

CHAPTER 17. The Physiology of Growth: Parasitic Species

It is more than mere convenience of format to give equal time to the parasitic species in an account of the growth and reproduction by the Saprolegniaceae. To be sure much of the work on these representatives has been directed toward solving practical problems, but to leave these water molds out because of that approach is to give a distorted view of the family. Much is to be learned from these fungi that do not conform to the habits of their saprotrophic counterparts even though all parasitic forms in the family are facultatively so and their nutritional requirements do not seem to be unique.

In this chapter we treat what is known of the chemical and physical parameters that influence the vegetative growth of those water molds implicated as disease-producing fungi. Subsequent chapters on the physiology of reproduction will consider further the more injurious ones. With respect to *Aphanomyces euteiches* and *A. cochlioides* the substance in the accounts to follow has been included in part in a review by Papavizas and Ayers (1974). It seems reasonable to consider each of the major cases of parasitism or pathogenesis separately.

APHANOMYCES EUTEICHES

Root rot of peas (*Pisum sativum* L.) has been the most extensively studied of the plant diseases caused by water molds. The fungus, *Aphanomyces euteiches*, grows both in natural extract or elution media (F. R. Jones and Drechsler, 1925) and in chemically defined ones (Papavizas and Davey, 1960a; Yang and Schoulties, 1972). Data from the precisely formulated substrates are of course the most meaningful for an understanding of the physiology of the fungus.

CHEMICAL PARAMETERS FOR GROWTH

CARBON AND NITROGEN NUTRITION: -- Geach (1936) was among the first to explore the nitrogen requirements of *Aphanomyces euteiches*. He found that growth of the mycelium was retarded by urea, ammonium sulfate, and sodium nitrate. Because these determinations were made from cultures growing in a pea decoction medium, they are of questionable value. Diametrically opposed in part to these results were those reported by Carley (1969). He determined that both ammonium and nitrate nitrogen could be used by an isolate of *A. euteiches*, but it grew better in the presence of the former. A very significant fact emerges from Carley's experimental work: one isolate of *A. euteiches* could derive adequate essential nitrogen from calcium nitrate. This surely needs confirmation.

According to R. T. Sherwood (1958), *Aphanomyces euteiches* required organic nitrogen for growth, and Papavizas and Davey (1960a) demonstrated that growth of this species occurred in a very broad spectrum of sources. Both *dl*-methionine and *dl*-

glutamic acid stimulated the hyphae of *A. euteiches*, and a mixture of amino acids also increased dry weight yields of mycelium. Ammonium nitrogen likewise could be used by *A. euteiches* (if the pH was properly adjusted and controlled) but nitrate nitrogen was not assimilated. Papavizas and Davey (1960a), mention a nutritional factor overlooked in most instances by investigators. Their data leave little doubt that the *l*-isomer of glutamic acid has greater growth-promoting ability (for *A. euteiches*) than the *d*-isomer. Moreover, the *dl* form is superior to both. Those reports on nutrition of water molds that fail to record the specific optical or structural isomer of organic constituents are deficient.

In their study, J. E. Mitchell and Yang (1966) found that *Aphanomyces euteiches* grew negligibly in media with amino acids incorporated in the proportions supplied by peptone, yet this derived extract supported good mycelial development with or without glucose. Casein hydrolysate likewise was a poor nitrogen source for *A. euteiches*. It is evident that single amino acids are less favorable for growth than mixtures unless, of course, the amino acid medium is supplemented with, for example, a reduced sulfur source.

Most of the detailed information on nitrogen nutrition of *Aphanomyces euteiches* comes from the work by Yang and Schoulties (1972) who attempted to develop a chemically defined medium. They used twelve nitrogen sources (organic and inorganic) in single substitution experiments. Their isolates of this species utilized ammonium nitrate, ammonium sulfate, and potassium nitrate, but maximum growth occurred in cultures supplied with nitrogen in the form of *dl*-aspartic acid. The organic nitrogen sources *dl*-asparagine, *l*-proline, *l*-leucine, and *l*-glutamic acid were about 50% as efficient in their growth-supporting capability as *dl*-aspartic acid.

The amino acid β -methylaspartic acid (MAA) has been implicated as a fungal inhibitory agent, and Lumsden *et al.* (1970) explored this property in some depth with *Aphanomyces euteiches*. In a basal medium containing nitrogen in the form of ammonium chloride, MAA prevented growth of this water mold. Inhibition could be counteracted if the nitrogen source was replaced by glutamic acid or aspartic acid, or by asparagine, valine, leucine, or proline. The mechanism by which MAA interrupts growth is not known, but it seems not to be through an influence on enzyme action, protein synthesis, or amino acid uptake (Lumsden *et al.*, 1970). The action of the amino acid evidently was fungistatic, because if the mycelium of *A. euteiches* was removed from media containing MAA, hyphal growth resumed.

The work of Papavizas and Ayers (1964) provides the best insight into the carbon requirements of *Aphanomyces euteiches*. Using two basal media differing in the sulfur source (*dl*-methionine and sodium thiosulfate), these investigators tested the growth-supporting capabilities of nine monosaccharides, five disaccharides, six polysaccharides, one trisaccharide, five pectic substances, 13 carbohydrate derivatives, and the TCA cycle intermediates pyruvate, acetate, citrate, and succinate. Table 31 is a qualitative summary of some of the quantitative results obtained by Papavizas and Ayers. They found that *d*-glucose was superior to all other carbon compounds tested, but appreciable mycelium of *A. euteiches* also developed in the basal

media supplied with maltose. Galactose was likewise a suitable carbohydrate for the fungus, but was about three times less efficient than glucose. Except for glycerol, no sugar alcohols, methyl esters of sugars or sugar acids were utilized by *A. euteiches*. Of the TCA cycle acids only pyruvate (as the sole carbon source) supported the fungus, and pectic substances (or their derivatives) were universally very poor suppliers of this element. Some sugars were unsuitable as sole carbon sources in the medium, but could be used if they were supplied in conjunction with hexoses. In terms of vegetative growth, therefore, it appears that *A. euteiches* has a very narrow spectrum of utilizable carbon sources. Glucose (*d*-) and fructose were clearly superior, Yang and Schoulties (1972) reported, to any other carbon source tested for the ability to support *A. euteiches*. Neither lactose nor mannose were proper suppliers of carbon for this species, but sucrose was used after prolonged incubation [Papavizas and Ayers (1964) had found that this sugar was not an adequate carbon compound for this water mold].

Temp (1966) demonstrated that glucose was superior to other sugars in insuring that mycelium of *Aphanomyces euteiches* would synthesize cellulolytic and pectinolytic enzymes. Data in his account show quite clearly that pectic substances (linear polymers of *d*-galacturonic units in α -1, 4 linkages) are not suitable carbon sources for the pea root rot fungus.

SULFUR NUTRITION: -- Failing to get growth of *Aphanomyces euteiches* in a medium containing magnesium sulfate, R. T. Sherwood (1958) suggested that the fungus could utilize only organic sulfur. Haglund (1960) and Haglund and King (1962) in essence confirmed Sherwood's report, finding that in some concentrations *l*-cystine, *l*-methionine, thiourea, and sodium thiosulfate were adequate suppliers of this element. The sodium salts of metabisulfite, dithionate, persulfate, bisulfite, and sulfate either did not support *A. euteiches*, or were toxic to it. The report by Haglund and King (1962) is particularly important because it demonstrates that some isolates of this species are quite uniform with respect to their sulfur requirements. Reduced sulfur evidently is mandatory for *A. euteiches* in a basal medium with micronutrients (Papavizas and Davey, 1960a). Davey and Papavizas (1962) found that there were measurable differences in growth among 12 isolates of *Aphanomyces euteiches* tested for their ability to utilize thiosulfate, *dl*-methionine, thioglycolic acid, and elemental sulfur. Furthermore, they determined that the oxidation state dictated which sulfur compounds could be used by the isolates. Elemental sulfur (oxidation state 0), and compounds with a valence of -2 would support growth of the fungus but those with oxidation states of +4 or +6 would not.

MINERAL NUTRITION: -- No systematic study of the mineral element requirements of *Aphanomyces euteiches* has been attempted. Concentrations of calcium chloride above 50 $\mu\text{g mL}^{-1}$, J. A. Lewis (1973b) noted, decreased growth of the fungus. Yang and Schoulties (1972), however, included calcium in their chemically defined medium, evidently concluding that it is an essential element. Magnesium, iron, and zinc also are necessary; in the absence of these elements in the medium, *A. euteiches* fails

to grow (Papavizas and Davey, 1960a). It is very likely that trace elements are in sufficient quantities as impurities in most synthetic media to provide for the needs of this watermold.

VITAMINS: -- There is no evidence in the literature to contradict the observations by Papavizas and Davey (1960a) and Winner (1966c) that *Aphanomyces euteiches* is autotrophic for vitamins. None of the following vitamins is known (Fowles, 1976) to stimulate mycelium production by this species when added to a synthetic, defined medium: biotin, folic acid, *p*-aminobenzoic acid, pyridoxamine·2 HCl, pyridoxin·2 HCl, nicotinic acid, calcium pantothenate, thiamin·HCl, and cyanocobalamin.

ANTIBIOTICS AND TOXIC COMPOUNDS: -- It is logical that plant pathogenic species of *Aphanomyces* should have been tested for their response to various inhibitor, suppressant, or toxic substances. Most of the work of this nature has been on the pea root rot fungus.

Antibiotics: -- Streptomycin and griseofulvin only slightly deter the growth of *Aphanomyces euteiches*, but at 1 ppm (1 mg L⁻¹) both actidione and oligomycin prohibit its growth and development (R. T. Sherwood, 1958). Filipin is ineffective at this same level, but does check the fungus at 10 ppm. Bacitracin and vancomycin in concentrations of 30-1000 ppm have no apparent growth retarding effect on the mycelium of the pea root rot fungus, Sundheim and Wiggen (1972) reported. They also found that neomycin was inhibitory at 1000 ppm. Schäufole and Beiss (1973) noticed that *A. euteiches* was retarded by pimarinin but in elevated concentrations of this compound, the fungus showed improved mycelial development. Streptomycin was hardly effective in reducing its growth even at a concentration of 400 ppm.

The response of *Aphanomyces euteiches* to streptomycin (Schäufole and Beiss, 1973) is of interest because sensitivity differences exist among strains. Vörös (1965) found that one strain of this species was actually stimulated by a 400 ppm concentration of this antibiotic, and the mycelium of this insensitive strain absorbed measurably less of the chemical than did its streptomycin-sensitive counterpart.

Isoflavonoid Phytoalexins: -- Phytoalexins have been defined (K. O. Müller, 1961) as antibiotics formed as the result of an interaction between the metabolic system of a host and that of an invading organism. These compounds then presumably prevent spread of the pathogen, but perhaps do not function solely in this regard (Lisker and Kuć, 1977). A somewhat broadened view of the function of phytoalexins prompted Kuć (1972) to regard these flavonoids as responses to an infectious agent, to its products, or even to an injury or physiological stimulus. In any case, two views of the function of phytoalexins (Van Etten and Pueppke, 1976) persist. These isoflavonoids may act as a mechanism of resistance by accumulating and operating at the site of disease induction, namely, at the interface between the hyphal surface and the invaded plant tissue. On

the other hand, perhaps it is as Pueppke (1978:118) has tentatively concluded that "... pisatin accumulation bears no relationship to the determination of compatibility in the *A. euteiches*-pea system." Independently, Hadwiger *et al.* (1977) have suggested that basic pea proteins rich in lysine and arginine may be potentially more important than pisatin in pea tissue resistance. This view, however, was based on evaluation that did not include *Aphanomyces*.

Pisatin (6a-hydroxy-3-methoxy-8, 9-methylenedioxypterocarpan) is one of five isoflavonoids produced by *Pisum sativum*, and is toxic to *Aphanomyces euteiches* (Van Etten, 1973). Pueppke and Van Etten (1974) assayed *in vitro* the biological activity of pisatin in modifying the growth (radial and dry weight measurements) of this water mold. They determined the ED₅₀ value to be at about 40 µg mL⁻¹, and growth of the fungus was stopped by a pisatin concentration of 100 µg mL⁻¹ (Pueppke and Van Etten, 1976). Although Pueppke and Van Etten (1976) postulated that *A. euteiches* degraded pisatin (*in vivo* at the hyphal surface) to noninhibitory metabolites, this change certainly seemed not to occur *in vitro*. The effect of the isoflavonoid *in vivo* is quite different (*see* Chapter 27, and Pueppke, 1975) than it is *in vitro*.

Both planar and aplanar isoflavonoids have antifungal properties. Van Etten (1976) bioassayed six pterocarpan and 11 structurally related isoflavonoids (6a, 1la-dehydropterocarpan, isoflavans, coumestans, and isoflavone). All compounds tested restricted radial growth of *Aphanomyces euteiches*, but (+)-tuberosin and (-)-phaseollinisoflavin were most effective, with 6a, 1la-dehydropisatin being the least consequential. Some flavonoids like glyceollin stringently moderated the radial growth of *A. euteiches*, but had little effect on spore viability; in the case of other compounds, the reverse was true.

A second phytoalexin of note in its effect on the mycelium of *Aphanomyces euteiches* is kievitone (2', 4', 5, 7-tetrahydroxy-8-isopentenylisoflavanone). This compound has been studied most extensively for its effect on the pea root rot organism by D. A. Smith (1976, 1978), and D. A. Smith, *et al.* (1975). Supplied to the growth medium of *A. euteiches*, kievitone had an ED₅₀ of 10 µg mL⁻¹, and the fungus ceased growing when exposed to 100 µg mL⁻¹. However, when the mycelium was washed free of the phytoalexin, hyphal development resumed. Kievitone derivatives (tri- and tetramethylated, cyclized, and hydrated forms) have also been tested (D. A. Smith, 1978) for their effectiveness in retarding *A. euteiches*. If the phenolic hydroxyls of kievitone are masked by methylation, the antifungal property of the isoflavanone is arrested.

Other Chemicals: -- Some herbicides, not unexpectedly, are highly toxic to the hyphae of *Aphanomyces euteiches*. Grau (1975, 1977) assayed dinitramine (N³, N³-diethyl-2, 4-dinitro-6-trifluoro-methyl-*m*-phenylene diamine) and trifluralin (α, α, α-trifluoro-2, 6-dinitro-N, N-dipropyl-*p*-toluidine) for possible antifungal activity against this species. Both compounds in concentrations from 0.25 to 1.0 µg mL⁻¹ retarded radial growth of the pea root pathogen.

Volatile compounds containing sulfur, and vapors from decomposing cabbage (*Brassica oleraceae* var. *capitata* L.) reduce or prohibit growth of *Aphanomyces euteiches* (J. A. Lewis and Papavizas, 1971a). It has been demonstrated by Lewis and Papavizas (1970,) that vapors from cruciferous residues decomposing in soil contain carbon disulfide, methanethiol, dimethyl sulfide, and dimethyl disulfide. These were shown to be extremely effective growth retardants as were fumes from methylisothiocyanate and allylisothiocyanate.

Chitosan is a deacetylated derivative of chitin. Allan and Hadwiger (1979) have shown that this derivative, but not the parent compound, is fungicidal to *Aphanomyces euteiches*.

Oxygen: -- Only R. T. Sherwood (1958) and R. T. Sherwood and Hagedorn (1961) have investigated at all extensively the part played by oxygen in the growth of *Aphanomyces euteiches*. Using modified van Tieghem cells Sherwood and Hagedorn passed various mixtures of oxygen and nitrogen over agar discs bearing surface mycelium of the fungus. Assaying the effects of the mixtures by linear growth measurements over a one-hour time span, they found that oxygen tensions of 0.01, 0.62, 5.0, or 20.0% had no perceptible effect on hyphal elongation. Other experiments indicated that the growth of *A. euteiches* was reduced at levels below 1.25% oxygen, and, of course, there was no growth when oxygen was absent. Sherwood's data suggested that this *Aphanomyces*, while aerobic, grows more favorably in a low oxygen tension (5%) than in a higher one (20%).

PHYSICAL PARAMETERS FOR GROWTH

The limits of temperature and PH within which a few isolates of *Aphanomyces euteiches* grow have been determined (Table 32). The available data do not suggest anything unique about these two physical conditions in stimulating or limiting the mycelial development of this fungus.

APHANOMYCES COCHLIOIDES

Beta vulgaris L. (among other Chenopodiaceae) is economically the most important host for *Aphanomyces cochlioides*, a species described in 1929 (mentioned, in abstract, in 1928) by Drechsler. In spite of the organism's importance as a phytopathogen, its growth physiology has been little explored.

CHEMICAL PARAMETERS

Like other species in the genus, *Aphanomyces cochlioides* grows well on a variety of natural product media (Herold, 1952). Only Herr (1973) and Fowles (1976), however, have contributed anything substantial to the knowledge of the carbon and nitrogen requirements of the beet blackroot organism.

Aphanomyces cochlioides uses glucose “preferentially” over sucrose, as does *A. raphani* (Ghafoor, 1964). Dry weight measurements of the mycelium of the former in synthetic media with carbon and nitrogen source substitutions yield three facts. First, it is the carbon level that in part affects nitrogen utilization. Herr (1973) found that *dl*-glutamic acid at low levels supported the highest dry weight yields. If increased concentrations of carbon were incorporated into the medium, *l*-asparagine was a suitable nitrogen source for *A. cochlioides*. Second, growth of the sugar beet pathogen rises in culture in response to a gain in exogenous nitrogen and a reduction in carbon. Trace elements, however, play a significant role in the response of this water mold to the carbon:nitrogen ratio. In the presence of trace chemicals plus methionine, isolates of *A. cochlioides* achieve increased growth as the available carbon level is augmented. If carbon is increased in the medium without trace elements, dry weight measurements reflect a corresponding drop in growth (Herr, 1973). Third, *A. cochlioides* displays very little ability to use carbon supplied by amino acids, a condition in sharp contrast to some of the saprotrophic water molds. For example, *A. cochlioides* cannot grow in mineral element media with *d*-asparagine alone or in combination with *dl*-asparagine (Herr, 1973). The experiments reported by Fowles (1976) do not convey quite so strongly as Herr’s (1973) data the limited ability of the sugar beet pathogen to use amino acids. Fowles reported that the following amino acids promoted growth of *A. cochlioides*: *dl*-glutamic acid, *dl*-aspartic acid, *l*-glutamine, *l*-asparagine, *l*-arginine, threonine, and serine. Methionine actually inhibited growth of the mycelium of the pathogen.

Herr (1973) incorporated *dl*-methionine as the sulfur source in various chemically defined media prepared for propagating *Aphanomyces cochlioides*. Winner’s (1966c) experiments demonstrated that both the *d*- and *l*-forms of methionine supported growth of this fungus, hence were suitable sources for sulfur. Neither sodium sulfate nor sodium thioglycollate provide sulfur for *A. cochlioides*, although Fowles (1976) found to the contrary for the thioglycollate. The latter investigator also noted that both *l*-cysteine and *l*-cystine satisfied the requirement for this element by the blackroot fungus. In common with other saprolegniaceous species, *A. cochlioides* can use a reduced sulfur source.

In his study of the influence of trace elements on nutrition in *Aphanomyces cochlioides*, Herr (1973) noted that growth (dry weight) of isolates increased if iron was incorporated into the medium, but decreased when copper ions were present. If the two ions were used together in a medium, however, the toxicity of copper was compromised. By itself, iron stimulated mycelium of the fungus in the presence of *dl*-glutamic acid and *l*-asparagine.

Like *Aphanomyces euteiches*, *A. cochlioides* also is sensitive to some antibiotics, and particularly so to pimarin (Schäufele and Beiss, 1973). Penicillin (but not streptomycin) has an inhibitory effect on the causal organism of blackroot in sugar beets. Incidental to determining something of the efficacy of antibiotics as culture adjuvants for the isolation of *Phytophthora* species, Eckert and Tsao (1962) uncovered certain reactions between antibiotics and the mycelium of *A. cochlioides* and *A. euteiches*.

These two species responded similarly. Their growth was not retarded on a medium containing penicillin or polymixin alone or in combination. Nystatin suppressed the mycelium, and pimaricin by itself or when in mixtures with other antibiotics prevented growth.

Lyda (1958) showed that there was a possible *in vitro* antagonistic effect on *Aphanomyces cochlioides* by fungi from such genera as *Alternaria*, *Chaetomium*, *Pythium*, *Stachybotrys*, and *Trichoderma*. He grew the test species in cultures in which a dialysis membrane separated the isolates from the agar. Blocks of medium subsequently removed from below the membranes contained in each instance diffusible substances that retarded the growth of *Aphanomyces*.

The suppressive effect of fungicides on blackroot of sugar beets is of course well known (Chapter 28). Niethammer (1931), however, experimented with toxic chemicals to determine their effect on the growth of *Aphanomyces cochlioides* in culture. One isolate of this species would grow in the presence of some fungicides, but obviously had been inhibited initially to some extent if its growth was compared to that of control cultures.

PHYSICAL PARAMETERS

Very little is known of the effects of physical conditions on the *in vitro* growth of *Aphanomyces cochlioides*. Experimentally, W. E. McKeen (1949) determined that isolates grew optimally at 30° and 32 °C, with no growth above 37 °C. Fowles' (1976) data are corroborative. These two independent observations suggest that *A. cochlioides* differs somewhat from most other water molds in having a high (and narrow) temperature optimum. Fowles (1976) has provided some information on the tolerance of this species to pH. The fungus grew at pH 4.0 but not at 8.0; the reaction of the medium giving optimum mycelial yield was pH 7.0. Herr (1971) demonstrated that colonies of the sugar beet root pathogen sporulated more efficiently in cultures that were rolled (to agitate the medium) than did mycelium grown in stationary vessels. Aeration of the fungus through shaking the culture containers resulted in reduced sporulation.

APHANOMYCES RAPHANI

Blackroot of radish (*Raphanus sativa* L.), and some other cruciferous hosts (Humaydan and Williams, 1975) is caused by *Aphanomyces raphani* (Kendrick, 1927). The bulk of what is known of the nutrition of this species has been provided by Ghafoor (1964), but Herold (1952), Humaydan and Williams (1978), and Ogoshi, Yokosawa, and Sakai, (1972) also have contributed.

Aphanomyces raphani grows in a wide variety of natural product media (Humaydan and Williams, 1978; Ogoshi, Yokosawa, and Sakai, 1972), including those prepared with a steep from radishes (Ghafoor, 1964). Such chemically undefined media (including peptone-containing agars: Herold, 1962) yield no knowledge about the specific nutritional requirements of the fungus. Ghafoor (1964), however, has provided some glimmerings of information.

Glucose and galactose were superior carbon sources for growth of *Aphanomyces raphani*, Ghafoor (1964) reported, and isolates of this species could not utilize fructose, lactose, or arabinose. Mycelium development was very poor when soluble starch was the sole supplier of carbon. Neither ammonium nor nitrate nitrogen could be metabolized by *A. raphani*, but four amino acids provided all the necessary nitrogen for the fungus: asparagine, histidine, tyrosine, and glutamic acid. High yields of mycelium resulted when sulfur in the culture medium was provided by cystine, cysteine, *dl*-methionine, or glutathione, but not when sulfates or sulfides were used. Although Ghafoor (1964) found that trace metals -- iron, zinc, copper, boron, manganese, and molybdenum -- were essential for the growth of *A. raphani* (*in vitro*), isolates were autotrophic for vitamins.

Little is known of the sensitivity of the radish blackroot fungus to exogenous chemicals. The mycelium of an isolate used experimentally by Humaydan and Williams (1978) was inhibited by boron, seemingly in contradiction to Ghafoor's (1964) report on the essential nature of this element. Streptomycin sulfate at 50 and 100 ppm evidently does not impede growth of *Aphanomyces raphani* -- at least it may be said that Ogoshi, Yokosawa, and Sakai (1972) recommended the use of this antibiotic at these levels in attempts at isolating the causal agent.

There is reasonable consistency among the various reports of the growth of *Aphanomyces raphani* with respect to temperature (Table 32). The single extant record of its tolerance to *in vitro* pH levels (Ghafoor, 1964; Table 32) is not unduly different from levels reported for other water molds.

APHANOMYCES SP.

In 1967 Fowles described *Aphanomyces aculeatus*, a water mold isolated from lesions on a dolphin. In publication (Fowles, 1976) this name was dropped since, as it turned out, the organism did not reproduce sexually, as originally thought, and therefore could not be properly identified. The only accounts of the physiology of the growth of this *Aphanomyces* sp. are those by Fowles (1967, 1976), although Unestam and his associates used Fowles' isolates briefly in some comparative biochemical studies with *A. astaci*.

It has been demonstrated by Fowles that the addition of 2.7 g L⁻¹ of glucose to a growth medium resulted in elevated mycelial production (dry weight) by *Aphanomyces* sp., but beyond this level, the hexose had no additive effect. Glutamate was a source of nitrogen superior to ammonium chloride at levels above 21.2 mg nitrogen L⁻¹, but below this concentration, the reverse was true. The following were adequate substitutes for glutamate: *dl*-glutamic and *dl*-aspartic acids, *l*-glutamine, *l*-asparagine, *l*-arginine, leucine, and cysteine. Threonine and serine were slightly inhibitory to *Aphanomyces* sp., and both ethionine and methionine noticeably retarded growth.

However, Fowles (1976) reported that the *l*-form of methionine was adequate as a sulfur source provided its concentration in the medium was less than the amount used when it was incorporated as the only supplier of nitrogen. Other organic sulfur

compounds were suitable for the *Aphanomyces* but sodium sulfate in the basal medium did not support growth. Vitamins added to the growth medium were neither suppressive nor stimulatory; this, of course, is quite in accord with the general physiology of the Saprolegniaceae.

Aphanomyces sp. has a substantial tolerance to high temperatures: Fowles (1976) reported that it grew, although feebly, at 40 °C. The mycelium developed optimally at 25 °C, with the lower limit for growth being at 10 °C. The fungus tolerated (*in vitro*) a pH of 8.0, but not 4.0; optimum yields of mycelium were obtained when the medium was at neutrality (other factors being supportive of growth).

APHANOMYCES ASTACI

The cause of crayfish plague (Nybelin, 1931, 1934; Unestam, 1968b), a serious disease of *Astacus astacus* L. in European waters, is an *Aphanomyces* that has defied all culture manipulations to induce it to reproduce sexually (Rennerfelt's view published in 1936, notwithstanding). Certainly all of the evidence assembled from studies on its pathology leaves little doubt that the fungus is physiologically adapted to its usual environment, the tissues of crayfish.

Until Nybelin (1934) successfully cultured *Aphanomyces astaci* from infected animals, the cause of the disease was often attributed to bacterial invasion (*see* Chapter 30). He propagated the fungus on a simple 3% nutrient agar, and stimulated its growth by incorporating crayfish blood into the medium. It is largely from the work of Unestam (1965a), however, that details of the physiology of the growth of *A. astaci* can be derived.

In general, *Aphanomyces astaci* is somewhat more fastidious than its phytopathogenic or saprotrophic kin. It does not grow on media containing yeast extract, casein hydrolysate, or tryptone (Unestam, 1965a). Rennerfelt (1936) who published the first experimental observations on nutrition of the crayfish plague fungus found that he could not rear it on semisolid substrates containing malt, peptone, or fruit extracts.

Unestam (1965a) experimented with two isolates of *Aphanomyces astaci* on a synthetic medium containing *l*-glutamic acid, *dl*-alanine, and ammonium chloride as suppliers of nitrogen. Of 25 carbon sources single substituted in the basic medium, glucose was adequate for the isolates if ammonium nitrogen was present. The only other sugar that supported growth of both isolates -- irrespective of the nature of the nitrogenous supply -- was maltose. Cellobiose was used by one isolate of *A. astaci* if *dl*-alanine was present, and by the other if nitrogen was supplied as the ammonium ion. Starch was a suitable substrate for one isolate, but only in the presence of NH₄Cl; α -ketoglutaric acid and *l*-glutamic acid also supported growth of one of the isolates. It has been shown by Söderhäll and his associates (1978) that the germ hyphae from spores of *A. astaci* do not have detectable chitinase activity until the hyphae branch.

Aphanomyces astaci in common with other Saprolegniaceae cannot utilize nitrate nitrogen. Unestam determined that nitrogen supplied by amino acids with low amide

concentrations (for example, *l*-asparagine, *l*-glutamine, *dl*-alanine, *dl*-proline, and *l*-glutamic and *l*-aspartic acid) encouraged growth of this species of *Aphanomyces*. At concentrations of 20-40 mg L⁻¹, however, amino acids such as *dl*-methionine and *l*-valine were toxic (but supported growth at nontoxic levels). Even at 3 mg L⁻¹ *dl*-norleucine was toxic to *A. astaci*. When used together in the growth medium, however, *l*-arginine and *l*-histidine counteracted the restraining effect of *dl*-methionine. Among the sulfur sources tested by Unestam (1965a) *l*-cysteine was superior to other organic forms, but *A. astaci* readily could use either elemental or reduced organic sulfur. No oxidized sulfur compounds, on the contrary, permitted colonies of the watermold to develop *in vitro*. Calcium, magnesium, potassium, and iron are essential for the growth of the crayfish plague fungus (Unestam, 1965a).

The only physical factors that have been tested for their influence on vegetative growth of *Aphanomyces astaci* are temperature and pH. Two sources of information are extant. Unestam (1965a) reported that isolates of *A. astaci* developed in culture within the pH range of 4.8-7.4, and Rennerfelt (1936) stated that his isolate grew, although sparingly, at pH 10.2 and above. The optimum for growth is pH 6.0 -7.0 according to Unestam. Both investigators noted that the crayfish plague fungus died at 30 °C, but found 20-25 °C to be the optimum range for its development.

SAPROLEGNIOSIS

Several species of watermolds have been at times implicated in killing fish and their eggs. The taxonomy of the causal agents, particularly in the early years of study on freshwater fish diseases, is far from clear in some instances, and remote from accuracy in others. Our attempt to sort out the systematics of the watermolds associated with fish is properly left for the taxonomic section. In the paragraphs to follow, the species names applied by the authors of the papers cited are retained.

NUTRITION

Historically, it is Robin's (1853) early account that supplies the first glimpse of the physiology of a watermold growing on fish. He was of course caught up in the popularity of the humor theory of disease (and in watermolds as algae, incidentally), hence theorized that liquid or semi liquid "humors" secreted to the animal's surface provided a suitable nutrient condition for the development of the parasitic growth (*Saprolegnia ferax*). To explain the ability of *S. ferax* to develop in water, Robin pointed to the fact that putrefaction of nitrogenous substances occurred in this "milieu," and so, on "altered places" (that is in wounds) on animals, the organism could "vegetate."

Although Kanouse (1932) first induced *Saprolegnia parasitica* (= *diclina*) to reproduce sexually in culture -- by manipulations of the nutrients -- the information she provided on sources of carbon and nitrogen suitable for its growth is of little value. She incorporated singly into media a variety of appropriate compounds, but the basic medium was a chemically undefined broth. The use of such a nutrient base would in

part explain her report that glucose in the broth alone was ineffective in supporting growth of *S. parasitica*, but when used in the presence of peptone and leucine (isomer?) it was a most satisfactory carbohydrate. The study by Hoshina and Ookubo (1956) on *S. parasitica* isolated from diseased eels (species?) only demonstrated that natural product media were the most satisfactory ones on which to encourage the fungus to grow (agar medium containing eel flesh, for example).

Powell *et al.* (1972) have provided most of the substantive information on the physiology of growth of *Saprolegnia parasitica*. They measured the mycelium production by isolates in a standard mineral salts medium with glucose, *dl*-methionine, and *l*-sodium glutamate as organic substrates, and compared the resulting growth with that attained by the same isolates in the same medium in which single substitutions and deletions of compounds were made.

Glucose, glycogen, and dextrin were suitable carbon sources for *Saprolegnia parasitica*, according to Powell and his associates (1972), as were cellobiose, fructose, glycerin, sodium lactate, and starch when glucose was deleted. In the basal medium without glucose, but containing (singly substituted) one of the following sugars *S. parasitica* did not grow: arabinose, galactose, lactose, mannose, sucrose, rhamnose, dulcitol, inulin, mannitol, sorbitol, and xylose. Powell and his associates also replaced sodium glutamate in the basal medium with other nitrogen sources. Casein hydrolysate and gelatin supported higher dry weight yields of mycelium of *S. parasitica* than did the basal medium alone. There were modest levels of growth of the isolates when cysteine, ammonium chloride, leucine, serine, or urea were supplied but poor development if the glutamate fraction had been replaced by glycine, isoleucine, lysine, methionine, phenylalanine, threonine, valine, or tryptophan. Potassium nitrate and sodium nitrate, similarly, were inadequate sources of nitrogen for *S. parasitica*, but four amino nitrogen sources were suitable, namely, alanine, arginine, histidine, and aspartic acid. Cystine and cysteine, when substituted for the *dl*-methionine, proved to be the most favorable sulfur sources for *S. parasitica*, but isolates of this species could only utilize reduced sulfur compounds.

Chong's (1973) study of nutrition in *Saprolegnia diclina* isolated from *Oncorhynchus kisutch* (Walbaum) gave data not far removed from that reported by Powell *et al.* (1972). The amino acids *l*-asparagine, histidine, and *dl*-aspartic acid were propitious suppliers of nitrogen, corroborating results obtained by Powell and his associates with *S. parasitica*. However, Chong (1973) reported rapid growth of *S. diclina* on a synthetic medium with valine; this was a poor source of nitrogen for *S. parasitica*. Chong (1973) also showed that endogenous phosphate influenced considerably the *in vitro* development of *S. diclina*. When the magnitude of growth was determined as dry weight of mycelium produced, high molarities of phosphate incorporated into the medium obviously had stimulated mycelial production. Radial growth measurements, on the contrary, did not reflect any differences in the molarity of phosphate used.

OTHER CHEMICAL FACTORS

There are but four chief accounts that mention the effect of oxygen on growth of water molds isolated from fish. In two instances (Duff, 1929; Rucker, 1944), the fungi were put into vessels of water that had been boiled then capped to exclude air. The results of this crude experimentation indicated that the isolates were strict aerobes. Observations by W. N. Tiffney (1936) suggest that *Saprolegnia parasitica* is quite tolerant of low oxygen tensions. During their study of saprolegniosis of eels, Hoshina *et al.* (1960) were unable to grow *S. parasitica* in an oxygen-free atmosphere.

Duff (1929) reported that *Saprolegnia* sp. grew optimally in medium containing 5% seawater, with an upper limit of tolerance at 20%. He found that a non-sexual *Achlya*, also isolated from fish, was much less tolerant of a saline environment: this fungus grew optimally at 0.5% salinity, but was inhibited if the salinity exceeded 8%. The data published by Stuart and Fuller (1968a) show quite clearly that growth of *S. parasitica* at 3.5% sodium chloride was about half that of the controls (Dick, 1968b, commented on these data, and Stuart and Fuller, 1968b, responded). According to Hoshina and Ookubo (1956) this same species of *Saprolegnia* would not produce hyphae in a medium where the concentration of NaCl was 3% or more.

The most informative data on salinity tolerance by *Saprolegnia parasitica* come from the work of J. L. Harrison and Jones (1971). Isolates of this species retained viability in water culture in a salinity of 24.5%, or in media with a seawater concentration of 70%. At incubation temperatures above 20 °C, the fungus actually grew in 70% seawater, and at all temperatures from 10-30 °C, it also produced mycelium in media containing 60% seawater. *Saprolegnia parasitica* tolerated higher concentrations of seawater when propagated on a glucose-yeast extract medium than when grown on hempseed. Harrison and Jones suggested that nutrition influenced salinity tolerance -- which, to be sure, it does -- but this may not be a permissible conclusion if data are obtained from growth measurements made on isolates propagated in two different physical environments, that is, on agar on the one hand and in water on the other.

There is of course ample literature dealing with chemical agents for the control or eradication of saprolegniaceous fungi *in situ* on invaded animals (Chapters 29, 30), but far less information on the effect of such compounds on growth of these organisms *in vitro*. Metallic copper and zinc (but not tin) inhibited *Saprolegnia parasitica*, Rucker (1944) reported, but the sulfate salt of copper in a concentration of 1×10^6 actually stimulated its growth. Hoshina and Ookubo (1956) noticed that both malachite green and potassium permanganate were highly toxic to the mycelium of *S. parasitica* in culture. Even in a concentration as low as 0.0001% malachite green prevented growth of a *Saprolegnia* sp. isolated from *Salmo salar* by Hodkinson and Hunter (1970b). *Saprolegnia diclina* does not grow in a concentration of this compound of 0.2 mg mL⁻¹ or above (Hatai and Egusa, 1977).

Potassium iodide, on the contrary, evidently is not very effective in obstructing the growth of water molds from fish, nor are zinc chloride and methylene blue, among other compounds. The latter chemical, however, is said (Hatai and Egusa, 1977) to interrupt mycelial development of *S. diclina* -- isolated from *Oncorhynchus rhodurus* -- at

a concentration of 100 $\mu\text{g mL}^{-1}$. Ross and Smith (1972) immersed colonies of *S. parasitica* in each of two iodine-containing proprietary disinfectants, but found that neither chemical solution was effective (up to 300 seconds submersion of the mycelium) in the concentration selected for testing. Both chromium chloride and sodium chromate in concentrations of 0.1-3.0 ppm and 0.1-0.3 ppm, respectively, stimulated the growth of an unidentified watermold isolated by Draggan (1977) from eggs of carp. Above these concentrations, these chromium compounds were toxic, as was chromium acetate at 20-30 ppm. The latter had a retarding effect on vegetative growth at 3-15 ppm.

PHYSICAL PARAMETERS

The known temperature and pH ranges at which the principal species of Saprolegniaceae associated with diseased fish or their eggs will grow are given in Table 33. No startling implications emerge from the data on these alleged parasitic species save, perhaps, that they have a commonality in their tolerance to low incubation temperatures. Olah and Farkas (1978), however, have evidence that *Achlyas* isolated from fish rearing areas have less of a tolerance (in terms of mycelial growth) to low water temperatures than do species of *Saprolegnia* from the same habitats.

There appears to be but one report on the effect of illumination on the growth of a watermold from fish. Duff (1929) found that light neither hindered nor augmented vegetative development in *Saprolegnia* sp. and *Achlya* sp. isolated from infested eggs of carp.