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Oxygen and Morphological Changes in *Allomyces macrogynus*

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Abstract

The supply of oxygen to cultures of *A. macrogynus* influenced the time of hyphal emergence, the angle of the hyphal tube to the rhizoid and the hyphal diameter. Reduced aeration favoured the release of both zoosporangia and resistant sporangia in cultures. Spores exhibited chemotaxis and hyphae exhibited chemotropism to oxygen.

Introduction

It was known that the oxygen requirement for vegetative growth of *Allomyces* species was less than the requirement for the production of gametangia (Kobr and Turian 1967) and of zoosporangia (Youatt *et al.* 1971). The production of resistant sporangia was favoured relative to zoosporangia at reduced levels of aeration (Youatt 1982) and was associated with the formation of *O*-ethylhomoserine, indicating that there had been fermentative production of ethanol (Youatt 1983). Increased hyphal extension at lowered oxygen tension had also been recorded (Youatt 1982).

In a recent study (Youatt 1985) hyphal emergence was delayed until after the third nuclear division. One possible explanation was the inhibition of emergence by oxygen. The relationship to nuclear division could then be explained by the period of accelerated growth which followed each nuclear division. Another recent observation which suggested oxygen inhibition of hyphal emergence was that well-aerated cultures in media containing thioacetamide exhibited prolonged inhibition of emergence. These cultures contained methionine sulfone in the amino acid pools, indicating highly oxidative conditions. A change to unshaken culture conditions permitted hyphal emergence after 30 min (Youatt 1986).

Times of hyphal emergence of *Allomyces* species have not often been given. Bruce and Mascarenhas (1977) suspended zygotes in glucose buffer solution before inoculating a rich medium with a high spore density and growing the culture in a petri dish. The hyphae emerged in 60–85 min and drawings indicate a single nucleus at this stage. Olson and Fuller (1971) used shaken cultures with zoospores of *A. neomoniliformis* in a rich medium. Hyphae had not emerged at the binucleate stage.

The effect of oxygen on all stages of development has been investigated here for the diploid growth cycle of *A. macrogynus*.

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Methods

A. macrogynus Burma 3·35 stock cultures were maintained in a liquid medium with 40 ml volume in a 250-ml conical flask shaken at 90 strokes/min, 50 mm amplitude at 30°C. The medium contained 2 mM $MgSO_4.7H_2O$, 0·2 mM $CaSO_4$, 1 mM each of L-methionine (excess), L-aspartic acid, L-histidine and L-proline, 4 mM glucose, 10 mM phosphate buffer pH 7 (added after sterilization), and 2 mg/l thiamine.HCl.

When large numbers of separate organisms were required conditions were modified and these cultures will be referred to as bulk cultures. Inorganic salts and thiamine were maintained at the same concentration, glucose was increased to 12 mM, aspartic acid, histidine and proline were increased to 3 mM each and methionine was reduced to 300 μM. A volume of 400 ml of this culture was shaken in 2-litre flasks on the reciprocal shaker. The inoculum was all the spores which were released from 60 ml of 2–3-day stock cultures by replacing medium with sterile tap water. To assist germination, spores were sometimes pre-incubated with 0·2% (w/v) casein hydrolysate for 20–30 min before the bulk medium was inoculated. Further detail concerning culture methods has been described by Youatt (1982).

For cultures incubated at measured air pressures Thunberg tubes were used. The pressure was reduced using a vacuum pump and the tubes were closed at the required pressures. Beckman scintillation vials containing 10 ml of medium inoculated and incubated without shaking permitted hyphal emergence in the minimum time. Unshaken cultures were maintained at the normal temperature of 30°C. Other procedures used to reduce the oxygen available to the cultures are described as required in the Results.

Chemicals were obtained from the Sigma Chemical Co., U.S.A. Dissolved oxygen was measured with a Yellow Springs YSI model 57 meter.

Results

Germination

Zoospores of *A. macrogynus* were able to germinate at 13·5 kPa pressure (equivalent to 1 p.p.m. O_2 in the medium described above) and in the presence of ascorbic acid. When the oxygen supply was not then sufficient for further growth the organisms survived to grow later when oxygen was admitted. Germination required less oxygen, therefore, than hyphal growth.

Hyphal Emergence

The favourable effect of reduced oxygen concentration on hyphal emergence was simply demonstrated. A mixture of spores and 0·4% (w/v) casein hydrolysate was spread over a microscope slide which was incubated in a horizontal position for 30 min and then drained and rinsed. This gave an even spread of germinated spores attached to the glass. Slides prepared in this way were then incubated in a vertical position in liquid culture in a pomade jar and withdrawn for inspection. Hyphae emerged in about 4 h at a depth of 25–30 mm and later at both greater and lesser depth. In order to obtain a quantitative estimate of oxygen requirements, lightly inoculated cultures in defined medium were shaken in Thunberg tubes at pressures ranging from 13 to 100 kPa at 30°C for 4–5 h and the earliest hyphal emergence was found to occur at 26 kPa pressure (i.e. 2·1 p.p.m. O_2).

Angle of Emergence

All descriptions found in the literature describe the emergence of the hyphal tube at 180° to the rhizoids but when cultures were incubated in low concentrations of oxygen without shaking other angles of emergence were observed. Tracings from enlarged photographs are shown in Fig. 1. Emergence at an angle of 90° was more commonly seen than emergence beside the rhizoid and a less commonly seen variation had two germ tubes. These forms of emergence were seen in deep, unshaken liquid

cultures or on solid media where oxygen access was restricted by covering germinated spores with sections of microscope slides. The proximity of other spores was not a requirement for these unusual forms of emergence and they were seen when several diameters separated the germling from its nearest neighbour. It is probable that there is an element of chemotropism involved here. The unusual forms were not seen when the cultures were shaken under reduced pressure, nor were they seen in conditions where the hyphae could not exhibit chemotropism to oxygen.

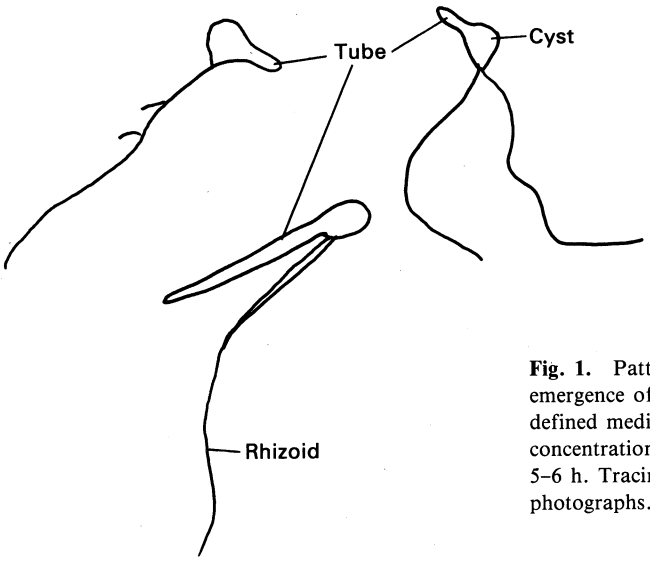

Fig. 1. Patterns of hyphal emergence of *A. macrogynus* in defined medium at an oxygen concentration of 2·1 p.p.m. at 5–6 h. Tracings from enlarged photographs.

Chemotropism to Oxygen

The procedure selected involved using ascorbic acid to generate the oxygen gradient. Filter papers dipped in ascorbic acid and then dried were laid in a petri dish in a T arrangement before the medium was poured. The overlying medium was sufficiently well buffered to maintain pH 7. A light spore inoculum was spread and checked microscopically. The spores were allowed to germinate (30 min) and a section overlapping the filter papers was then covered by a segment of microscope slide. Hyphal emergence was inhibited immediately adjacent to the ascorbic acid strips but eventually occurred more than 24 h later. Hyphae emerged early (4–4½ h) in other regions. Within 1 mm of the edge of the slide hyphae had turned towards that edge in 9 h. At this time organisms 2 mm from the edge were still growing in random directions and clumps showed the normal radial pattern. At 20 h hyphae within 2 mm of the edge of the slide had also turned towards that edge. This effect could not be attributed to a negative reaction to ascorbic acid since at the 2-mm distance the growth had been good and the concentration of ascorbic acid was higher than at the edge of the slide. The unusual forms of hyphal emergence of Fig. 1 were only seen in the region 1 mm from the edge of the slide.

Hyphal Growth with Restricted Oxygen

The pattern of growth observed with restricted oxygen varied according to the experimental conditions which influenced the supply of oxygen—the depth

of the medium and the density of the population. In severely limiting conditions (2 p.p.m. O_2) the hyphal tube which emerged and grew out was extremely narrow, estimated to be about 5 μm. In the defined medium within about 2 h from emergence the length observed was 200 μm approximately and the hyphal contents were concentrated in the upper 50–60 μm. The organisms in a scintillation vial continued to grow up to 2 mm in length before encountering adequate oxygen supplies. The response was then a widening of the hyphal tip to normal diameter (10–20 μm) and the growth of new rhizoids in the same region leaving the rest of the organism looking empty.

A culture was grown with poor aeration (in a scintillation vial) until the dimensions were approximately 5 by 200 μm and then transferred to a shaker (10 ml in 100 ml conical flask) to ensure ample aeration. Hyphal elongation ceased and within 30 min the hyphal tip had widened. Over a period of 4–5 h the widening proceeded back to the junction with the rhizoids and the rhizoids also became wider. During this process the hyphal contents which had been concentrated at the tip were distributed backwards towards the base.

An alternation of 30-min periods of shaken and still incubation of deep liquid cultures (scintillation vial) with narrow hyphae produced variations in diameter and a corrugated outline which was subsequently evened out by a period of shaking. The hyphal diameter therefore has a direct dependence on the supply of oxygen in a given medium.

Oxygen as an Inhibitor of Hyphal Growth

In the example above, the oxygen-deficient organisms transferred to the shaker showed no hyphal elongation until the hyphal diameter had been increased and there was, therefore, no growth at the apex. In the earlier study (Youatt 1985), hyphal emergence was delayed by shaking until after the third nuclear division.

In such experiments, with ungerminated spores for inoculum, the time of emergence was 9–10 h. The earliest time of emergence in the present study with low oxygen and enriched medium was 100 min and with defined medium $3\frac{1}{2}$–4 h. The longest delay, with thioacetamide in bulk cultures, was more than 24 h. By varying the depth of medium and the density of spores a range of times has been observed between these extremes, confirming that the supply of oxygen controls hyphal emergence.

A highly aerated bulk culture was sampled at intervals and the 10-ml samples were transferred to unshaken conditions in a vial at 30°C. The times of hyphal emergence and of nuclear divisions were noted. A sample removed at 30 min produced the hyphal tube at 4–$4\frac{1}{2}$ h after the first nuclear division. A sample removed at 100 min also produced the hyphal tube at 4–$4\frac{1}{2}$ h. Samples removed at 3 and 4 h produced their hyphal tubes at 5–6 h at the second nuclear division. Plants removed at 5 and 6 h produced the hyphal tubes at 6 and 6 h respectively. The times when marked spurts of growth occurred were at 4 and 6 h. The main shaken culture produced the hyphal tube at 9 h after the third division. While oxygen clearly seems to inhibit emergence, the relation to DNA synthesis and nuclear division cannot be clearly differentiated from the oxygen effect because of the growth spurts which follow divisions.

Spore Release

Throughout the literature the normal procedure for initiating spore release in *Allomyces* species has been to transfer the sporangia to dilute salt solutions or water. All that is actually required is to transfer shaken cultures to unshaken conditions at the same temperature. Liquid cultures in defined medium are regularly maintained in this laboratory and shaking prevents the release of zoosporangia for 12–14 days. Viable spores are released on standing. It seemed that this system offered a way of estimating whether the time taken to respond to a change in oxygen concentration was less than the 30 min at which morphological change was observable. Samples of culture with mature zoosporangia (40 ml in 250-ml conical flasks) were transferred to stationary conditions at 30°C and then returned to the shaker at 4, 9, 14, 19 and 24 min. Cultures returned at 4 and 9 min did not release spores when shaken again (later, after 40 min on the bench, they were still able to release normal spores). Some spores were released on the shaker after being stationary for 14 min and all spores were released after 19 min. This shortened the estimate of the time required to effect chemical change within the organism to about 15 min. At this time the dissolved oxygen had fallen to 5–6 p.p.m. O_2 (range given because of the requirement to stir).

Resistant sporangia were produced in 7-day cultures (Youatt 1982). Samples were transferred to water in shallow or deep layers and the deep suspension was lightly gassed with nitrogen for 30 s. Spores were released in less than $1\frac{1}{2}$ h under low oxygen tension (as above, estimated to be about 2 p.p.m.). In the shallow layer spores had not been released in 6 h but had been released overnight. Cultures transferred to unshaken conditions also released meiospores from resistant sporangia. Reduced oxygen concentration therefore favoured spore release from both types of sporangium of the diploid cycle of growth.

Chemotaxis of Spores to Oxygen

When the concentration of oxygen was too low spores were immobilized and eventually disintegrated. In order to observe chemotaxis spore motility had to be maintained. Spores are known to exhibit chemotaxis to nutrients so these were either avoided or kept uniform throughout. A light suspension of spores was used to avoid interactions between organisms or their metabolic products. Two kinds of experimental conditions of many tested are described.

In the first method small clumps were taken from liquid cultures 6–10 days old. At this age after the clumps had been washed, carry-over of nutrients was minimal. The clumps were placed individually on microscope slides. A 15-mm coverslip was placed over the clump and all but 2–3 mm of the perimeter was sealed with a mixture of melted wax and lanoline. Water was introduced through the gap and the slide was tapped to remove any trapped air bubbles. Spore release took about 40 min at 16–20°C (room temperature). The clump of plants served to define the origin of the spores as well as to assist in establishing an oxygen gradient. It required about 30–60 min for viable spores to accumulate near the air–water interface, the time depending on the distance between the clump and the opening. After 1–2 h an examination of the perimeter of the coverslip established that spores had only congregated near the source of oxygen. Room temperature was used because spores retained their motility better than at 30°C. For a control, killed stained spores were included in such experiments. They remained evenly distributed under the slides.

In the second method, nutrient agar was autoclaved and plates were poured immediately before use to minimize the dissolved oxygen. Plates were inoculated lightly with spores, spread evenly on the surface. Coverslips were placed firmly on the medium and most of the perimeter was sealed with melted agar leaving 3–4 mm open to the air. The areas under the coverslips were examined and found to have actively motile spores throughout the area. After 2–3 h spores could only be found at the edges of the coverslips and further inspection after 8 h found no germinated organisms under the coverslips in any other region. It was clear from the accumulation of spores near the edges of the coverslips that some spores were able to pass the edge and germinate immediately outside. The oxygen gradient with the agar seal and the 3–4 mm space was not severe since there were no organisms at 8 h with the long thin hyphae typical of low oxygen concentrations and hyphal emergence was only beginning. Killing stained spores remained evenly distributed.

Discussion

From the evidence given above *A. macrogynus* might well be described as microaerophilic. The oxygen content (100 kPa) of media in equilibrium with atmospheric oxygen has been unfavourable to hyphal emergence, hyphal elongation in some conditions, the development of resistant sporangia and the release of spores from both zoosporangia and resistant sporangia. Oxygen, therefore, has an important role in the control of development. It is also a major factor in determining the diameter of the hyphal tube.

In the general literature on the fungi the role of oxygen is not often emphasized but two papers of direct interest are those of Bourret (1985) and Robinson (1973). Bourret described the inhibition of tip growth by excess oxygen in *Pilobolus crystallinus* associated with continuing development in other parts of the fungal mass. In *A. macrogynus* organisms with narrow hyphae did not undergo tip growth when the culture was shaken until the hyphal diameter had been increased. In highly aerated media *A. macrogynus* exhibits alternation of tip and base development which is described in the following paper (Cleary *et al.* 1986).

Robinson's (1973) paper was of interest because it described response of fungi to oxygen. This response seems to have been, to a large extent, denied or ignored by other workers. The angle of emergence from adjacent pairs of arthrospores of *Geotrichum candidum* was such as to give maximum separation of the hyphae. Robinson suggested a chemotropism for oxygen and was able to demonstrate such an effect. The unusual angles of hyphal emergence in *A. macrogynus* (Fig. 1) were not due to adjacent spores, being observed best in well-separated organisms and only at low oxygen concentration. There are two possible explanations: one is that chemotropism to oxygen is involved but is readily masked by other factors and the other that the positioning of the nuclei is abnormal in oxygen deficiency.

By varying the density of cultures and the means of aeration it was possible to demonstrate hyphal emergence in *A. macrogynus* shortly after each of the first three nuclear divisions but the shortest time observed was still after one DNA replication. It is not possible to say why there is an apparent link between hyphal emergence and DNA replication but the time of emergence also coincides with a spurt in growth and it seems most likely that there is an increased consumption of oxygen which is the real controlling factor. Bruce and Mascarenhas (1977) showed

hyphal emergence without nuclear division but without specific comment on this observation. Zygotes often seem to grow more vigorously than zoospores and in addition the prior incubation in the presence of glucose may have provided more substrate for reduction.

The conditions required to demonstrate chemotaxis and chemotropism differed to the extent that the phenomena were not readily demonstrated together. This was because chemotropism required an oxygen gradient in a range so low that spores were rendered immobile and eventually disintegrated. It is possible to see curving of hyphae of normal diameter but much easier to observe it in the long thin hyphae of oxygen-deficient organisms. For both phenomena light suspensions were required to avoid other effects and make observation simpler. Chemotaxis to oxygen may be involved in the floating seed method for primary isolation of *Allomyces* species. The survival value of chemotropism was well illustrated by growth out of the region of oxygen deficiency.

The rapid release of spores from resistant sporangia under reduced oxygen tension may make it easier in future to understand the maturation process. In other studies release has taken times of $4\frac{1}{2}$–6 h and attempts have been made to speed maturation. Machlis and Crasemann (1956) improved release by prior drying of the sporangia which they thought might have rendered the wall more permeable. Now it might be suggested that a labile enzyme or intermediate was lost, changing the balance of oxidation and reduction in the sporangium. A recent observation that resistant sporangia with acid-resistant walls take up dyes like neutral red and methylene blue suggests that they are quite permeable and has replaced the need for the use of concentrated H_2SO_4 as a test when there is uncertainty about the type of sporangium present. Zoosporangia and healthy hyphae do not take up these dyes (Youatt, unpublished data).

Kobr and Turian (1967) studied some biochemical changes in *A. arbuscula* after 3 h of anoxia. The results described in the present paper show that further studies of biochemical change should involve shorter time intervals. The time for commitment to spore release suggests that the relevant time scale is as short as 15 min. In the various ways that oxygen influences the development of *A. macrogynus* visible morphological change follows as early as 30 min from a change in the oxygen concentration.

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