Hyphal Emergence and Outgrowth of
*Allomyces macrogynus* in Aerated Cultures

*Ann Cleary,* A,C  *Jean Youatt* A,D  and  *T. P. O’Brien*B

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

**B** Department of Botany, Monash University, Clayton, Vic. 3168.

**C** Work presented in partial fulfilment of the requirements for B.Sc. (Hons), Monash University.

**D** To whom correspondence should be addressed.

**Abstract**

Hyphal emergence was delayed in aerated cultures of *A. macrogynus*. Continuous observation of individual organisms revealed alternation of growth at the tip and at the base, including an incorporation of part of the primary rhizoid into the hyphal region. Electron microscopy of sections confirmed the deposition of additional wall during the stages of development of the base. These sections also showed changes in wall structure during growth, in particular a readily removable component of early walls. Nuclei were positioned just below the emerging tips at hyphal emergence and at branching. The apical zones of exclusion contained few, if any, vesicles and multivesicular bodies. In other regions the mitochondria were observed in close proximity to the plasmalemma.

**Introduction**

In highly aerated cultures of *Allomyces macrogynus* in a chemically defined medium hyphal emergence did not occur until 8–9 h, after the third replication of DNA. The process of hyphal development was associated with changes in the colour and water repellance of the walls (Youatt 1985). In a separate study the time of hyphal emergence has been linked to the supply of oxygen (Youatt 1986b).

The present study was carried out because intermittent observation of *A. macrogynus* in highly aerated conditions had suggested that the formation of the hyphae involved constriction at the top of the pre-existing cyst. By time-lapse photography of developing germlings it has been possible to record a complex process of growth. In addition it had been suggested that there might be a relationship between nuclear divisions and developmental stages including hyphal emergence and branching (Youatt 1985). In the present study use was made of Nomarski and phase-contrast optics to observe the nuclei of germlings in aerated cultures and of electron micrographs showing wall sections to cast further light on the changes in wall structure.

Gooday (1971) commenced a paper with ‘It is generally accepted that a fungal hypha grows by deposition of material at its tip’ and in a symposium report on fungal walls and hyphal growth such a view was widely accepted by the contributors (Burnett and Trinci 1979). A general statement from these sources would be that walls are deposited primarily at the apex of the growing hypha, that the apical region is characterized by an accumulation of cytoplasmic vesicles associated with the synthesis of wall components, that enzymes of lysis and synthesis may remain embedded in an inactive form within wall structures, that such enzymes are involved in branching
of the hyphae, and that some forms of osmotic shock or inhibitors can induce wall synthesis in areas other than the apex.

What we have found in young *A. macrognus* germlings is that wall development occurs at the apex and at the base, both in the prehyphal cyst and after hyphal emergence. There was a notable absence of vesicles in the apical zone of exclusion which contrasts with the presence of vesicles in older cultures of *A. arbuscula* (Roos and Turian 1977), though a recent paper by Turian *et al.* (1985) shows relatively few vesicles in the apical zone of *N. crassa*.

**Methods**

*Culture Methods*

Culture methods have been described previously (Youatt 1982, 1986a). Bulk cultures with thioacetamide contained 2 mm thioacetamide and 1 mm alanine in place of methionine.

*Analysis*

Glycogen was analysed as in Youatt (1980). Chemicals were obtained from Sigma Chemical Co., U.S.A.

*Well Slides*

Valwax was prepared by mixing equal weights of paraffin wax, lanoline and Vaseline. Two 18-mm coverslips were placed about 14 mm apart and fixed to a microscope slide with Valwax. The volume of the well was determined by the amount of wax used and approximated 0.22 ml. A drop of suitably diluted culture of approximate volume 0.07 ml was covered by a third coverslip and the edges were sealed all around with Valwax. The dilution of the culture was adjusted to give 10-15 germlings in the drop.

*Light Microscopy*

A Zeiss photomicroscope was set up in a 30°C room. Bright-field, Nomarski-interference and phase-contrast optics were used and photographs of the developing germlings in the well slides were taken with Kodak Panatomic-X film (ASA 32). Time-lapse photographic records were made on Kodak-plus X reversal film using a Paillard-Wild variometer control unit MBF-B and timer MBF-C.

*Electron Microscopy*

Samples of cultures in the medium were fixed in 2% (v/v) glutaraldehyde for 1 min and post-fixed in 1% (w/v) OsO₄ (added to the glutaraldehyde) for a time not exceeding 15 min. The fixed organisms were washed thoroughly with culture medium and embedded in 0.7% (w/v) agar. Embedded samples were dehydrated by ethanol in a series of concentrations from 5-100% and several times in 100% ethanol. Samples were then embedded in Spurr's resin and sections were cut following O'Brien and McCully (1981). Sections were stained with 0.3% (w/v) toluidine blue in 0.1% (w/v) borax, pH 9, for light microscopy. For electron microscopy sections were mounted on Formvar-coated slot grids and stained with saturated uranyl acetate solution in 50% (v/v) ethanol for 15 min and post-stained with lead citrate for 5 min. The sections were observed with the Jeol JEM-200CX transmission electron microscope at 80 kv.

*Results*

*Sequence of Stages of Development*

Well slides did not provide sufficient oxygen to follow the complete stages of development from spore to fully hyphal fungus without departure from the pattern observed in a shaken culture. The major difference was hyphal emergence after the second rather than the third nuclear division. The early stages of development were filmed from the time of inoculation with pregerminated spores and the later stages by transferring organisms from a shaken culture to the well slide. On these occasions the bulk shaken culture was monitored to ensure that there was parallel development
Hyphal Emergence in *Allomyces macrogynus*

Fig. 1. Sequence of the 17 main stages of development of *A. macrogynus* grown from spores in defined medium under highly aerated conditions (see Methods). X indicates that a nuclear division occurred between the two stages and times of nuclear division are given in the text. Hyphal emergence begins at stage 10. Bar approximately 20 μm.
in the two systems. Drawings in Fig. 1 show the 17 major recognizable stages of development from the combined sequences.

Stages 1–4 (Fig. 1) show an initial elongation and widening at the apex followed by a widening of the base of the cyst. Fig. 2a shows tracings from a time-lapse film in which the base expansion (stages 2–4) is clearly shown. Stage 3 is like the binucleate germling of *A. neomontiformis* of Olson and Fuller (1971) which was grown in shaken culture. Stages 5–7 show a second alternation of tip widening followed by base widening so that in stages 3 and 6 there is a brief achievement of parallel walls. In stages 6 and 7 the development of the base also involves widening of the rhizoid back to the first branch. Tracings of time-lapse photographs show this is Fig. 2b.

---

**Fig. 2.** Tracings from time-lapse photographs. 
*a*, First period of backward development of a germling grown from the pregerminated spore in a well slide. Times for the three tracings are 30 min, 1 h 10 min and 2 h 20 min and they correspond with stages 2, 3, and 4 of Fig. 1. *b*, Second period of backward development showing expansion of the rhizoid. Times are 7 h 30 min, 7 h 55 min, 8 h 30 min, and 8 h 55 min from inoculation with a pregerminated spore and using a well slide. *c*, Germling from a well-aerated culture transferred to a well slide just before emergence of the hyphal germ tube. Times are 10 h, 10 h 15 min, and 10 h 30 min. Bars = 2 μm.

---

**Fig. 3.** *a*, *A. macrogynus* germling with hyphal germ tube emerging. Nucleus (arrow) visible just moving into the germ tube. *b*, Hyphal germling showing even distribution of nuclei (arrow) down finely granulated hyphal germ tube. Vacuoles (V) restricted to cyst region. *c*, Mature hypha showing two nuclei (one indicated by arrow) below the flattened hyphal tip immediately prior to branching. *d*, Same hypha as shown in e later, with one nucleus positioned below each branch apex. *e*, Failed branch (bold arrow) on mature hypha of *A. macrogynus* with pseudo-septum (S) formed in the branch which continued to grow. [Note: septa only form at branch points and below reproductive structures in *Allomyces* species.] *a* and *b*, Normarski optics; *c*-e, phase-contrast optics. Bars = 10 μm, that in *a* also applicable to *b*, *c*, and *d.*
Hyphal Emergence in *Allomyces macrogynus*
In stages 8–10 there is elongation and widening of the tip. Stage 10, described as club-shaped, is the typical last stage before hyphal emergence and the stage found in bulk cultures in which growth is inhibited by thioacetamide. In normal cultures this stage is maintained for variable times depending on the culture density while in the inhibited thioacetamide culture it is maintained for 24 h or more.

When conditions become favourable for hyphal emergence, stage 11 is limited to the formation of an apical zone of exclusion, best seen in electron micrographs (see Fig. 4). Stages 11–16 proceed rapidly, being visible even with low-power magnification within 30 min. Fig. 2c from traced photographs makes it clear that development at this stage is all at the tip and does not involve constriction of the existing cyst. Figs 3a and 3b show the finer granulation of the hyphal tube and the vacuoles of the cyst region, described earlier (Youatt 1985).

Even after hyphal emergence there is still no total apical domination. As the germ tube elongates there is again a widening at both the junction of the cyst with the hyphal germ tube and at the junction of the cyst with the rhizoids which eventually gives largely straight-sided hyphal walls of mature fungi, though traces of the original cyst region may still be visible as in Fig. 1, stages 15 and 16. In these final stages the vacuoles of the cyst region are reduced or disappear and the granulation of the hyphal contents becomes finer down to the rhizoid junction. The final hyphal diameter is variable and controlled largely by the oxygen supply (Youatt 1986a), and hence is also affected by the density of the culture.

**Nuclear Divisions**

It was not possible to see all the nuclei in every organism of a sample because, as the electron micrographs show, the cytoplasm was densely granular. The estimated times of nuclear division were the times at which two, more than two, more than four or more than eight nuclei could be seen. The stages at which nuclear divisions occurred are indicated in Fig. 1 and the times were approximately 3, 5–6 and 9 h for the first three divisions. The fourth division, which was not observed as often, was about 12–13 h.

At the time of hyphal emergence a nucleus was positioned immediately below the pointed apex of the cyst (Fig. 3a) and moved into the extending germ tube. A similar positioning and movement of nuclei was often observable at the hyphal tip during branching (Figs 3c and 3d). The absence of two nuclei at this stage was followed by failure of one branch to extend (Fig. 3e). As the emerging hyphal tube elongated, further nuclear divisions occurred and the spacing of the nuclei within the hyphae was remarkably regular (Fig. 3b). In electron micrographs microtubules were found in association with the nuclei and may have been responsible for their movement.

**Mitochondria**

Mitochondria were abundant throughout the cytoplasm and adjacent to the cell membrane (see Fig. 6). In sections an apical exclusion zone devoid of mitochondria, vacuoles and nuclei was detectable before visible hyphal emergence (Fig. 1, stage 11; Fig. 4).
Other Features
Where fungal walls are believed to develop primarily from the apex, vesicles and multivesicular bodies have been a feature of the apical exclusion zone. These were notably absent from well-aerated cultures of *A. macrogynus* (Fig. 4).

Fig. 4. Transmission electron micrograph of apex of hyphal germling showing distinct apical zone. Apical zone of exclusion bounded by mitochondria (*M*). Small aggregates of glycogen (*G*) and some endoplasmic reticulum (*ER*) present. Note absence of vesicles. Bar = 0.5 µm.

Endoplasmic reticulum and ribosomes may be seen and lighter aggregates, believed to be glycogen granules. Glycogen had not previously been assayed in such young
plants but was assayed and found to be present in two fractions, as in older cultures, as acid-soluble and acid-insoluble but alkali-soluble glycogen (Youatt 1980). Concentric granules were rare in the earliest stages but increased in numbers and size in the hyphal fungi. Some are seen in Fig. 7 at low magnification and were confirmed by viewing at higher magnification.

Walls

Electron micrographs showing walls at different stages of fungal development were prepared to see whether they could explain the differences in physical properties recorded previously. In Figs 5 and 6 are recorded longitudinal sections of the cyst region of prehyphal germlings and in Fig. 7 that of a hyphal germling. Enlargements of wall sections at various distances from the apex are shown for each. There are two wall layers, the inner chitin layer and the outer, more electron-dense, non-fibrillar matrix. The appearance of these walls was found to vary down the length of the germlings and also between germlings at different developmental stages.

The germling of Fig. 5 corresponds with stage 5 of Fig. 1. There is a large amount of outer electron-dense material relative to the inner chitin layer and this wall material is firmly attached in the upper regions a, b of Figs 5A, 5B. In the lower section c of Figs 5A, 5B the outer wall material is less electron-dense and has been lost in many areas. Fig. 6A shows a prehyphal organism just before hyphal emergence (cf. Fig. 1, stage 11). The wall contains more chitin all around the cyst region and the outer matrix wall layer at the apex has far less electron-dense material than in the binucleate germling of Fig. 5A. This change, which occurred only at the apex, could indicate wall softening prior to the emergence of the germ tube. Note in the wall region below (Figs 6A, b; 6B, b) that the wall is thicker and more of the outer layer is missing (detail not shown). Fig. 7 (cf. Fig. 1, stage 14) shows a hyphal organism with wall sections. At the apex there was only a thin, chitin layer which thickened and decreased in electron density from the inner surface to the outer surface below the apical dome (Figs 7A, b; 7B, b). At about 10-5 μm below the top a thin, more electron-dense layer was visible over the chitin (Figs 7A, c; 7B, c). This layer increased in thickness and electron density down the hyphal germ tube. In the region shown in Figs 7A, d; 7B, d, a thin but firmly adherent electron-dense layer covered the chitin. This is the only remaining region of the original cyst wall as the original outer layer has been lost in many places in the lower part of the cyst. This lower region (Figs 7A, e; 7B, e) has a greatly thickened chitin layer. These observations suggest that the outer layer of the hyphal germ tube is deposited over the chitin layer below the apical region.

Discussion

Four aspects of the previous paper (Youatt 1985) require comment in the light of this new study. They are (1) that nuclear positioning might be important in branching; (2) that there appeared to be a relationship between DNA replication

Fig. 5. A, Longitudinal section of binucleate (Nu) prehyphal germling (stage 5, Fig. 1) showing central distribution of vacuoles (V) and mitochondria (M) evenly distributed throughout densely granular cytoplasm. Bar = 1.0 μm. B, Wall segments (a–c) from corresponding areas of A (see text). Bar = 0.5 μm.
and new structural change in the growing organisms; (3) that the development of the hyphal form in aerated cultures appeared to involve a constriction working back from the tip to the base; and (4) that young organisms have a wall component which is very easily removed.

The evidence of Fig. 3 supports the idea that nuclear positioning is important in hyphal branching and also in hyphal emergence. It is clear that there is no essential relationship to the third nuclear division. This aspect is discussed more fully in Youatt (1986b). It is suggested that a balance of oxidative and reductive metabolism is concerned in hyphal emergence. The link to DNA synthesis may be that hyphae cannot emerge while nuclear division is in progress and that the spurt in growth which follows division also increases the oxygen demand. It is clear that hyphal emergence does not involve a constriction of the tip; the backward change in the granularity of the hyphal contents is confirmed and an extraordinary alternation of development at the tip and at the base is discussed in greater detail below.

The electron micrographs show a wall layer which is readily lost from the original cyst region and it is reasonable to equate this with the brown galactan-protein layer removed by homogenization (Youatt 1985). As the organisms developed, a new layer was formed which was electron-dense and firmly adherent to the underlying chitin layer. It was suggested above that this layer was deposited over the chitin layer in the region just below the apical zone. From the chemical study we suggest that this layer contains glucan and protein and probably lipid. Fultz and Sussman (1966) by their immuno-fluorescent antibody technique for galactan in mature organisms detected galactan at the hyphal tip and through cracks in the outer wall. From this we conclude that the galactan that is synthesized at the later times forms part of the cementing substance in which the chitin fibres are embedded.

This study has demonstrated clearly that aerated cultures of *A. macrogynus* deposited wall at the tip and at the base of the organism during growth to the stage of the mature hyphal organism. The time-lapse photography and the wall sections establish wall synthesis at the base and even into the initial rhizoid region. In addition the organisms did not have the vesicles normally associated with wall synthesis in the fungi. The 'spitzenkorper' associated with multivesicular bodies is known to be absent from *Allomyces* species but Roos and Turian (1977) described the presence of vesicles in *A. arbuscula* hyphae. Their cultures had an age of at least 1½ days. In *A. macrogynus* the young cultures which lacked vesicles were growing vigorously with a measured growth of 10 μm per hour during hyphal emergence. We have to conclude that accumulations of vesicles are not involved in this process. Morrison (1977) also noted an absence of vesicles and abundance of ribosomes in 18–22 h cultures of *A. macrogynus*.

In an investigation of the effects of oxygen on early growth of *A. macrogynus* it became clear that high oxygen concentration favoured backward development, perhaps by inhibiting development at the apex and low oxygen content favoured apical development. Bourret (1985) has also recorded a possible example of oxygen inhibition of apical growth in *Pilobolus crystallinus*. In *A. macrogynus* grown in

**Fig. 6.** *A*, Longitudinal section of prehyphal cyst (stage 11, Fig. 1) showing mitochondria (*M*) distributed throughout the cytoplasm and adjacent to the plasmalemma except at the apex where a zone of exclusion is being formed. Note loss of outer wall layer in lower region of the cyst and the reduction in electron density and thickness at the apex. Bar = 2·0 μm. *B*, Wall segments (a, b) taken from corresponding areas a, b of *A*. Bar = 0·25 μm.
Fig. 7. A. Longitudinal section of hyphal germling (stage 14, Fig. 1). Large vacuoles (V) restricted to original cyst region and widened rhizoid. Nuclei (Nu) evenly spaced in rhizoid region and found below the hyphal tip. Concentric granules (CG) located throughout the germling. Bar = 5·0 μm.
well-aerated conditions we were not able to demonstrate a reducing zone at the apex by the use of methylene blue as described in *A. arbuscula* (Turian 1976).

In other fungi wall development at the apex is thought to require the establishment of a potential gradient along the hyphae (Jennings 1979). If such a gradient is involved in *A. macrogynus* then it must undergo several reversals of polarity during the alternation of tip and base development. There is evidence of excessively oxidative conditions in aerated cultures of *A. macrogynus* from the observation of methionine sulfone in cultures containing thioacetamide (Youatt 1986a). For the present we conclude, as did Bourret (1985), that above a critical concentration, oxygen is an inhibitor of growth at the hyphal tip. This is not incompatible with the idea of a potential gradient if under such conditions the gradient cannot be established. In such conditions the organism achieved sides which approximated to parallel on two occasions before hyphal emergence (Fig. 1, stages 3 and 6), when this state was finally achieved. We have no knowledge at present as to how the hyphae tend to the parallel sides but we do know that the diameter is highly dependent on the available oxygen (Youatt 1986b).

References


---

*B*, Wall segments (a–e) taken from corresponding areas of *A*. Observe reduction in electron density and increase in thickness of the inner chitin layer from a to e and gradual appearance of outer matrix from a to c. Original region of sharply defined outer wall is shown in d. Outer wall diffuse and often lost (e). Bar = 0·25 μm.

Manuscript received 3 February 1986, accepted 28 May 1986