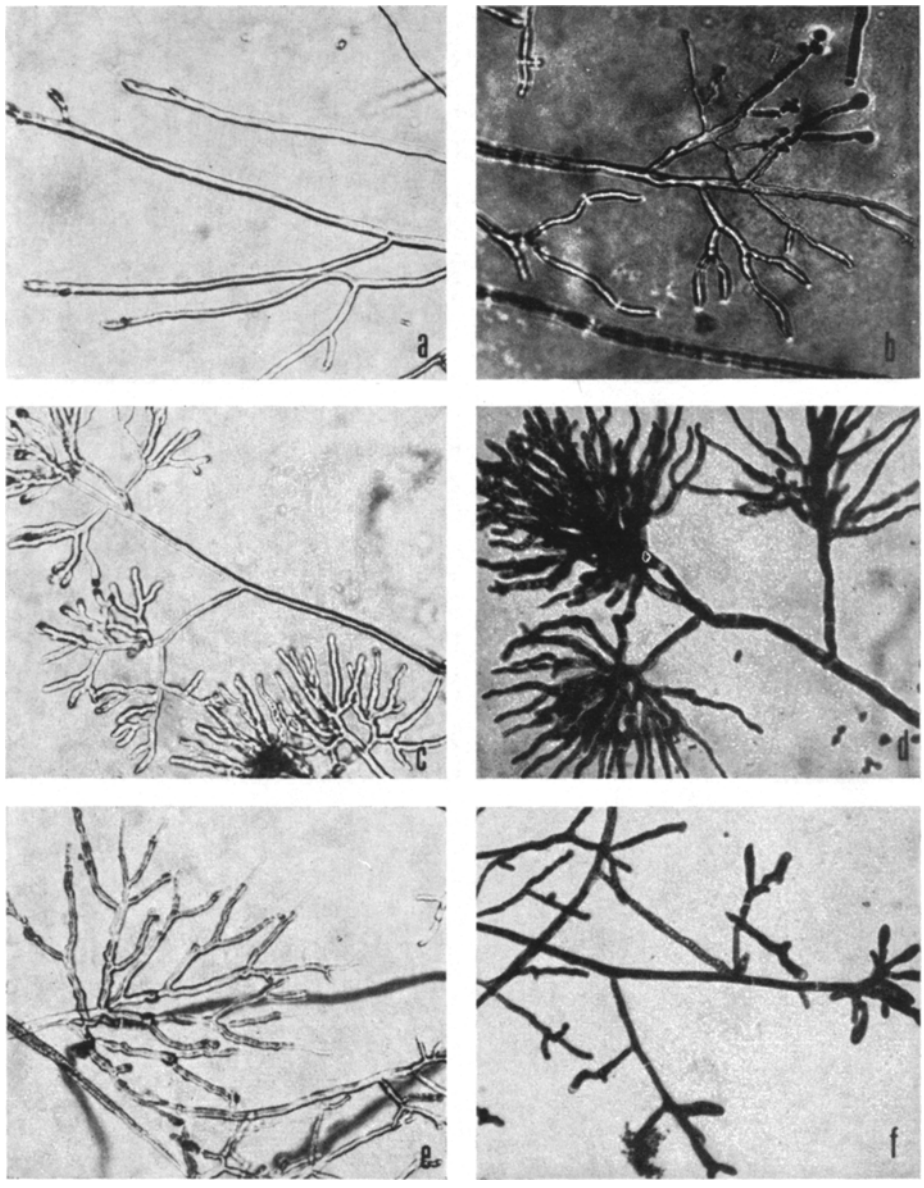

PLATE 2. Dynamics of changes in myceliar growth of *Neurospora crassa* induced by antibiotics. a, Control without antibiotics; b, cyanein 5.6 μg/ml after a 6-h treatment; c, ramihyphin A 0.1 μg/ml after a 6-h treatment; d, ramihyphin A 0.1 μg/ml after a 10-h treatment; e, scopathricin 14 μg/ml after a 6-h treatment; f, scopathricin 14 μg/ml after a 10-h treatment. Magnification approximately 540×.