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Selective Development of Resistant Sporangia in Growing Cultures of *Allomyces macrogynus* and *A. arbuscula*

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Abstract

The culturing of *A. macrogynus* and *A. arbuscula* was re-investigated with particular reference to the selective development of either zoosporangia or resistant sporangia in diploid cultures. The latter required an excess of glucose and a balance of concentrations of methionine and thiamine together with a reduced availability of oxygen. Results suggest that regulatory controls may be exerted by variations in the enzymes and coenzymes involved in the metabolism of pyruvate.

Extra keywords: synthetic media

Introduction

Control of differentiation in *Allomyces* species is achieved by variations in the environment of the cultures. In the diploid plants these variations determine whether sporangia are produced and whether the sporangia are zoosporangia or resistant sporangia. Some studies have already been reported in which controls were exerted on growing cultures (Youatt 1973; Sandstedt and Aronson 1978) or on plants in incomplete media (Youatt 1980*a*, 1980*b*, 1980*c*). Sandstedt and Aronson used a temperature control with the production of zoosporangia at 22°C and resistant sporangia at 12°C. Borkhardt and Olson (1979) have shown that *A. macrogynus* Emerson is tetraploid when grown at 25°C and diploid when grown for a period of time at 35°C. It was preferred, therefore, to concentrate on the control of diploid plants where development and maturation of resistant sporangia were achieved in 4–7 days.

In the earlier study (Youatt 1973) it was found that resistant sporangia developed preferentially when an excess of glucose was present after the supply of amino acids was exhausted. A supply of glucose was also essential with plants in incomplete media where the development of resistant sporangia was associated with the accumulation of glycogen and trehalose (Youatt 1980*c*), with reduced degradation of RNA and with conservation of phosphate (Youatt 1980*b*).

It was noted by Khandjian and Turian (1976) that *A. arbuscula* Butler required several transfers in liquid media before consistent growth patterns were observed. This was confirmed for *A. arbuscula* and described for *A. macrogynus* (Youatt 1980*a*) using defined media with amino acids and glucose. There appeared to be no published studies in which either species had been grown through several transfers in the more demanding ammonium salts and glucose media. With the advent of genetic studies

of *A. macrogynus* (Nielsen and Olson, personal communication) and the development of auxotrophic mutants, this aspect of the nutrition of *A. macrogynus* seemed to require further study. In this laboratory there were unexplained variations in the development of sporangia in some media which indicated that the knowledge of the control of differentiation was incomplete.

In this paper some of the variables in the growth of *A. macrogynus* and *A. arbuscula* are re-investigated. Experimental variables are first taken individually and then interactions between variables are described in the context of designing media for special purposes. One of the aims of the present study was to obtain resistant sporangia in the terminal positions.

Methods

Cultures

A. macrogynus Burma 3·35 and *A. arbuscula* Ceylon 1 were obtained from Dr L. Machlis, Department of Botany, University of California, at Berkeley. Both cultures have been maintained at 30°C for 10 years in casein hydrolysate–glucose (CHG) medium and in fully defined 4AAG medium (Table 1). Culture conditions have been described by Youatt (1973, 1980*a*). *A. macrogynus* 3·35 (35°C), the diploid culture and auxotrophic mutants of it were obtained from Dr L. Olson, The Institute of Genetics, Copenhagen.

Table 1. Media used in the culture of *Allomyces* species

Designation	Mg^{2+} (mM)	Ca^{2+} (mM)	Thiamine-HCl (mg/l)	L-Methionine (mM)	Nitrogen source (mM)	Glucose (mM)	Phosphate (mM)	Other
Machlis B	0·5[A]	0·5[A]	0·15	0·33	10 (ammonium phosphate)	30	15	Trace elements
NH_4G	2·0[B]	0·2[C]	2·0	0·30	9 (ammonium (citrate)	9	10	100 μM His
4AAG	2·0[B]	0·2[C]	2·0	1·0	1 each of His Asp Pro	4	10	
3AAG	2·0[B]	0·2[C]	2·0	0·125	6 each of His Asp	12	10	
CHG	2·0[B]	0·2[C]	0·15	—	—	27	10	Casein hydrolysate (4 g/l)

[A] As chloride. [B] As sulfate. [C] As sulfate or chloride.

Media

The compositions of several media used in this study are given in Table 1. Phosphate buffer was added after other components had been autoclaved together.

Aeration

Either a reciprocal shaker of 50 mm amplitude and 90 strokes/min or a rotary shaker of 120 rpm was used. The volume of medium and the size of conical flask used are indicated as 40/500, for example, where 40 ml of medium was in a 500-ml flask.

Standard Inoculum

The percentage of spores which germinate in culture media is variable, being affected by osmotic shock and attachment to glass surfaces (Youatt 1976). The inoculum is therefore defined purely

in terms of the procedure used which was standardized as follows. Cultures were grown in 40 ml of 4AAG medium in a 250-ml flask for 2–3 days at 30°C. A small plug of sterile cotton wool was placed in the medium to hold back the plants as the spent medium was removed by pipette and replaced by 8 ml of sterile water. The spores were released within 60 min. The suspension of spores was separated from the plants by pipette, again using the cotton wool plug to retain the plants. The percentage inoculum is then defined as the number of millilitres of this suspension used to inoculate 100 ml of medium.

Assays

Rhomboid crystals were produced in the Machlis medium (Table 1) with high concentrations of magnesium ion. The crystals were washed with distilled water, dissolved in dilute perchloric acid and analysed for ammonium and phosphate ions.

Ammonium ion was determined by Nessler's method (Oser 1965) and phosphate by Allen's (1940) method. Glucose was estimated enzymically (PGO assay kit; Sigma Chemical Co., St Louis, Missouri, U.S.A.). Amino acids were estimated in some experiments by automatic analysis; in others, chromatograms were developed in 80% (v/v) isopropanol–water or 80% (w/v) phenol with 3% (v/v) NH_3. Fructose was assayed by the cysteine–sulfuric acid procedure (Ashwell 1966).

Uptake of Mannose

D-[^{3}H]Mannose and D-[^{3}H]glucose were obtained from the Radiochemical Centre, Amersham, U.K. Suspensions of plants were exposed to each sugar at concentrations of 2 mM for periods ranging from 30 min to 10 h. Plants were washed rapidly and dried on Whatman GFA filters and counted in 2,5-diphenyloxazole–toluene scintillation fluid in a Beckman counter.

Reagents

Amino acids, sugars and thiamine-HCl were obtained from Sigma Chemical Co., St Louis, Missouri, U.S.A.

Results

Microscopic Observations of Developing Plants

In normal growth media spores germinated and developed hyphal tubes which underwent symmetrical *hyphal branching*. When nutrients were exhausted a *terminal sporangium* was walled off at the hyphal tip and below this a second *subterminal sporangium* sometimes developed. Alternatively a single *sporangial branch* sometimes emerged below the septum of the terminal sporangium. In deficient media *abnormal branching* was observed which included asymmetric branching and deposition of a partial septum without bifurcation. The microscopic observation of excessive rhizoid development was an early sign of the exhaustion of essential nutrients while an increased extension of the hyphae was associated with an inadequate supply of oxygen.

Prior History of the Culture

The inoculum for all the experiments was liquid-adapted by several transfers in 4AAG medium. Even so, the first transfer to a poorer medium often resulted in abnormal growth.

In this laboratory plants, when first transferred, have often matched the description of Machlis and Crasemann (1956) in having excessive rhizoid development and discoloured mycelium and yet on further transfer in the same medium have adapted and grown normally. Auxotrophic mutants supplied by Olson showed this behaviour in defined liquid media with amino acids and glucose. *A. arbuscula* grew for one

transfer only on NH_4G medium (Table 1). *A. arbuscula* and both strains of *A. macrogynus* required thiamine for vegetative growth in 4AAG medium on first transfer but not in liquid-adapted cultures. These examples illustrate the importance of knowing the prior history of the culture and it is strongly recommended that repeated transfer in liquid media be adopted as a regular practice.

Size of the Inoculum

The size of the inoculum has been an important consideration in media with suboptimal concentrations of methionine, for example. Only with heavy inocula has it ever been possible to obtain single unclumped plants. Both aspects will be discussed further.

Surface–Volume Ratios of Shaken Cultures

The pattern of differentiation has proved to be dependent on the surface–volume ratio when suitable media were selected. Thus resistant sporangia in the terminal position were produced at all times with 40 ml 3AAG medium in a 100-ml flask but zoosporangia were produced as well with 40 ml of the same medium in a 500-ml flask.

In small flasks shaking provides the necessary oxygen for sporangium formation (Kobr and Turian 1967; Youatt *et al.* 1971). Unfortunately shaking also caused spores to clump just before the emerging rhizoids were visible microscopically. Clumping was more pronounced with a rotary than with a reciprocal shaker.

Buffers and pH of Media

The optimum pH for dry weight yield was pH 5·8 and the optimum pH for resistant sporangium formation was close to 7 (Youatt 1973). This was also true for NH_4G medium (Table 1) where percentages of resistant sporangia were 0, 10, and 60 at pH 4·5, 5·3 and 6·8–7·2 respectively. Failure to control the pH of media during growth modified both the yield and the pattern of differentiation.

In the present study the ammonium phosphate of Machlis' medium was replaced by ammonium citrate (Table 1). This extended the buffer range to cover pH 2–8 (McIlvaine 1921) and avoided problems of phosphate precipitation described by Machlis (1953*b*) and discussed further below. Machlis (1953*c*) and Ingraham and Emerson (1954) showed that citrate was not used. There was no growth in the present medium when glucose was omitted.

There was no decline in pH in NH_4G, 4AAG or CHG media with 10 mM phosphate buffer when inoculated with spores from liquid-adapted cultures.

Effect of Magnesium Ions

Machlis (1953*b*) described inhibition of growth in media with a high concentration of magnesium ions and showed that this inhibition was associated with separation of small crystals. This observation was confirmed in the present study and the crystals were identified as magnesium ammonium phosphate. The problem was by-passed by the use of ammonium citrate. It was confirmed that 0·5 mM Mg^{2+} was sufficient for vegetative growth but sporangium development improved up to 2 mM and no toxic effect was observed at 8 mM.

Effect of Calcium Ions

Machlis (1953*a*) and Ingraham and Emerson (1954) defined a requirement for Ca^{2+} in *A. macrogynus* and *A. arbuscula* respectively. Youatt (1973) found that several transfers without added calcium were required to demonstrate a requirement for calcium with *A. macrogynus*. The presence of calcium up to 0·2 mM slightly improved differentiation in both species in the present study and ionic concentrations of 0·8 mM were not inhibitory.

Carbon Sources

The present study confirmed that *A. macrogynus* used D-mannose. D-[^{3}H]Mannose was rapidly incorporated and the replacement of D-glucose by D-mannose resulted in the same patterns of differentiation with zoosporangia in 4AAG medium (Table 1) and resistant sporangia with an excess of either glucose or mannose (see below).

Up to 10% of the glucose was converted to fructose when glucose was sterilized in the medium. A comparison of media for which glucose was sterilized separately showed that fructose did not produce any observable effect on growth or differentiation. However, the presence of fructose made it necessary to estimate residual glucose by an enzymic method. Above 20–22 mM glucose became inhibitory with longer growth lags and increased acid production.

Different combinations of amino acids in the absence of glucose allowed the development of zoosporangia only. This is consistent with observations that resistant sporangia develop only when there is an excess of glucose or mannose.

Nitrogen Sources

Commercial samples of casein hydrolysate have proved to be variable in composition. Sigma's enzyme-hydrolysed casein favoured the development of resistant sporangia at lower glucose concentrations than British Drug Houses acid-hydrolysed casein. The presence of peptides may reduce the total nitrogen available to the cultures and the composition may vary with respect to individual amino acids.

When supplied with all the common amino acids (e.g. in hydrolysed casein) both species at the end of the growth period had used all the amino acids. Culture media of many varied compositions are therefore possible. The 4AAG medium was originally developed to support *A. macrogynus*, *A. arbuscula* and hybrids of these (Youatt 1973). As neither species requires proline the 3AAG medium contains only methionine, histidine and aspartic acid.

Some of the amino acids are effective inhibitors of the development of zoosporangia (Youatt 1973) and in conjunction with an excess of glucose favour the development of resistant sporangia. In the 3AAG medium, for example, aspartic acid was able to be replaced by leucine which was a better inhibitor. Other aspects of nitrogen sources involve other variables and are discussed further below.

Methionine Requirement

In the absence of added methionine a 3% inoculum of spores (see Methods) developed to the stage defined as binucleate by Olson and Fuller (1971). In the same experiment a 0·6% inoculum produced plants with short, unbranched hyphae

and with one zoosporangium and a mass of rhizoids. To achieve the same stage of development with a 3% inoculum 8 μM L-methionine was required.

The L-methionine concentration of the 4AAG medium became a growth-limiting factor when it was reduced below 250 μM while maintaining all other nutrients unchanged. With a 0·6% inoculum and with concentrations of 60 μM and less the abnormal branching patterns described above were observed. With methionine concentrations ranging from 8 to 100 μM, resistant sporangia developed and assays showed that an excess of glucose was present. Thus the required excess of glucose was achieved with an amino acid : glucose ratio of 1:1·3. Ruiz-Herrera and Starkey (1969) showed that other soil fungi also used glucose during the metabolism of methionine. In the present study it was also observed that aspartic acid was used simultaneously with methionine when glucose was absent. These observations thus provided an alternative method for ensuring the excess of glucose required for the development of resistant sporangia. This was particularly convenient in view of the inhibitory effects of a high concentration of glucose already described.

When the 4AAG medium was used at four times normal strength, supplying 4 mM methionine, the initial growth was excellent with a 2% inoculum. At 21–22 h abnormal rhizoid development commenced and up to 46 h no further development occurred. At 22 h plants already contained high levels of methionine in their contents, the external supply of histidine had run out but glucose, aspartic acid and proline were still available. At 46 h the glucose, aspartic acid and proline had been metabolized but a high internal concentration of methionine still remained. In other media in which only the concentration of methionine was reduced, cultures differentiated normally and produced zoosporangia only with 1 mM methionine and mixtures of zoosporangia and resistant sporangia at 100 μM. Thus variation in the supply of methionine offers considerable scope for development of media for special purposes.

Thiamine Requirements

Various authors (Quantz 1943; Machlis 1953*b*; Ingraham and Emerson 1954) have described the requirement for thiamine by *A. macrogynus* and *A. arbuscula*. It was a surprise to find that liquid-adapted cultures of *A. macrogynus* 3·35 and 3·35 (35°C) as well as *A. arbuscula* did not require thiamine for vegetative growth in NH_4G, 4AAG or CHG media.

In the present study a different effect of thiamine at a higher concentration range was observed. In CHG medium without thiamine a dense growth was almost completely vegetative. The plants grown on 4AAG and NH_4G media still produced zoosporangia but 3AAG medium (40/100) allowed close to 100% development of resistant sporangia. At a thiamine concentration of 4 μg/ml the resistant sporangia developed more rapidly and more glucose was consumed in the first 24 h. Alanine accumulated in the thiamine-free culture but not in those with thiamine at 2 or 4 μg/ml, suggesting that other conversions of pyruvic acid were perhaps blocked since the enzymes require thiamine diphosphate.

Designing Media for A. macrogynus

It will be apparent from the results so far that the selective development of either zoosporangia or resistant sporangia does not depend on a single factor. Those

factors which are expected to make a medium more favourable to the production of resistant sporangia are (1) reducing aeration by the use of 40/100 conditions; (2) ensuring the maintenance of pH 6·7–7·0 throughout growth; (3) supplying 2 mM magnesium sulfate and 0·2 mM calcium sulfate; (4) ensuring an excess of glucose when growth ceases; (5) including amino acids which are effective in delaying production of zoosporangia (Youatt 1973); (6) using methionine concentrations which are suboptimal; and (7) supplying thiamine at concentrations of 2–4 μg/ml.

The original 4AAG medium with 1:1 ratio (equimolar glucose and amino acids) gave only zoosporangia because the amino acid and glucose supplies were exhausted simultaneously. An excess of glucose was required for the development of resistant sporangia and reduced aeration helped to ensure that these were produced first. Following the present investigation this medium was modified to give the 3AAG medium. The modifications included (1) omitting proline which does not delay zoosporangium development (Youatt 1973); (2) reducing the concentration of methionine to a suboptimal level so that (3) glucose was in excess even with a 1:1 ratio of glucose to the combination of histidine (which is used rapidly) and aspartic acid (which is used slowly). When growth ceased, glucose and aspartic acid were still present and favourable to the production of terminal resistant sporangia with 40/100 conditions. This medium could be converted to produce zoosporangia by increasing the methionine content to 1 mM and using 40/500 conditions.

In media containing ammonium salts and glucose, glucose is the precursor of amino acids as well as the source of energy which complicates controls in this medium. When the ammonium citrate medium was varied in composition with respect to glucose, formation of resistant sporangia was rare with 9 mM NH_4^+ and 3 mM glucose and absent with 1·5 mM glucose. These results are similar to those of Sandstedt and Aronson (1978) at 22°C. From analysis of the residual ammonium ion present it was calculated that approximately 2 moles of glucose were metabolized for each mole of ammonium ion. Experimentally it was found that 22·5–27 mM glucose provided an excess for the metabolism of 9 mM ammonium citrate in this medium. Amino acids were barely detectable in the media and differentiation began before the nutrients were exhausted. Clearly in this medium the trigger to differentiation was not an exhaustion of the nitrogen supply. However, microscopic inspection of plants growing in NH_4G media with varying glucose concentration showed normal hyphal development and branching with 9 mM ammonium citrate and glucose concentrations below 9 mM. At 9 mM glucose the development pattern changed and in 10/100 conditions a zoosporangium was produced followed by sporangial branching. With 27 mM glucose successive sporangial branches produced resistant sporangia. Sporangial branching had been observed with suspensions of plants in glucose–glutamate solution (Youatt 1980*a*) so that one condition leading to sporangial branching was an incomplete medium. An alternative explanation of inhibition of normal hyphal branching follows from observations of growth on 4AAG medium to which 16 mM tyrosine was added. Plants in this medium showed no hyphal branching. Instead, a succession of sporangial branches produced 8 or more sporangia, mainly resistant, over several days in the presence of adequate concentrations of histidine and glucose. For the NH_4G medium an inhibition is the preferred explanation since optimal levels of other nutrients had been provided. The question arises whether this pattern of development could be switched to 100% production

of resistant sporangia in the terminal position. When aeration was reduced and in 40/100 conditions a medium with 9 mM ammonium citrate and 27 mM glucose produced resistant sporangia in the terminal position. Finally, 4AAG, 3AAG and NH_4G media may all be prepared from 40-fold concentrates, lacking only the phosphate buffer which is added after sterilization. These concentrates have been stored over chloroform at room temperature for several months without adverse effects.

Discussion

Results described in this paper differ in some respects from earlier reports. The chief reason for the differences lies in the earlier practice of maintaining cultures on solid media of rich and undefined composition. These cultures were transferred to liquid media which were more exacting and adaptation to these conditions did not take place.

Carry-over of nutrients with the spores would account for the fact that *A. arbuscula* was able to grow initially on transfer to Machlis medium but could not be maintained in serial transfer. Ingraham and Emerson (1954) made only one transfer from rich to demanding medium. In contrast, the first transfer may be less successful than subsequent cultures in the same medium when the organisms have adapted to produce more biosynthetic enzymes. Cultures described by Machlis and Crasemann (1956) with excessive rhizoid development and yellow mycelium have been seen in the first transfer of cultures which subsequently grew in normal fashion. This was the behaviour of the auxotrophic mutants supplied by Dr Olson.

Quantz (1943) distinguished between a concentration of thiamine which permitted vegetative growth and one which allowed the development of gametangia. Now it appears that the two species of *Allomyces* used in this study are able to make the thiamine they require in favourable conditions.

Acid production in cultures was a problem in Machlis' medium (Machlis 1953*b*; Ingraham and Emerson 1954). Unbuffered suspensions of *A. macrogynus* metabolized glucose without acid production provided they were sufficiently well aerated (Youatt 1980*a*). However, the present study showed that acid could be produced when the concentration of glucose was too high. Machlis' conditions combined 30 mM glucose with reduced aeration and this would account for the problems of pH control.

In Machlis' medium the appropriate molar concentrations for the precipitation of magnesium ammonium phosphate were reached before the optimum concentration of magnesium. The use of ammonium citrate avoids the problem. Although the species of *Allomyces* will use ammonium salts as their sole source of nitrogen, growth is improved by small additions of amino acids. Those used have been glutamic acid (Machlis and Crasemann 1956; Sandstedt and Aronson 1978). Quantz (1943) had good results with asparagine and histidine. Nielson and Olson (personal communication) used asparagine, whereas in this laboratory histidine has always been the amino acid which was most readily used.

Despite differences in the media, the optimum concentrations of methionine determined by Machlis (600 μM DL-methionine) is in good agreement with 250 μM L-methionine. Experiments have indicated that methionine has a role in hyphal branching and that excess tyrosine inhibited normal branching. These observations may be of future interest in the study of the process of branching.

Possible Mechanisms for the Control of Differentiation

The results described in this paper confirm the previous observations that the development of resistant sporangia requires the availability of glucose. There are several reasons for directing attention to the metabolic pathways which diverge from pyruvic acid. Controls of metabolism in this area have been discussed by Larner (1971). Many enzyme reactions involved in the conversion of pyruvic acid require thiamine diphosphate. Exceptions are amination or transamination of pyruvic acid to alanine; hence the suggestion made earlier that the appearance of alanine in thiamine-deficient cultures, and not in cultures with thiamine at concentrations of 2–4 μg/ml, was due to an accumulation of pyruvic acid when other pathways of conversion were not available.

Various competing pathways exist for pyruvic acid. One involves gluconeogenesis and glycogen formation. Another leads into the citric acid cycle. The enzyme which converts pyruvic acid to oxaloacetic acid is of interest because (1) it requires acetyl CoA; and (2) it is a known regulatory enzyme in other systems. In addition thiamine is involved in production of acetyl CoA. The interest in CoA arises from the fact that methionine is the usable source of sulfur from which *A. macrogynus* must synthesize its coenzyme A and hence plants with limiting methionine may be limited in this coenzyme. Further, *A. macrogynus* accumulates acidic intermediates with amino acids that require this coenzyme for their catabolism—leucine, isoleucine, valine, methionine, threonine (Youatt, unpublished observations).

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