

CHAPTER 6. Morphogenesis: Vegetative

Members of the Saprolegniaceae are constructed of coenocytic aggregations of tubular hyphae having septa only where reproductive structures are formed. Although the individual filaments may taper distally, they are not constricted or segmented.

In this and the chapters to follow, structural features of water molds are considered, beginning with the earliest known experimental and observational accounts. For the most part, such aspects of reproduction as the effect of nutrition on sex cell formation are treated in depth in other chapters. Similarly, cytological and ultrastructural observations are only touched upon in passing in these chapters on morphology.

Since morphology and growth are inextricably interwoven in these chapters, it is well at the outset to define two terms (as we use them). By growth is meant a gain in hyphal mass, measured, for instance, by an increase in dry weight. The structural and functional changes that accompany growth are expressions of development. Thus, the apical elongation of a hypha is growth; the appearance of a terminal sporangium is development.

As has been noted (Chapter 1), the 18th- and early 19th-century naturalists encountered water mold hyphae, but generally were not aware of their true nature. Arderon (1748) remarked that he could press out from the filaments on an infected roach a brownish liquid, and thus (without knowing so) showed that hyphae contain cytoplasm. A "contagium" of a salamander occupied Hannover's (1839) attention. He observed that each filament of "*Conferva*" -- as he thought the fungus to be -- had a conical apex, and together the mass of threads made up a slimy "efflorescenz." From Hannover, then, comes the concept of the hyphal apex in water molds. Obviously puzzled by hyphal mass seen on frogs, Stilling zu Cassel (1841) concluded that he had material resembling the "efflorescenz" of Hannover, and that the specimens were probably plant-like. His illustrations (Stilling zu Cassel, 1841: Pl. 11, figs. 1-3) convey an impression of branched, coenocytic hyphae, and he evidently was struck by the "Körnchen" (refractive oil bodies, very likely) in these filaments. Simple or branched, sometimes inflated, contorted, or sinuous hyphae were also observed by various biologists in calcareous parts of aquatic animals. Duncan (1876b, 1881) believed the slender borings in sponge spicules were filaments of *Achlya penetrans*. The tubes which Wedl (1859) found in recent and fossil (Devonian) shells were identified as hyphae of *Saprolegnia ferax*. Indeed, Bornet and Flahault (1889) even described two new genera of fungi from calcareous materials solely on the basis of hyphal characteristics.

THE HYPHAL WALL

According to A. Fischer (1892), the "membran" (wall) of all saprolegniaceous fungi consisted only of cellulose, and this same conclusion was to appear in later papers by Wisselingh (1898), F. Wettstein (1921), Hopkins (1929), Nabel (1939), and Frey (1950). Even by the application of sophisticated analytical methods (chromatography, infrared

and pyrolysis mass spectrophotometry (Weijman, 1976) investigators were to report a measurable cellulose fraction in the hyphal wall of water molds. Still, not all early investigators reported finding cellulose only in the wall of the saprolegniaceous fungi they examined. Mangin (1890, 1893), for instance, noted that plant cell walls were made up of two fundamental substances, as he called them, and one of these, which he named callose, was to him the basic constituent in the wall of species of water molds. This concept of the one-compound nature of the wall remained firmly established in mycology well into the mid-20th Century (see Aronson's 1965 review of cell wall chemistry; also Bartnicki-Garcia, 1968).

Beginning in 1962, several experimental studies were published showing that the wall chemistry of the Saprolegniaceae was incorrect. Chromatographic and electrophoretic analyses demonstrated beyond doubt that the filament wall in *Saprolegnia ferax* was a complex of neutral sugars, hexosamines, and amino acids (Crook and Johnston, 1962). Parker and his associates (1963) compared the hyphal wall chemistry of eight species -- *Achlya flagellata*[†], *A. racemosa*, *Brevilegnia bispora*, *B. unispërma* var. *delicat*[†], *Dictyuchus* sp., *Saprolegnia ferax* (including *S. monoicat*[†]), and *S. litoralis* -- utilizing x-ray diffraction, chromatography of hydrolysates, and transmission electron microscopy. For the various analyses, they prepared several fractions such as hot water soluble, alkali soluble, or alkali insoluble ones. Glucose was the dominant sugar, with mannose and glucosamine in fractions from the walls of the *Saprolegnia* species (confirmed by Crook and Johnston, 1962, and Thomas, 1966). Parker and his colleagues did not find glucosamine in species of *Achlya*, but it is a minor constituent of the wall of hyphae of *A. ambisexualis* (Thomas, 1966). In this same *Achlya*, Aronson and coworkers (1967) recovered polysaccharide fractions from the hyphal wall, and determined that the major constituents were non-cellulosic glucans of β -(1-3) and β -(1-6) glucoside linkages. Only a very small proportion of the filament wall of those water molds examined by Parker's group consisted of native cellulose (cellulose I), and there was no evidence of chitin in them. It is of interest to note that hot water-extracted polysaccharide fractions of the water mold hyphae were not crystallographically identical to those from the wall of specimens of *Vaucheria*. Nearly 85% of the wall preparations from the various species of Saprolegniaceae were non-cellulosic polysaccharides. In species of Vaucheriaceae, to the contrary, about 90% of the filament wall was cellulose (Parker, et al., 1963), with other polysaccharides being only minor constituents.

The presence of glucosamine in the hyphal wall of some water molds (Crook and Johnston, 1962; Parker, et al., 1963; Thomas, 1966) has led to the supposition that chitin could be a component of the total wall chemistry in these fungi. Dietrich (1973) found that when walls from the hyphae of *Achlya flagellata*[†], *A. orion*, *A. pseudoradiosa*[†], and *Dictyuchus monosporus* were treated with a crude chitinase preparation (from snail gut) n-acetylglucosamine was liberated. In a later comparative study of these and three additional water molds Dietrich (1975) found glucosamine present consistently, although in small amounts. When the antibiotic polyoxin D, a specific inhibitor of chitin synthase, was added to preparations of hyphae of *A. radiosa*, the formation of

n-acetylglucosamine was substantially reduced (Dietrich and Campos, 1978). Evidence for the presence of chitin in the hyphal wall of saprolegnians rests on Dietrich's work, for Parker and his associates (1963) did not find it in the hyphae of the water molds they tested. If this polysaccharide is present in these fungi it is a very minor component.

Working with *Saprolegnia ferax* and *Dictyuchus sterile*† Sietsma *et al.* (1969) demonstrated that the hyphal wall is quite complex chemically. A major portion of the wall of these fungi is composed of amorphous glucans: laminarin, a β -D-(1-3)-linked polymer (linear), and a mixed linkage β -D-(1-3) and β -D-(1-6) polymer (branched) plus a minor fraction, cellulose, a β -D-(1-4)-linked component. About 80-90% of the wall consisted of carbohydrates, 1-3% of hexosamines, 3-8% lipids, and 4% proteins, (the percentages of these constituents may vary greatly depending upon the method of preparation, specifically the "cleaning" of the wall fractions). Save for specific amounts, a similar chemical constituency was determined by Novaes-Ledieu *et al.* (1967), and D. S. Cameron and Taylor (1976) for the water molds they analyzed. The only sugars present in hydrolyzed wall preparations (Sietsma *et al.*, 1969) were glucose and traces of mannose; glucosamine also was detected. Parker *et al.* (1963) found some ribose in the walls of hyphae they examined, and in the fractionated wall of *S. ferax* filaments Novaes-Ledieu and his associates (1967) found traces of laminaribiose, rhamnose, and ribose in addition to the predominating glucose. The analysis of the hyphal wall in *S. diclina* also showed glucose to be the dominant neutral sugar; among the total sugars were small fractions of arabinose, ribose, fucose, xylose, and mannose.

Perhaps the study by D. S. Cameron and Taylor (1976) of *Saprolegnia diclina* best illustrates the complex array of chemical components in the hyphal walls of members of the Saprolegniaceae. They reported the following quantities ($\mu\text{g mg}^{-1}$ of dry wall material): anhydro-neutral sugars (725.5), anhydro-amino sugars (8.9), anhydro-uronic acids (13.3), n-amino sugar (84.9), anhydro-amino acids (13.3), lipid (119.5), and ash (24.8). Cysteine was not present in wall preparations, although 18 other amino acids were detected. Sixteen amino acids (assayed by thin-layer chromatography) were found by Vaziri-Tehrani and Dick (1980a) in nine isolates of *Achlya*.

The reviews by Bartnicki-Garcia (1968, 1969, 1973) illustrate the complex chemistry of the hyphal wall in Oomycetes, of which the Saprolegniaceae are a part. He recognized eight groups of fungi, based on fundamental differences in wall chemistry. The Oomycetes fall into category II: the hyphal wall consists largely of noncellulosic, alkali-insoluble glucans [β -(1-3) and β -(1-6)-linked polymers] plus cellulose.

At least in one water mold, the pathogen *Aphanomyces astaci*, wall glucans perform a substantive function in the interrelationship between it and the host (crayfish). It is known (Unestam and Beskow, 1976) that phenol oxidase in crayfish blood and cuticle is specifically activated on contact with purified hyphal wall fragments of *A. astaci* (and some other fungi). When the fragments are treated with exo- β -(1-3)-glucanase, however, their capacity to activate this crayfish blood serum enzyme is destroyed (Unestam and Söderhäll, 1977). Thus it appears that the prime constituents of the hyphal wall in *A. astaci* β -(1-3)-glucans are the specific elicitors of the crayfish response to invasion.

These glucans also activate prophenol oxidase in crayfish serum (Söderhäll and Unestam, communication).

Preparations from the mycelium of a Basidiomycete have been shown by Jiménez-Martínez and Novaes-Ledieu (1969) to liberate protoplasts from the hyphae when applied to the mycelium of *Saprolegnia litoralis*. The preparation contained β -(1-3) glucanase, but neither this enzyme alone nor β -(1-6) glucanase by itself digested the hyphal wall of the watermold.

Excellent accounts of some aspects of hyphal wall structure and biosynthesis in fungi appear in papers by Farkâs (1979) and Hunsley and Burnett (1970). The former devotes welcome speculative attention to possible regulatory mechanisms. A significant advance in application of enzyme-dissection technology by Hunsley and Burnett resulted in a reconstruction of the chemistry of hyphal walls (*Phytophthora*) superimposed on their physical architecture. The work of these investigators could be extended profitably to watermolds.

APICAL GROWTH

It has long been accepted that hyphal elongation is apical rather than intercalary. Berthold (1886) professed his belief in this principle, and J. H. Smith (1923) confirmed it with a series of very detailed measurements on hyphae. Hine (1878), too, had measured hyphal growth, and expressed his surprise over the rapidity with which filaments of some watermold species elongated. Much later, mycologists were to notice that rates of hyphal extension vary with individual species, as might be expected. For instance, Bret (1971) calculated that the maximum elongation rate in *Saprolegnia ferax* was 1000 mm hour⁻¹; in a female strain of *Achlya ambisexualis*, it was only $340 \pm 45 \mu\text{m}$.

The hyphal tip where apical growth occurs is assumed to be cylindrical. Trinci and Saunders (1977), however, formulated a different view (though they did not work with any watermold). From a direct examination and mathematical analysis of growing hyphae they concluded that there is a stable pattern in both circumferential and longitudinal aspects of hyphal tip growth. The extension zones where elongation takes place are not hemispherical but are more nearly half ellipsoids of revolution.

The mycelium of those watermolds studied carefully for the dynamics of growth -- *Achlya ambisexualis* (C. O. Warren and Sells, 1971, for example -- follows essentially the pattern in bacterial colonies although in prokaryotes, to be sure, the times over which the various phases occur are very short. In *A. flagellata*[†] (Barbier, 1971b) it is impossible to recognize the early stages of growth except microscopically; hyphae are initially sinuous, and have a small diameter. Such a mycelial configuration may be viewed as essentially comparable to the lag phase in the population development of bacteria. It also is possible that the lag phase resulting when hyphae are transferred is in part a reflection of the time required for the filament to overcome the traumatic shock of being broken off during the transfer process. Barbier's (1971b) data show that as the acidity of the medium is decreased the lag phase in the growth of *A. flagellata*[†] increases. He proposed that in an acid environment the mycelium contains all the constitutive

enzymes necessary for growth, and the lag period is accordingly shortened. In an alkaline culture, however, the fungus must first synthesize adaptive enzymes before the lag phase is terminated. Since phenotypic changes can be seen to take place in the hyphae during the latent and actively growing phases, Barbier (1971b) postulated that pH must modify both metabolism (such as protein synthesis) and cytoplasmic structure.

Griffin *et al.* (1974) propagated *Achlya ambisexualis* in submerged culture where it followed the kinetics of exponential growth. Not surprisingly, the culture medium -- the nitrogen source particularly -- influences the growth rate of hyphae in this species, and in *A. bisexualis* the method of agitating the mycelium during its growth also has an effect. Consistently higher growth rates occur in cultures subjected to rotary rather than reciprocal shaking, irrespective of the nitrogen source. Protein, DNA, and RNA increase exponentially during vegetative growth to maintain a constant proportion in the mycelium. A significant linear correlation seems to exist between specific growth rate, and radial increase in the size of the colony, and there is a very rapid doubling time: 51 minutes for filaments of *A. bisexualis* propagated at 24 °C on a medium with casein hydrolysate. Griffin and his associates concluded that oxygen supply was not the limiting factor in the growth of this fungus; the growth rate data supported the notion of constant ribosome efficiency in protein synthesis associated with growth. This latter idea has since been questioned.

A detailed study of the effect of agitation on the growth of watermold mycelium (*Achlya bisexualis*) is that by Montant and Darnaud (1971). Agitation favored colony growth up through the seventh day of incubation, but after about 10 days of shaking hyphal inhibition commenced. In stationary culture at three days the hyphae were "typical", but those developed in agitated vessels for the same period were tortuous, irregular, evaginated at points, and provided with rounded rather than tapered apices. Montant and Darnaud (1971), and Darnaud (1972a) proposed that localized metabolic changes, augmented by mechanical agitation modified hyphal morphology in these regions.

Successful apical growth in hyphae implies the existence of apical dominance (Barbier, 1975) and the presence of a difference in hydrostatic pressure between the interior of the tip and the external medium (Park and Robinson, 1966a, b; Bartnicki-Garcia and Lippman, 1972). Furthermore, filament elongation depends upon a balance between rate of synthesis and insertion of new wall substances (Chapter 13) and the maintenance of this pressure differential. If one factor changes without a corresponding adjustment in the other, elongation does not occur (Park and Robinson, 1966b). When the extension of a hyphal apex is checked by a hypertonic solution, slight but noticeable morphological changes result: the tip expands, followed by some degree of hyphal renewal expressed in lateral branching (see also Darnaud, 1972b).

That discontinuance of apical growth is not simply the result of maladjustment between two factors is shown in experiments by Bartnicki-Garcia and Lippman (1972). They demonstrated that various treatments -- sharp increases in temperature, application of neutral salts, detergents, or alcohols -- caused bursting of the hyphal tip,

and a measurable temperature coefficient was associated with this phenomenon. The implication of course, is that rupture is not solely an osmotic phenomenon. There is circumstantial evidence, then, that hyphal tips have a substantial potential for lysis. For hyphal extension to occur, the release of this lytic potential must be gradual and in harmony with wall synthesis (Bartnicki-Garcia and Lippman, 1972). Hill and Mullins (1979a) have suggested that in *Achlya ambisexualis*, at least, the lytic effect may be the rate-limiting step in hyphal wall synthesis.

On the basis of perfusion experiments with *Achlya flagellata*[†] Barbier (1975) concluded that hyphae of this fungus displayed apical dominance. Washing the mycelium with distilled water, he found, had the same effect as sporangial development: suppression of apical dominance followed by the onset of branching. Under the influence of continued perfusion, multiple sympodial branching occurs, and apical cymes are formed at hyphal tips. Cymose branching in *A. flagellata*[†] is alleged to be rare (T.W. Johnson, 1956b), but Barbier's observations (1975) suggest that this type of ramification may only be one expression of the amount of water surrounding the colony.

Suspension of apical dominance does not necessarily result simply in branching, (see following section). Modifications in hyphal morphology may issue from a variety of factors, and readily can be induced artificially. For example, kievitone (2', 4', 5, 7-tetrahydroxy-8-isopentenylisoflavone) is a phytoalexin (D. A. Smith *et al.*, 1973; Van Etten and Pueppke, 1976), produced by diseased plants of *Phaseolus vulgaris* L., that substantially reduces mycelial growth of *Aphanomyces euteiches* (D. A. Smith, 1976; D. A. Smith *et al.*, 1975). Hyphae propagated in the presence of certain concentrations of kievitone branch frequently, giving a prominent compactness to colonies on agar. Within the individual hyphae, cytoplasmic streaming ceases, vacuoles increase in number (and prominence), and the cytoplasm becomes granular.

Even the nature of natural-product growth media can have a profound effect on the texture displayed by watermold hyphae (Hodkinson and Hunter, 1971). Moreau and Moreau (1937b) reported multiple vesicle formation by hyphae of saprolegniaceous fungi whose growth was "suspended" by such substances as glycerin or mineral salts. The vesicles, they believed, were the expression of a pathological condition brought about by the external medium. These vesicles (sometimes catenulate) contained parietal protoplasm in which the nuclei aggregated and eventually degenerated.

Höhnk (1957a) isolated some non-sexual specimens of Saprolegniaceae from mesohaline waters, and separated them into four recognizable growth forms. In watermolds of three of these categories there were no terminal sporangia, and the hyphal tips were variously formed. The apex of a hypha could be lobed, bulbous, dichotomously branched, and sharply tapering or rounded, and in some instances even delimited by a septum. Around portions of the hyphae of *Achlya colorata* Moreau (1946) detected massive aggregations of bacteria. Within such an encrustation the hypha was swollen, and in time, would branch. Moreau contended that this hyphal configuration was, in fact, a bacterial gall.

Using culture filtrates and preparations from the mycelium of *Achlya ambisexualis* (male strain), Hill and Mullins (1979a) assayed for the activity of nine enzymes. Each enzyme exhibited some activity in the various samples, but only cellulase and ATPase gave a higher response in the filtrate than in direct extracts from the mycelium. An increase in fresh weight of the mycelium accompanied wall synthesis, and early in mycelial growth glucose was increasingly incorporated into the hyphal walls. The secretion of cellulase was correlated both with hyphal growth and wall synthesis, Hill and Mullins found, but under nutritional stress these processes were inhibited.

BRANCHING

Hyphal extension, when it ceases, often forecasts branching, an integral part of morphogenesis. Branching may be of two types (Park and Robinson, 1966a), subapical (from immediately below the apical "dome") or lateral, in which case the hyphal continuation is at some distance behind the apex, and is initially at a right angle to the main axis. Much of the knowledge of the morphological and physiological expressions of branching in the hyphae of water molds comes from the thorough studies by Bret, Fèvre, and Larpent.

Lateral branching (Larpent, 1970; Larpent, *et al.*, 1973) involves two stages: emergence of the branch initial, and growth (apical elongation) of this incipient secondary hypha. Thus, it appears that growth and branching are not separate, unlinked processes, and that apical dominance is a functional part of ramification. However, not all lower groups of plants exhibit apical dominance. From a comparative study of enzymes and branching in *Saprolegnia monoica*[†], a *Podospora*, a moss protonema, and an alga, Auvity *et al.* (1974) concluded that there is apical dominance in fungal hyphae and in Bryophytes, but not in algae.

In 1962(a, b), Larpent showed that hyphae of a female strain of *Achlya bisexualis* and *Saprolegnia monoica*[†] (Larpent, 1966, 1972b) propagated in the presence of methionine alone did not branch, although they did so in the presence of griseofulvin and histidine. In *A. bisexualis* (Larpent *et al.*, 1971), the effect of griseofulvin was intensified as its concentration was raised. Principal hyphae in the presence of this antibiotic were supplemented by secondary axes that elongated to precisely the same degree and length that the principal axis did. When the concentration of griseofulvin was increased additional secondary axes were stimulated to form such that a compact cyme developed at the hyphal tip. Sorbose and nystatin (Larpent, 1962b) inhibited elongation of hyphae in *A. bisexualis*. In the presence of griseofulvin or colchicines, hyphal elongation ceased and branching commenced (Larpent, 1963a); erythromycin suppressed branching, but colchicine counteracted this response. Thus, two responses are evident in the mycelium of at least these two species of the family: hyphal tip inhibition resulting in loss of the filament's ability to elongate, and branch stimulation.

In *Saprolegnia monoica*[†] it is possible to change the branching pattern of hyphae without at the same time modifying their rates of elongation, and the reverse is also true (Larpent, 1963a). In the presence of sodium fluoride, for example, extension of the

hyphae is inhibited, but their branching is not affected. In mycelium grown in the presence of casein, for instance, elongation and branching are together stimulated in the individual hyphae. Chloramphenicol in a yeast extract medium induces an opposite reaction in the hyphae: reduction in the rate of apical growth, and a proportional decline in the frequency of lateral branching. Filaments of this fungus in the presence of the antimetabolite canavanine have a lowered branching frequency, but if arginine is added to the medium in a concentration equal to that of the canavanine, the growth rate is restored and branching accelerates. To explain the selective effects of inhibitory agents Larpent (1966) suggested two alternatives. Some inhibitors (ones deficient in nitrogen or sulfur, for example) block metabolism to such an extent that both elongation and branching are retarded. Other suppressants such as erythromycin, colchicine, and sodium fluoride counter only particular metabolic pathways, consequently interfering either with extension or branching but not both.

Apical dominance seems to be fortified, and lateral branching correspondingly repressed, when *Saprolegnia monoica*[†] and *Achlya bisexualis* are cultivated in nutritively deficient media (Larpent, 1966). Furthermore, where branching is not prevented, the compounds in the medium have some influence over the distance between successive offshoots. Hyphae in a medium containing leucine tend to show greater distances between laterals than do those grown in the absence of this compound. Valine, arginine, alanine, and some concentrations of methionine, on the contrary, stimulate ramification such that there are shorter distances between branches on hyphae in media with these compounds than in cultures not supplied with them. When the rate of elongation is reduced, apical dominance is augmented, and branching is accordingly suppressed (Larpent, 1966). In *A. bisexualis*, at least, hyphal branching is influenced to a pronounced degree by the nitrogen source in the culture medium. Griffin *et al.* (1974) found that in mycelium propagated in a casein medium, the number of primary branches in the peripheral zone of colonies was 41; in the presence of glutamate, the number was 8.2, but there was an average of only 1.5 branches per filament when the fungus was grown in a medium with ammonium tartrate. Glycine did not augment branching. Casein hydrolysate, but not individual amino acids purified from this compound, is said to elicit branching in *A. bisexualis* (Musgrave *et al.*, 1977).

It has also been demonstrated in some Saprolegniaceae that although hyphal elongation may be inhibited by certain chemicals, branching may be hyperstimulated. Both cytochalasin B and D (Larpent, 1974) are effective in this respect, and are able to counteract the branch-inhibiting effect of erythromycin. Mitomycin C, an antibiotic that blocks DNA replication, also retards hyphal elongation in water molds, suggesting (Larpent, 1971; Larpent *et al.*, 1971) that there is a correlation between nuclear division and hyphal extension. Puromycin, an inhibitor of protein synthesis, likewise reduces longitudinal growth in hyphae. The hyphae of *S. monoica*[†] seem to be affected differently by actinomycin D and puromycin. If actinomycin D is in the medium, abridgement of hyphal growth rate and the filament's loss of ability to form lateral branches are proportional to the log of the concentration of the antibiotic. The velocity

of hyphal prolongation in the presence of puromycin diminishes proportionally to the log of the concentration of this compound as well, but in some concentrations lateral branches are suppressed and elongation commences.

In the presence of certain chemicals both longitudinal extension and branching of hyphae are affected equally (Larpent and Chabroulet, 1963). One such compound is 8-hydroxyquinone. In mycelium propagated in the presence of $1500 \mu\text{g L}^{-1}$ of this compound, both growth rate and frequency of branching in hyphae of *Saprolegnia monoica*[†] are curbed. If casein or sulfates of zinc or iron are added to the medium, however, the obstructional effect of the quinone is cancelled.

Apical dominance in hyphae of certain water molds is reinforced (Larpent, 1963c, 1966) by culturing specimens in a nutrient-deficient medium or by adding inhibitors to a nutrient-rich substratum on which the fungi are growing. Larpent propagated hyphae of *Saprolegnia monoica*[†] on agar blocks in van Tieghem cells. To one surface of a coverslip, he fastened two agar blocks separated from one another such that the gap between them was a barrier to hyphal growth. One block contained 4 g L^{-1} of yeast extract, the other only 0.2 g L^{-1} . The richer medium was inoculated, and the hyphae allowed to grow across the gap to contact the "poor" medium; the low nutrient block was also inoculated. There was rapid growth and abundant branching of the hyphae on the agar with 4 g of yeast extract, but filaments on the reduced nutrient medium elongated but did not branch. However, when hyphae from the block containing the higher concentration of yeast extract contacted the deficient substratum they grew onto the low-nutrient agar and there commenced to branch. The same discontinuity apparatus tested compounds inhibitory to growth or branching. Hyphae growing on the agar block without the chemical, but in contact with the block containing the agent reacted as if the compound were in fact present in both media. Hence, there was a translocation from the older parts of the hyphae to the younger portions (Larpent, 1964, 1966) of the "stimulus" responsible for suppressing or augmenting apical dominance. The reverse was evidently not possible. Larpent postulated (1974) that cytoplasmic movement through contractile microfibrils and filaments might in some fashion contribute to the observed stimulus-transporting phenomenon.

In sum, the work of Larpent (1966, 1971, 1972a, b) and his associates (Larpent *et al.*, 1973) has explored branching -- or lack thereof -- as a function of externally applied stimulators or inhibitors. The observational and experimental data suggest that in members of the Saprolegniaceae nutritional deficiencies in the medium and the presence of protein synthesis inhibitors suppress lateral branching as would be expected.

Chemicals that inhibit protein synthesis also influence the rate of hyphal extension (Larpent, 1966; Fèvre, 1972, 1976). Borrod *et al.* (1970) tested the effect of *dl-p*-fluorophenylalanine on hyphae of *Saprolegnia monoica*[†]. Concentrations of this inhibitor up to 100 mg L^{-1} hampered elongation, but above this limit longitudinal growth progressed at a steady rate although at a level about 48% of that of hyphae in control cultures. Bret (1972a) noted in the mycelium of *Achlya ambisexualis* (female strain) that the rate of hyphal elongation diminished in cultures when the

concentrations of *dl*-*p*-fluorophenylalanine were raised. However, in the hyphae of this same species there is a time-dependent reaction. In media lacking nitrogen or ones containing the protein synthesis inhibitor some of the aging hyphae developed a prelethal excess of branching at the apex -- "hyperbranching" as Bret termed it. Other filaments, on the contrary, simply formed terminal sporangia.

Upon closer examination of the phenomenon of hyperramification Bret (1972b) found some cases to be illusory. Reduction in the elongation rate in hyphae of *Saproegnia ferax* is proportional to the log of the concentration (0-400 mg L⁻¹) of *dl*-*p*-fluorophenylalanine. When the level of the inhibitor is increased, the distance between successive branches diminishes. However, when the number of branches is considered, it is evident that after a certain concentration of the inhibitor is reached, the degree of branch inhibition in the mycelium lags behind that of extension, hence the branching only appears to be excessively stimulated (Bret, 1971). Larpent (1966) detected essentially the same "quasi" branching in *S. monoicat*.

Darnaud (1972b) studied the effect of osmotic pressure on hyphal growth of *Achlya bisexualis*. With an increasing concentration of glucose or NaCl in the medium, there was a corresponding decrease in the weight of the resulting mycelium, a reduction attributed to endogenous metabolic adjustments in response to the internal concentration of the hyphae and not to nutritional deficiency or failure of nutrients to enter the system. Within 15 minutes after 36-hour-old colonies were transplanted from a 3% (4.5 atm) glucose solution to a 0.45% (1.1 atm) solution, changes in the hyphal tips occurred. At first the tip of the hypha bulged outward. Some filaments then also branched dichotomously with one lateral elongating, and the other not growing, or both growing, or one becoming irregular and sinuous. According to Darnaud the abrupt changes in hyphal tip morphology resulting from increases in external osmotic pressure are expressed in a sudden dilation of the cytoplasm at the filament extremity followed by a retraction that ruptures the cytoplasmic contact with the wall. Using polyethylene glycol as the osmoticum, and antheridiol-induced cellulase (assayed viscometrically), Thomas (1970) discovered that decreased turgor prevented the secretion of a hydrolytic enzyme said to be required for localized softening of the hyphal wall and, as a consequence, branching did not occur (confirmed by Mullins, 1979). Water stress thus is another factor that influences the branching response.

ENZYMES AND HYPHAL MORPHOGENESIS

It is now known that hydrolytic enzyme induction in water molds is a prerequisite to branching (Thomas and Mullins, 1969; Fèvre, 1979b) and can be initiated by such compounds as the sex hormone antheridiol, casein hydrolysate, or metabolites containing amino acids or mixtures of pure amino acids (Mullins, 1973). When cycloheximide is incorporated into a growth medium, cellulase production in *Achlya ambisexualis* is inhibited as is protein synthesis (Mullins, 1973). This type of cellulase is an endo- or random-splitting enzyme that cleaves the cellulose substrate into polymers of varying lengths, but does not release the monomer glucose. It is assayed by the

viscometric method. Furthermore, any treatment that prevents either synthesis or secretion of cellulase also checks lateral branch formation. Puromycin and *dl*-*p*-fluorophenylalanine also are effective arrestors of cellulase production as is *dl*-leucine in certain concentrations. If *dl*-phenylalanine is added to cultures of *A. ambisexualis* simultaneous with *dl*-*p*-fluorophenylalanine, the inhibitor effect of the latter is negated (Thomas and Mullins, 1969). Casein hydrolysate is known to induce cellulase release in *Saprolegnia parasitica*[†] and in a male strain of *Dictyuchus monosporus* (Mullins, 1973). It has been shown (Timberlake and Griffin, 1973) that puromycin and cycloheximide obstruct the incorporation of *l*-proline into acid-insoluble proteins of *A. bisexualis*; *dl*-*p*-fluorophenylalanine, however, prevents the synthesis of the functional enzyme alkaline phosphatase without directly affecting *l*-proline.

Independent of Thomas and Mullins (1967), Fèvre (1969, 1972) demonstrated that *dl*-*p*-fluorophenylalanine reduced the cellulolytic ability of the mycelium of *Saprolegnia monoica*[†], and the fungus rapidly lost the ability to form lateral branches. Erythromycin and puromycin also forestalled branching, and, as Fèvre viewed it, thus reinforced the apical dominance of hyphae. Sodium fluoride, on the other hand, inhibits branch elongation but not the number of laterals formed (Fèvre, 1969) suggesting (Fèvre, 1972) that cellulase activity is not diminished in the presence of this compound. Glucanase activity and branching in the hyphae of *S. monoica*[†] are also correlated (Fèvre, 1972): when protein synthesis is inhibited by *dl*-*p*-fluorophenylalanine the activity of β -1, 3-glucanase decreases and simultaneously branching is prevented.

Both glucanases and cellulases (Fèvre, 1976) act on cellulose and glucans in the hyphal wall (Sietsma *et al.*, 1969) to permit branching. In watermold hyphae where a reduction in the enzymes capable of modifying wall structure develops -- depletion of β -1, 3-glucanase, cellulase, glucan synthetase, and protein disulfur reductases -- there is an accompanying loss of ability to branch. Fèvre (1979a) further demonstrated that intracellular glucanase activity is the result of at least two enzymes. One of these, exoglucanase (active on laminarin but not on modified laminarin), is weakly bound to the hyphal wall. The second enzyme system, endoglucanase (active on laminarin and an oxidized form), is liberated when the wall undergoes autolysis, but its activity may be blocked by treating the mycelium with glucono- δ -lactone. Hyphae when so treated display reduced growth, but branching commenced concomitantly. Digitonin solubilizes glucan synthetases in the hyphae of *Saprolegnia monoica*[†], and some part of this enzyme system can be activated by proteolysis (Fèvre, 1979c).

The enzyme activity of hyphal extracts from *Saprolegnia monoica*[†] has been related to morphogenesis, and particularly to ultrastructure of the filaments. Miele and Linkins (1978), for example, showed that the hyphal walls of *Achyla bisexualis* (male strain) are disrupted if mycelium is grown in medium containing cellulose. Such walls are much thinner than are those of colonies grown in the presence of glucose or cellobiose. Fèvre (1976) has represented schematically a concept of the biochemical constitution of the hyphal apex superimposed on the ultrastructural architecture as proposed by Grove *et al.* (1970). In effect, he suggests that enzymes associated with elongation and branching are not localized in a single organelle (*see* Chapter 13). Fèvre postulates that the

distribution of enzymes in the hyphal tip requires that there be a region for synthesis (endoplasmic reticulum), a transport site (dictyosomes and vesicles) and a locus for response, (plasmalemma). Accordingly, growth and branching are thought to take place in the following manner.

Glucanases, cellulases, and synthetases are assembled in the endoplasmic reticulum (ER) which cuts off vesicles that enlarge to become the dictyosomes. These vesicles incorporate the enzyme complexes. In turn, blebs from the dictyosome enlarge into other vesicles (which now contain the enzymes) that migrate to the hyphal tip where they fuse with the plasmalemma and release the enzyme complexes to the outside of this membrane. Branching is accomplished (Fèvre, 1976) by the same sequence of events, starting with the activity of the ER. Fèvre assumes that in the process of branching, however, an excess of vesicles accumulates at the hyphal tip, and as the tip elongates, some of these wall vesicles (under the action of turgor) are left in isolated groups against the lateral wall. At each of the points where these vesicles adjoin the wall they enzymatically create a weakened zone (wall softening), and the lateral wall bulges to initiate a new growing tip. The wall then, does not control morphogenesis in the hypha (Fèvre *et al.*, 1975), but it is at the level of the cytoplasm and plasmalemma that apical differentiation takes place. There is always an accumulation of vesicles at the tip of a growing hypha (*Saprolegnia monoica*[†]) and it is here also (Fèvre *et al.*, 1975) that β -1,3-glucanase and protein disulfide reductase are active. In unbranched hyphae the level of these enzymes is low, but they are detectable in elevated quantities in branched hyphae. If Fèvre's concept of ultrastructural involvement in branching is correct, then Istvánffi's (1895) observation some 80 years earlier was not without foundation. He concluded that subapical branching in hyphae occurred only in close proximity to a nucleus.

The role of enzymes in hyphal branch induction has also been studied experimentally (Thomas, 1966, 1970; Thomas and Mullins, 1967, 1969) with antheridiol (Arsenault *et al.*, 1968; Barksdale, 1970; Horgen, 1977a; see also chapter 21). When mycelium of *Achlya ambisexualis* is treated with antheridiol, there is a detectable rise in cellulase activity to a peak that coincides in time with the appearance of lateral branches (Thomas and Mullins, 1967). Casein hydrolysate also elicits branching, but there is no increase in cellulase -- and consequently no branching -- when *dl-p*-fluorophenylalanine or puromycin are added to mycelial cultures. Since these compounds inhibit protein synthesis, it appears that cellulase activity is dependent in some fashion on protein synthesis (Thomas and Mullins, 1967, 1969; B. E. Kane *et al.*, 1973; Timberlake *et al.*, 1973). Thomas (1966) reported that the extraction properties of hormone-induced cellulase differed somewhat from "vegetative" (noninduced) cellulase. The latter is a metabolically labile endoenzyme released into the medium, and is probably involved in normal apical growth. The induced enzyme possesses a different thermal stability from that of the vegetative cellulase. Moreover, the cellulase in *A. ambisexualis* differs from that of other fungi in that it is not elicited by cellulosic substrates (but see Miele and Linkins, 1978), and is ineffective in degrading such substrates. Protease activity is higher in noninduced hyphal preparations, Thomas found, than in hyphae treated with

antheridiol. It is at least possible (Thomas, 1966) that the production and action of vegetative and induced cellulase are controlled differently in the hyphae of this *Achlya*. Cellulase in the male strain of *A. ambisexualis* can be recovered from both particulate and soluble fractions of mycelial homogenates. It appears, however, that the particulate-bound cellulase is not derived from the same pool of molecules as the soluble-fraction enzyme, suggesting that the former is possibly an integral membrane protein (Hill and Mullins, 1979). Hill and Mullins (1980b) have attempted to isolate and characterize cellulase-containing membranes in *A. ambisexualis* by differential and density gradient centrifugation. Maximum cellulase activity occurred at an isopycnic density of 1.19 g cm⁻³; these were similar maxima for IDPase, ATPase, UDPG transferase, and sedimentable carbohydrates. The particles in which the enzyme activity was localized have not been positively identified (Hill and Mullins, 1980b), but possibly are the large vesicles (150 nm diameters) described by these same authors (Hill and Mullins, 1980a).

Freeze-fracture preparations of antheridiol-induced branching in *Achlya ambisexualis* show membrane configurations (indentations in the overlying wall) that are consistent with exocytosis (Mullins, 1979). This would indicate that the structural integrity of the wall -- at least in this species -- resides in the microfibrillar cellulose moiety (Novaes-Ledieu, 1967; Parker *et al.*, 1963). Hyphae subjected to water stress show no membrane profiles of exocytosis, but if the mycelium is allowed to recover from that stress the membrane evaginates and causes wall indentations at the sites of antheridial branch initiation.

In a study of the relationship between cellulase secretion and cytoplasmic streaming in *Achlya ambisexualis*, Thomas *et al.* (1974) employed cytochalasin A and B, Colchicine, and vinblastin, compounds known to disrupt microtubule function. They discovered that cytochalasin A inhibited both cellulase production and streaming; cytochalasin B was a mild inhibitor of, or, in some concentrations a stimulant to, the synthesis of cellulase. Neither colchicine nor vinblastin had an effect on streaming or enzyme production. It appears that in this one watermold, at least, there is a coupling between secretion of cellulase and streaming.

Auvity *et al.* (1974) compiled evidence to suggest that endoenzymes in hyphae of *Saprolegnia monoica*[†] may have different functions. They interpret their experimental data to indicate that reductase reacts only very feebly on unbranched hyphae, but has a marked effect on branched ones. Enzymes such as glucose-6-phosphate dehydrogenase, and malate and glutamate dehydrogenase are involved in hyphal extension, but not in branching.

The foregoing account makes no attempt to appraise the accumulated information on hyphal wall chemistry and structure in the watermolds. The current knowledge strongly accentuates the conclusion that these aspects of the hypha are very complex and as yet not fully understood. Neither the function nor the interrelationships of the various wall components are entirely identified and evaluated. It is essential, moreover, that in future work a careful assessment of methods of preparation be made, and results be interpreted within any limitations imposed by

methodology. Experimentation with a “chemically clean” wall fraction may in fact not actually represent the functional wall. Certainly differences in reported percentages or amounts of individual components of the hypha’s external boundary may well be traceable to preparation of samples. If a criticism is to be leveled at past investigations of wall chemistry in the water molds, it is that assessment of techniques has not been made.

APICAL DOMINANCE AND REPRODUCTION

The array of information on hyphal elongation in the Saprolegniaceae leaves no doubt that apical dominance can be enhanced or inhibited by endogenous substances. The appearance of sporangia and oogonia on the mycelium likewise influences apical dominance, and suppress the hyphae much as if their tips were excised (as Larpent, 1966, has done). Even in the species of *Saprolegnia* where percurrent proliferation occurs (S. J. Hughes, 1971a, b), sporangial renewal appears to alternate with retardation and initiation of apical dominance. If no terminal sporangium develops, apical dominance is retained, and monopodial growth continues (Barbier, 1975). Where a sporangium forms and sympodial growth occurs, as in *Achyla flagellata*[†], perfusion (Barbier, 1975) leads to the induction of repetitious helicoidal cymes measuring several millimeters. It must be recognized, however, that in water cultures where the fungus is not necessarily subjected to exposure to concentrated inhibitors, the production of laterals can be simple or multiple so that it is difficult to determine if the branching is in fact sympodial or dichotomous. This situation occurs also where the hyphae are exposed to chemical inhibitors (Larpent, 1971; Larpent *et al.*, 1971).

THE COLONY

Although several authors have referred to colony characteristics in their papers on Saprolegniaceae, there appears to be but one publication devoted entirely to this subject, that of Karl Otto Müller (1922). In the two species with which Müller worked, *Saprolegnia monoica* and *S. thureti* (both are now recognized as *S. ferax*), the characteristic colony shape was said to be determined by hyphal diameter and number of branch hyphae (“daughter” filaments). Chemotropism evidently also plays a part in determining colony shape; Müller proposed that the hyphae in a colony, at least on agar media, curved toward the highest concentrations of nutrients. Although he claimed to have demonstrated mathematically that the mass of the colony increases proportionally to the nutrient concentration, it must also be recognized that colony mass is linked to hyphal diameter and degree of ramification. Even when hyphae in Müller’s experimental cultures were “forced” to grow through narrow apertures, the emergent filaments subsequently formed a circular colony.

In the early stages of colony development, Karl Otto Müller (1922) found, the mycelium grows in an irregular pattern. As the marginal hyphae contact a homogeneous nutrient source (and concentration?), a regular rate of growth ensues,

and the circular pattern becomes evident. Griffin *et al.* (1974) detected a significant linear correlation of colony radial growth with specific growth rates in mycelium of *Achlya bisexualis*. In a somewhat less precise fashion, Müller had earlier determined that such a ratio existed for the species he studied.

According to Karl Otto Müller (1922) there is a demonstrable relationship between the velocity of increase in colony diameter and thickness (diameter) of the hyphae; both temperature and osmotic concentration of the surrounding medium influence each of these factors. As to the function of branching in colony development, Müller suggested that the branches developing behind the apices of hyphae in a colony grew, in the main, in a radial-centrifugal direction. This pattern, he believed, was the direct result of negative chemotropism: filaments growing away from their own exogenous metabolic products. The germ tube from a settled spore, on the contrary, exhibits positive chemotropism, Müller argued, and thus curves or grows toward "new" nutrient sources. Larpent (1970) also recognized the curvature or oblique orientation of lateral branches, but could not account for such a pattern. To be sure, repeated branching from below hyphal tips inhibited by some external force (griseofulvin in the medium, for example) occurs much as Müller demonstrated (but had attributed to negative chemotropism). That branching -- contributing to the circular colony pattern -- is a more complex process than simple tropic responses seems firmly established (Larpent, 1966; Larpent *et al.*, 1971; Fèvre, 1968).

For a thorough general account of hyphal morphogenesis, see Bartnicki-Garcia (1973).

GEMMAE

Distended, densely-cytoplasmic regions are sometimes delimited from parent hyphae singly or in a catenulate fashion. Such segments are gemmae or chlamydospores. Not all members of the Saprolegniaceae produce these cells, and, in those that do, the function is sometimes obscure. Gemmae may germinate directly into new hyphae, convert into sporangia (Galloway, 1891, among others), or, in rare instances (Maurizio, 1894; T. W. Johnson, 1956b) produce oogonia.

For the most part, gemmae generally have been ignored by mycologists save where terminology appears to have been the troublesome point. Unger (1843, 1844) regarded these hyphal segments as abnormalities, and summarily dismissed them. To Schröter (1869), gemmae were gonidia that appeared on hyphae of *Achlya proliferata* when the fungus was grown in bog or well water. These cells converted without a resting period directly into new hyphae. According to A. Fischer (1892), gemmae developed a function (converting to sporangia, for instance), in oxygen-rich waters, but Swan (1898) regarded the densely cytoplasmic vegetative cells of *Leptolegnia* specimens as chlamydospores: abnormalities elicited as a response to unusual environmental conditions. Quite the opposite view was taken by Kanouse (1932:441), who regarded the chlamydospores (gemmae) in cultures of *S. parasitica*[†] richly supplied with nutrients

as "... a special type of reaction of the fungus to transform the almost unlimited food supply into protoplasmic reserves."

That gemmae -- at least for some water molds -- may be abnormal structures is not an idea without foundation. The study of *Saprolegnia* sp. by C. J. A. Berkeley (1944) is a case in point. Like Maurizio, he found hyphal segments that were intermediate in structure between gemmae and sporangia (percurrent renewal, for instance). Moreover, in Berkeley's isolate the gemmae that functioned as sporangia had a tendency to form abnormally large and sluggish planonts. In what he called "gemmae-sporangia", Berkeley also observed secondary spores emerging, but without leaving cysts within the confining cell -- an unlikely performance by a species of *Saprolegnia*! From some gemmae-sporangia, elongate spores escaped that, after commencing to swim, became pip-shaped (that is, assumed the shape of primary spores). Either the cylindrical spores had reentered the gemmae-sporangia, Berkeley thought, or these vegetative cells did in fact produce secondary, reniform planonts. Such behavior recalls coincidentally the peculiar motile phase of an unidentified *Saprolegnia* discovered by T. W. Johnson (1975) in Norway.

Maurizio's view of gemmae (1894, 1896b) diverged prominently from prevailing thought at the time. He concluded that the hyphal segments of his *Saprolegnia rhaetica*[†] were neither conidia nor gemmae, but were chlamydospores. This decision was expanded upon at length in his 1896(b) paper, in which he proposed that chlamydospores in the Saprolegniaceae were significant phylogenetically. This was later to be denied by Weston (1918:164). In any case, Maurizio reversed his terminology of 1894, and considered gemmae to be conidia. His preoccupation -- to what now seems an excessive degree -- with these vegetative structures is nowhere more cogently illustrated than in his naming of gemmae (his "conidia") on the basis of function: conidial sporangia (gemmae converting to sporangia), conidial oogonia, and, finally, conidial antheridia. To Maurizio (1896b), the "sporangienanlage" were primitive, nonspecialized structures from which sporangia and oogonia had evolved.

Maurizio was not alone in his interest in gemmae, for Humphrey (1893) also recognized "types" of such cells. Sporangium-like bodies, arrested for a time in their development (presumably by some external conditions) but in which planonts were subsequently cleaved, Humphrey termed resting sporangia. Chlamydospores, on the other hand, were produced in addition to and in the same position as sporangia, yet on germination developed a hypha which then bore a terminal cell that formed motile spores.

Walz (1870a; a condensed version appears in Val'ts, 1870, in Russian), who observed pythiaceus discharge in fungi he identified (incorrectly) as *Saprolegnia monoica*[†] and *S. dioica*[†], illustrated thick-walled structures that he held to be conidia. One of the figures (Walz, 1870a: pl. 9, fig. 21) is of a structure suspiciously like a mucoraceous sporangium, but Weston (1917) thought some of Walz's drawings depicted cells very much like the "resistant spores" in *Achlya* sp. Sorokine (1876) reported thick walled "conidia" (gemmae) in *Aphanomyces stellatus*, but this is a very suspect observation. No species of Saprolegniaceae, let alone any of *Aphanomyces*,

produces catenulate gemmae separated by an isthmus-like thick wall as Sorokine figures the structures. He may well have seen conidia of some entomophthoraceous fungus in his preparations.

The nonsexual *Achlya* sp. with which Weston (1917) worked, developed swellings of various shapes at the hyphal tips. These bulbous portions were delimited by septa, and subsequently became thick-walled. Such swellings, he observed, occurred only under conditions that inhibited sporangium development. In water, these resistant spores (as Weston termed them) formed a germ tube bearing a terminal, achlyoid sporangium; on agar, their germination was entirely siphonoblastic. Because of their regular and distinctive appearance Weston argued that these resistant spores were not gemmae. He thought it was possible to divide the family into three groups on the basis of their ability to form sex cells: strongly sexual ones (even under adverse conditions), sexual ones (only when specimens were subjected to particular environmental conditions), and those species which had lost altogether the ability to reproduce sexually. He saw, moreover, a parallel situation in some of the leptomitaceous and blastocladiaceous fungi. The failure of these fungi to form sex cells coincided with their ability to form resistant sporangia. The resistant spores described by Weston (1917) are suspiciously like gemmae and oogonial initials of a dioecious (heterothallic) *Achlya* such as *A. ambisexualis*. Thus, it is entirely likely that Weston's *Achlya* sp. was a mating strain.

Little is known of the factors that induce gemmae. Griffin (1966) was able to induce hyphal segments in *Achlya* sp. by adding 40-80 mM of the chloride salts of calcium, magnesium, sodium, or potassium. A 5 $\mu\text{g mL}^{-1}$ concentration actinomycin D caused hyphal tips of the fungus to form gemmae rather than sporangia, suggesting (Griffin and Breuker, 1969) that since gemmae and sporangia have similar early development stages, the former may be partially aborted sporangia. Shake culture methods applied to *A. bisexualis* delayed the appearance of gemmae beyond the time of their development in stationary culture (Montant and Darnaud, 1971). Gemmae were formed by a *Saprolegnia* sp. isolated by C. J. A. Berkeley (1944) when the fungus was grown on boiled watercress and allowed to age. If gemma-forming colonies were propagated on a peptone medium, these structures produced hyphae on germination. When these gemma-bearing isolates then were transferred to a glucose solution, the gemmae produced spores. Patently, much must yet be done to determine the nature and origin of gemmae, and the factors influencing their function.