

DIFFERENTIATION IN SPECIES OF *ALLOMYCES*: THE PRODUCTION OF SPORANGIA

By JEAN YOUATT,* R. FLEMING,* and B. JOBLING*

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Abstract

Conditions have been defined for the production of sporangia by vegetative plants of *Allomyces* species. Diploid plants produced zoosporangia and released spores in 3–4 hr at 32°C. Haploid plants produced sexual spores in a similar period. The chief factor controlling differentiation to produce sporangia was the supply of amino acids. Amino acid deficiency induced sporogenesis but individual amino acids varied in their efficiency in delaying differentiation and ammonium salts were ineffective. The process of sporangium production required adequate aeration and plants had previously to have been cultivated with adequate thiamine and glucose or to have these nutrients supplied.

I. INTRODUCTION

Allomyces species have some advantages for the study of cell differentiation. The plants have a complex life cycle with diploid and haploid phases of growth. Two types of sporangium are produced on the diploid plant—zoosporangia and meiosporangia; and two types of sporangium are produced on the haploid plant—male and female gametangia. In this cycle of development there are structural changes which may be followed by a combination of microscopic and chemical methods. As this paper will show, differentiation to produce sporangia can be controlled through the external environment in a highly uniform manner.

The formation of zoosporangia after the transfer of diploid vegetative plants to dilute salts solutions was reported by Machlis and Ossia (1953). Kobl and Turian (1967) investigated the formation of gametangia on haploid plants. To avoid the necessity of producing meiospores for the inoculation of haploid liquid cultures, these authors used small plugs taken from the growing edge of haploid cultures on solid medium. They found that, on transfer to distilled water, sporogenesis occurred and was not prevented by additions of glucose and various inorganic components, including ammonium salts. They did not test amino acids in their system despite the fact that their cultures had been grown with casein hydrolysate, and not ammonium salts, as the source of nitrogen. No gametangia were produced under anaerobic conditions and, with reduced levels of dissolved oxygen, the process was considerably retarded. Kobl and Turian concluded, therefore, that oxygen was the “trigger mechanism”.

The present report shows that plants of *Allomyces* respond to a deficiency of amino acids by producing sporangia. While the process is aerobic, evidence will be produced that differentiating plants do not have an increased demand for oxygen, as postulated by Kobl and Turian.

* Department of Chemistry, Monash University, Clayton, Vic. 3168.

II. MATERIALS AND METHODS

Allomyces macrogynus R. Emerson (Burma strain), *A. arbuscula* E. J. Butler (Ceylon strain), and male and female hybrid strains were obtained from Dr. Machlis, Department of Botany, University of California, Berkeley, U.S.A.

Unless otherwise stated, the cultures were grown on a medium containing 5 g glucose, 4 g casein hydrolysate, 15 mg thiamine, 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.05 g CaCl_2 per litre. A phosphate buffer of pH 7 was prepared with 53.1 g KH_2PO_4 and 86.6 g Na_2HPO_4 per litre. After separate sterilization at 15 lb/in², 1 ml of buffer was added to 100 ml of medium. Solid media contained 2% agar.

The pH of the medium was tested after autoclaving and throughout the growth period using British Drug Houses (BDH) narrow-range indicator papers or a Beckman pH-meter. It was found that the pH remained in the range of 6.8–7.0 throughout the growth of the organisms.

For routine culture BDH or Oxoid acid-hydrolysed casein was used. These were not vitamin-free and to establish the dependence on amino acids they were replaced in some experiments by CalBiochem vitamin-free hydrolysed casein which was prepared from casein containing a maximum of 3% of other components. An artificial mixture of pure L-amino acids was also prepared to the published amino acid composition of casein (Block and Bolling 1947).

When an attempt was made to reproduce the conditions of Kobr and Turian (1967), 12 plugs of approximately 5 mm² were transferred from the growing edge of a culture on agar to 50 ml of Turian's medium (1963) contained in a 150-ml conical flask.

On all other occasions media were inoculated with spores. Zoospores were obtained by transferring 2–3-day-old diploid plants, bearing sporangia, to shallow layers of sterile distilled water. Spore release occurred within 1–2 hr. To obtain meiospores free of zoospores, plants bearing mature meiosporangia were first air-dried and then transferred to sterile distilled water. Spore release took from 4 to 6 hr. Meiosporangia resist desiccation (Machlis and Crasemann 1956). Normally a 1% inoculum of about 10^5 spores per millilitre was used.

Initially, cultures were incubated at 25°C following Machlis and Ossia (1953) and Turian (1963). Subsequently it was found that the optimum temperature was near 32°C which is in agreement with Quantz (1943) who used an incubation temperature of 34°C. Cultures were shaken at 90 strokes per minute on a reciprocal shaker with a 2-in. amplitude, taking 5 ml of medium in a 25-ml conical flask, 10 ml in a 50-ml flask, and so on.

A.R. grade L-amino acids were obtained from Calbiochem, Los Angeles, California. Separate amino acids were neutralized with sodium hydroxide where necessary and were autoclaved with the phosphate buffer but separate from the other medium components.

The residual amino acid concentration of the medium was estimated semiquantitatively by preparing a series of dilutions covering a range of 5–90% of the original medium concentration. The culture supernatants were compared with the standard series by spotting equal volumes of samples on paper and developing the spots with ninhydrin.

Most of the experiments described in this paper used 16–24-hr-old plants in which the hyphae had branched 3–4 times. At this size the whole plant was observed at $\times 100$ magnification in shallow liquid layers contained in small dishes of 1-in. diameter containing 0.5–1.0 ml of medium. Similar plants were sometimes observed on Cellophane placed over a solid medium. This arrangement allowed the development of rhizoids to be observed more readily. Culture plugs such as those used by Turian were less conveniently examined and it was not always easy to distinguish the new growth from that already present at the time of transfer.

III. RESULTS AND DISCUSSION

(a) Response to Amino Acid Deficiency

It was observed that growing cultures produced sporangia when the amino acid content had declined to about one-tenth of the initial concentration. Glucose was still present at this stage. To confirm that the response was related to the amino acid concentration, vegetative plants were transferred to fresh medium containing all the components except amino acids. Zoosporangia or gametangia formed within 4 hr.

The addition of normal concentrations of casein hydrolysate, or a mixture of amino acids corresponding to the composition of casein hydrolysate, suppressed sporogenesis.

The sequence of events in a plant deprived of amino acids was (1) an increased development of rhizoids; (2) swelling of the hyphal tip and the development of a granular appearance; (3) walling off of a sporangium; (4) development of papillae; and (5) the release of spores. In young plants, the process exhibited a high degree of synchrony. Sporangia developed on all the hyphae at similar rates and the release of spores occurred simultaneously from many sporangia. At 32°C spore release from haploid and diploid plants with good aeration occurred in 3–4 hr.

(b) *Attempts to Suppress Sporogenesis with Single Amino Acids*

It seemed that amino acid deficiency was likely to be equivalent to nitrogen deficiency and that observing the effect of an amino acid on sporogenesis might serve as a rapid test of its availability as a nitrogen source. Single amino acids were tested at 1 and 10 mM concentration. A range of response was observed from L-proline which had no delaying action at all to L-valine which delayed the formation of sporangia for several hours. Other amino acids with negligible delaying action included arginine, aspartic acid, cysteine, serine, and glutamic acid. Amino acids which were effective in delaying the formation of sporangia included histidine, isoleucine, methionine, phenylalanine, threonine, and tryptophan.

No direct relationship has been observed between the effect of an amino acid on sporogenesis and its ability to stimulate the growth of *Allomyces* species in chemically defined media. Nutritional studies, which are still in progress, have identified methionine, histidine, aspartic acid, proline, arginine, and lysine as good nitrogen sources for the organisms. This group contains amino acids which retard sporogenesis and others which do not. The apparent discrepancy may be due to the fact that single amino acids were used in experiments on sporangium formation while the organisms have an absolute requirement for methionine.

(c) *Glucose Requirement in Sporogenesis*

Young plants, under normal conditions, produce sporangia in the presence or absence of glucose. However, when a 12% spore inoculum was used a dense mass of small plants was produced in 16 hr which exhibited signs of nutritional deficiency, such as the presence of rhizoids sprouting along the hyphae. These plants either failed to make sporangia in the absence of glucose or produced abnormally small sporangia which released about four spores per sporangium. When glucose was supplied, the plants made normal sporangia. It was concluded that the normal conditions of growth allowed the plants to accumulate sufficient sources of carbon and energy.

(d) *Thiamine Requirement for Sporogenesis*

Quantz (1943) reported a thiamine requirement in *Allomyces*. The present study confirmed that thiamine-deficient plants grow poorly and are highly vegetative.

Addition of thiamine to deficient cultures stimulated the formation of sporangia but the time taken depended on the availability of amino acids in the medium. Plants from the normal medium presumably carried over sufficient thiamine and did not require an external source during sporogenesis.

(e) *Oxygen Requirement for Sporogenesis*

Sporogenesis was completely inhibited under nitrogen. However, in air the development of sporangia occurred at similar rates in shaken and unshaken suspensions between 25°–35°C and it was concluded that the oxygen requirement was met by diffusion into the shallow layers. Kobr and Turian (1967) had postulated an increased oxygen demand during the formation of sporangia. It was possible to test this hypothesis by measuring oxygen consumption in the presence of (1) an amino acid which suppressed the formation of sporangia but did not stimulate oxygen uptake (L-valine) and (2) an amino acid which permitted the development of sporangia without stimulating oxygen uptake (L-aspartic acid). A control contained no added amino acid. No increase in the consumption of oxygen was observed in the plants which produced sporangia and released mature spores.

TABLE 1

EFFECT OF TEMPERATURE ON SPOROGENESIS IN *ALLOMYCES MACROGYNUS*

Microscopic examination was made at time intervals to determine the percentage of plants with zoosporangia (ZS) and the percentage of sporangia released. No sporangia were present at the time of transfer to the amino acid-deficient medium

Temp. (°C)	1 hr		2 hr		3 hr		4 hr	
	ZS (%)	Released (%)	ZS (%)	Released (%)	ZS (%)	Released (%)	ZS (%)	Released (%)
26	<10	0	60–70	0	70–80	<5	80–90	5
28	<10	0	60–70	0	70–80	<5	80–90	20–30
30	20–30	0	70–80	0	80–90	<10	100	60–70
32	50–60	0	95–100	<5	100	20–30	100	95–100
34	20–30	0	60–70	0	80–90	<10	100	60–70
36	10–20	0	30–40	0	60–70	<5	70–80	10–20
38	<10	0	10	0	20–30	0	50–60	0
40	0	0	0	0	0	0	0	0

Cultures of diploid and haploid plants of the four cultures of *Allomyces* were produced according to the method of Turian (1963). While the amino acid content remained high no sporangia developed in either shaken or unshaken cultures. When amino acids were depleted sporangia developed in both stationary and shaken cultures. It was concluded that the differentiation process requires oxygen but that oxygen does not control the process in the sense of being the “trigger mechanism”.

(f) *Temperature Effect on Sporogenesis*

Table 1 shows the effect of temperature on sporogenesis. It was not possible to provide shaking at each of these temperatures simultaneously and the results are for stationary suspensions. The optimum temperature appeared to lie close to 32°C.

(g) *Reversibility of Commitment to Sporogenesis*

In a related organism, *Blastocladiella*, differentiation is triggered by the presence of bicarbonate and a point-of-no-return has been described (Cantino 1967). *Allomyces* did not exhibit such a commitment to sporogenesis and, when amino acids were added, recommenced vegetative growth by branching of the hyphae.

(h) *Other Responses of Allomyces to Amino Acids*

Allomyces species respond to amino acids in other ways. Machlis (1969) reported a chemotactic response to leucine and lysine. The same two amino acids hasten the encystment of spores of *Allomyces* (Cain, personal communication).

In the present investigation of *Allomyces* two further effects have been noted. Firstly, casein hydrolysate at 4 g/l inhibits the release of spores from mature sporangia while the remaining components of the medium allow spore release. Secondly, in diploid plants the proportion of glucose : casein hydrolysate determines whether the production of zoosporangia or meiosporangia is favoured. A medium containing 4 g casein hydrolysate and 2.5 g glucose per litre and normal levels of all other nutrients, produced zoosporangia exclusively. A medium with 2.5 g/l casein hydrolysate and 5 g/l glucose showed an increased production of meiosporangia and occasionally meiosporangia were produced exclusively.

The same phenomenon has been observed in media containing pure L-amino acids. At constant glucose levels and with varying amino acid concentration or with constant amino acid and varying glucose concentration the production of zoosporangia exclusively occurs at low molar ratios of glucose to nitrogen and the production of meiosporangia occurs when the glucose : nitrogen ratio is increased. Detailed studies of the amino acid requirements are in progress and will be reported elsewhere.

Amino acids, therefore, appear to exert controls at many stages in the life cycle of *Allomyces*.

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