Effect of Inhibitors on the Morphology and Growth of *Allomyces macrogynus*

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SUMMARY

The ability of 18 compounds to inhibit morphogenesis and/or growth of the diploid stage of *Allomyces macrogynus* was assayed. Of the three stages of development – germination, vegetative growth, and sporogenesis – germination appeared to be the most sensitive to inhibition and vegetative growth the least sensitive. Sporogenesis was sensitive to most of the inhibitors tested, but high concentrations were required for effective activity. Inhibitors could be grouped according to the stage of germination (cyst or rhizoid) at which they blocked development; those effective at the rhizoid stage, could be divided further on the basis of the resultant morphology of the germling.

INTRODUCTION

The phycomycete *Allomyces macrogynus* is well-suited for developmental studies of eukaryotic organisms. During its diploid life-cycle (spore germination, vegetative growth, and sporogenesis) it produces a number of well-defined, differentiated cell types (Lovett, 1967; Burke, Seale & McCarthy, 1972). The life-cycle can be conveniently reproduced on defined medium and large amounts of the cell types can be obtained by manipulation of growth conditions (Emerson & Wilson, 1954; Machlis & Crasemann, 1956; Machlis *et al.*, 1966).

This report examines the effect of inhibitors, known to have specific biochemical effects in other eukaryotic systems, on the morphogenesis and growth of the diploid life-cycle of *A. macrogynus*. Differential effects of inhibitors on morphogenesis and growth may allow the development of tools for examining specific events in the life-cycle. An extensive survey has been made of the effects of inhibitors on several phycomycetes, including *A. macrogynus* (Matsuma & Cantino, 1971). However, only the inhibition of the overall process of germination was measured: neither the stage of germination affected, nor the inhibitory effects on vegetative growth or sporogenesis were determined.

Several qualitative measurements of inhibition in phycomycetes have been reported. Cycloheximide blocks germination at the cyst stage in *A. arbuscula* (Burke *et al.*, 1972) and *Blastocladiella emersonii* (Lovett, 1968); and actinomycin D blocks germination at the rhizoid stage in both of these organisms. Actinomycin D blocks sporogenesis in *Achlya ambisexualis* (Griffin & Breuker, 1969), but not in *A. arbuscula* (Burke *et al.*, 1972) and *B. emersonii* (Murphy & Lovett, 1966).

METHODS

Strain. All studies were performed with the sporophyte stage of *Allomyces macrogynus* Burma.

Growth and inhibition studies. In studies of the effects of inhibitors on either germination or vegetative growth, mitospores, at a final concentration of 5×10^4 ml⁻¹, were inoculated in 25 ml of Machlis minimal medium (Machlis, 1953) modified by the addition of glutamic acid (500 µg ml⁻¹). The inclusion of glutamic acid greatly enhanced growth and increased the maximum mass reached by the culture. The mitospores were obtained as described previously (Burke *et al.*, 1972). In germination experiments, inhibitors were present in the medium at the time of spore inoculation; in vegetative growth experiments, inhibitors were added after either 16 or 24 h growth. All cultures were incubated at 28 °C in a rotary-shaking water bath operating at 125 rev./min. Spore induction was accomplished by filtering 16 h cultures on a stainless-steel mesh and then resuspending in 'dilute salt' solution (Machlis & Crasemann, 1956). Inhibitors were present from the time of spore induction, which was monitored visually for 6 h.

Measurement of inhibition. Inhibition was determined by: (i) following the interference in the normal morphological development of cultures during germination and sporangium induction; and (ii) measuring the dry weight of the culture. In all cases duplicate test cultures were weighed after 24 h drying at 60 °C, and inhibition was measured at least twice.

Inhibitors. Mitomycin C, emetine hydrochloride, chloramphenicol, tetracycline, 2,4-dinitrophenol (2,4-DNP), fluorouracil, *p*-fluorophenylalanine, and azaguanine were obtained from Sigma; acridine orange from Polysciences, Inc.; nalidixic acid, ethidium bromide, actinomycin D, rifampin, cycloheximide, puromycin, neomycin, and polymyxin from Calbiochem; and sodium azide from Eastman.

Light microscopy. A Leitz photomicroscope was employed for all observations. Wet mounts were made on glass slides. In some cases the slides were coated with 2 % (w/v) agar.

RESULTS

Effect of inhibitors on growth of spores

Eighteen compounds were tested for their ability to inhibit the growth of *A. macrogynus* in cultures started from mitospores. They included compounds which have been shown to inhibit DNA synthesis, RNA synthesis, protein synthesis, membrane synthesis, and mitochondrial function in eukaryotic systems.

Most of the test compounds inhibited growth, as measured by dry weight increase, by at least 50 % at a concentration of 20 µg ml⁻¹ or less; although some required up to 55 µg ml⁻¹ for 50 % inhibition and 200 µg ml⁻¹ for 90 % inhibition (Fig. 1, Table 1). There did not appear to be any relationship between the amount of inhibitor necessary and the probable function inhibited. For most compounds, a concentration of two to three times that producing 50 % inhibition yielded at least 90 % inhibition. A plot of the percentage inhibition versus the log of the dose generally gave a straight line, suggesting a zero order interaction, which may be indicative of a unique site for inhibitor sensitivity. The few exceptions to this relationship occurred at the highest levels of inhibition (over 90 %) where measurements were most difficult to make. There was a wide variation in sensitivity to different inhibitors.

Effect of inhibition on vegetative growth

In the above experiments, inhibition was measured by determining the dry weight of the cultures relative to the dry weight of an untreated control. Since inhibitors were present prior to germination and measurements were not made until the control culture reached its maximum vegetative growth, it was possible that we were measuring inhibition of vegetative growth rather than inhibition of germination. However, cultures inhibited by 90 %

Fig. 1. Inhibition of growth by various inhibitors. Cultures were started from spores as detailed in Methods. The percentage inhibition of dry weight production of the culture relative to an untreated control after 40 h incubation is plotted versus the concentration of inhibitor. ○, Cycloheximide; ●, fluorouracil; □, fluorophenylalanine; ■, rifampin.

Table 1. *The effective concentrations of inhibitors of growth and of sporangium formation in A. macrogynus, and the stage of germination that the inhibitors affected*

For determination of growth inhibition, cultures were started from mitospores and grown for 40 h as detailed in Methods: inhibitors were present at the time of inoculation. Results are expressed as the inhibitor concentration required to inhibit the increase in the dry weight of the culture relative to that of an untreated control by 50 % or 90 %. The stage at which germination was affected (cyst or rhizoid) was determined visually.

For inhibition of sporangium formation, results are expressed as the minimum concentration necessary to block sporangium formation.

Inhibitor	Concn (μg ml ⁻¹) for growth inhibition:		Germination stage blocked*	Concn (μg ml ⁻¹) for sporangium formation inhibition†
	50 %	90 %		
Cycloheximide	0.02	0.1	C	1.0
Fluorouracil	0.5	0.9	R	100.0(NE)
Ethidium bromide	1.1	2.5	R	50.0
Sodium azide	1.5	20.0	C	100.0
2,4-DNP	1.5	16.5	C	25.0
Tetracycline	2.0	38.0	R	500.0
Azaguanine	2.5	7.0	R	25.0
Actinomycin D	2.5	5.0	R	10.0
Fluorophenylalanine	5.0	9.5	R	100.0(NE)
Mitomycin C	6.0	12.0	R	100.0(NE)
Chloramphenicol	9.0	40.0	R	500.0(NE)
Emetine-HCl	9.5	16.0	R	20.0
Polymyxin	11.0	15.0	R	25.0
Nalidixic acid	15.0	25.0	R	50.0(NE)
Neomycin	20.0	200.0	R	100.0
Puromycin	20.0	50.0	R	50.0
Acridine orange	23.0	75.0	R	100.0(NE)
Rifampin	55.0	95.0	R	500.0

* C, Cyst; R, rhizoid. † NE, Inhibitor had no effect at this concentration.

Fig. 2. Inhibition by fluorophenylalanine. Spores germinated in the presence of fluorophenylalanine ($50 \mu\text{g ml}^{-1}$) after 40 h incubation. Bar marker represents $20 \mu\text{m}$.

Fig. 3. Inhibition by ethidium bromide. Spores germinated in the presence of ethidium bromide ($5.0 \mu\text{g ml}^{-1}$) after 40 h incubation. Bar marker represents $50 \mu\text{m}$.

Fig. 4. Inhibition by chloramphenicol. Spores germinated in the presence of chloramphenicol ($100 \mu\text{g ml}^{-1}$) after 40 h incubation. Bar marker represents $50 \mu\text{m}$.

or more were always in the germling stage, and those inhibited to a lesser extent were a mixture of germlings and vegetative growth of normal appearance: thus, the primary effect of inhibition appears to be on germination. When the compounds, at concentrations which caused 90 % inhibition in cultures started from mitospores, were added to cultures after 16 h growth, they had no effect on the dry weight increase of cultures relative to that of an untreated control. To measure the effect of inhibitors on vegetative growth, they were added after 24 h growth to untreated cultures and the dry weights of these cultures were measured

after another 8 h growth. During this time, the dry weight of the controls increased at maximum rate. Only rifampin, *p*-fluorophenylalanine and 2,4-DNP caused any significant inhibition of weight increase; all other compounds either did not inhibit the dry weight increase, or apparently stimulated it. Vegetative growth appeared to be much less sensitive to inhibition than germination since the three compounds giving significant inhibition were present at concentrations three to five times greater than those required for 90 % inhibition of growth when starting from spores (Table 1).

Effect of inhibition on morphology during germination

The specific morphogenetic event during germination which was most severely affected by each test compound was determined by visual observation of the treated cultures. Typically, spore germination consists of four stages: mitospore, cyst formation, rhizoid outgrowth, and hyphal formation. The inhibitors tested fell into two classes when considering their maximum effect upon germination: there was either no development beyond cyst formation or no development beyond rhizoid formation (Table 1).

High concentrations of cycloheximide, sodium azide, and 2,4-DNP, inhibited germination at the cyst stage; all other compounds inhibited germination at the rhizoid stage.

There were, however, significant differences in the appearance of the organisms blocked at the rhizoid stage. The addition of *p*-fluorophenylalanine and fluorouracil produced organisms that appeared to have several rhizoid roots rather than the normal single root (Fig. 2). Ethidium bromide inhibition gave rise to spherical cells with a straight, unbranched rhizoid – a ‘lollipop’ shape (Fig. 3), which is typical of the initial morphology of the normal rhizoid stage. In other cases, there seemed to be a definite elongation and thickening of the base of the rhizoid prior to cessation of growth (Fig. 4). Thus, although the majority of these inhibitors blocked development at the rhizoid stage, they exhibited a differential effect on the morphology of the inhibited germling. The examples shown in Figs. 2, 3 and 4 represent the majority of the population present and are not isolated instances.

Effect of inhibitors on sporogenesis

Many of the inhibitors were also able to block sporogenesis (Table 1). The concentrations necessary for effective inhibition were not always the same as those effective during germination. Some inhibitors, such as ethidium bromide, tetracycline and sodium azide, were only effective at considerably greater concentrations than those effective in blocking germination; actinomycin D, 2,4-DNP, and polymyxin were effective at concentrations similar to those blocking germination, whereas other inhibitors, such as fluorouracil, *p*-fluorophenylalanine, and mitomycin were not effective at the concentrations tested. Those inhibitors which were effective did not demonstrate any differential effect: all blocked the appearance of the first stage of sporogenesis.

DISCUSSION

Our studies have shown that germination of *A. macrogynus* mitospores is differentially susceptible to a number of inhibitors of known functions in other eukaryotic systems. Vegetative growth and sporogenesis were also sensitive to some of the inhibitors, but required much higher levels for inhibition to occur and did not display any differential inhibitory response.

None of the inhibitors stopped the encystment process. Apparently the cell-wall synthesis necessary for encystment does not require any function blocked by the inhibitors.

It has been previously demonstrated (Burke *et al.*, 1972) that cytoplasmic protein synthesis is required for differentiation from cyst to rhizoid. Cycloheximide, an inhibitor of cytoplasmic protein synthesis (Pestka, 1971), and 2,4-DNP and sodium azide (Harold, 1972), uncouplers of oxidative phosphorylation, inhibited growth beyond the cyst stage. However, inhibitors of protein synthesis, emetine and puromycin (Pestka, 1971), and *p*-fluorophenylalanine, a phenylalanine analogue, did not block the transformation of cyst to rhizoid, even at concentrations that were five to ten times higher than those resulting in 90 % inhibition of growth. Our experiments did not explain why these latter inhibitors of protein synthesis did not block germination at encystment.

Ethidium bromide and other inhibitors of mitochondrial function, i.e. nalidixic acid, rifampin, chloramphenicol, tetracycline, and neomycin (Kroon & DeVries, 1970), blocked germination at the rhizoid stage. It seems likely that mitochondrial function is required for growth beyond germination. Ethidium bromide inhibition was of further interest since it appeared to partition the germination process by inhibiting the dispersal of the nuclear cap. This structure, which contains all of the spore ribosomes, breaks down at encystment, dispersing the ribosomes throughout the cell (Lovett, 1963). In the presence of ethidium bromide many of the germlings had intact nuclear caps even when the rhizoid was present.

It is apparent that dry weight measurements cannot be used as an indicator of the inhibition of vegetative growth. While we were able to measure an effect on vegetative growth, the treated organisms actually weighed more than the controls in some cases, thus precluding a true measurement of inhibition. One possible explanation is that inhibition of growth stimulates accumulation of storage polymers such as glycogen or polyphosphate, and it is these materials that cause the weight increase. Other studies have shown that the organism does contain a hexose phosphate polymer which could be a candidate for this material (unpublished results).

Dry weight measurement, however, did seem to be a reliable indicator of inhibition, at least at greater than 90 % inhibition, in cultures started from mitospores. This was indicated by the observation that these cultures were all in the germination stage. Further, we have made some measurements of the DNA content of inhibited cultures relative to control cultures and have found that inhibition of dry weight and DNA content are similar (± 10 %) in the range of 30 to 80 % inhibition. The percentage of DNA per unit dry weight was very small, so these measurements cannot be done with enough precision to be of any use other than as a guideline.

The differential effects on germination imply that the developmental processes of this stage can be unlinked, and that chemical inhibition may be used to identify specific developmental processes. However, chemical inhibition by those inhibitors tested does not seem to be suitable for use in studying vegetative growth and sporogenesis. The high levels of inhibitors needed would make it difficult to be certain that one was measuring a specific effect of the inhibitor and the lack of a differential effect on either vegetative growth or sporogenesis reduces the usefulness of these inhibitors. The increased concentration of inhibitor required may be due to a change in permeability during vegetative growth and sporogenesis.

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