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This study examined the effects of pH, temperature, washing, and post-hydration drying on germination of Anisogramma anomala ascopores. Spores germinate most readily in the temperature range of 5 to 15 degrees C, with an optimal temperature of 10 degrees (1 sd=0.05). Growth was best in the pH range of 5 to 6. Washed ascopores germinate at significantly higher rates than unwashed spores (P=.05, .01, and .06 for three experiments). Germination rate declined sharply with increasing post-hydration drying, with 5 minutes of drying resulting in a 45% reduction in germination. After one hour of drying, spore germination was reduced by 98.5%. Those environmental factors which were optimal for germination in vitro reflected prevailing field conditions during the natural host infection period.

The growth of A. anomala on artificial meduium is described. The germination process includes the following series of events: (1) spore hydration and swelling, (2) emergence of the germ tube, (3) vesicle formation, (4)

production of hyphal initials, and (5) hyphal growth. Attempts to maintain cultures of A. anomala for longer than several months were unsuccessful.

STUDIES ON THE GERMINATION AND GROWTH OF *ANISOGRAMMA ANOMALA* Peck (Muller) IN VITRO

by Richard Lightfoot

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STUDIES ON THE GERMINATION AND GROWTH OF *ANISOGRAMMA ANOMALA* (Peck) Muller IN VITRO

GENERAL INTRODUCTION AND LITERATURE REVIEW

The ascomycete *Anisogramma anomala* (Peck) E. Muller causes a canker disease on European filbert (*Corylus avellana*), an orchard crop of increasing importance in the Pacific Northwest. Endemic to the northeast-central part of North America as a minor pathogen of native hazelnut (*Corylus americana*), this fungus is much more virulent on commercial varieties of filbert introduced from Europe. It has been suggested that *Anisogramma* canker was the main cause of failure to establish a filbert industry in the northeastern U.S. (Barss, 1930).

Advancing European settlement brought filbert culture to the west, and the Willamette Valley of Oregon now produces ninety-five percent of the domestic filbert crop. The importance of protecting the industry from the threat of *Anisogramma* canker was acknowledged in 1923 when a quarantine was established prohibiting importation into the Pacific Northwest of filbert stock from east of the Rocky Mountains. The Rockies act as a natural barrier to the spread of the fungus, as the wild hazelnut host is restricted to the areas east of the continental divide.

The quarantine worked well until 1973, when *A. anomala* was reported parasitising filberts in southwestern Washington (Davidson and Davidson, 1973). Subsequent

surveys found the disease to be very well established, and at the present time an estimated forty filbert orchards are infected, the majority of them in southwest Washington. Even if all infected orchard trees were to be destroyed, substantial reservoirs of A. anomala would still be present in the population of filbert trees scattered in woodlands throughout the infected area. Small animals and birds are efficient disseminators of cultivated hazelnuts, and a large population of wild C. avellana exists in Washington and Oregon.

As the gravity of the situation became apparent, efforts were made to study the disease (now termed eastern filbert blight to distinquish it from filbert bacterial blight caused by Xanthomonas coryli) and to develop methods to prevent its spread. These activities were hampered by difficulty in growing the fungus in vitro. While the epidemiology of eastern filbert blight and the development of the fungus in vitro have been studied (Gottwald and Cameron 1980a, Gottwald and Cameron 1980b, Gottwald and Cameron 1979), previous attempts to culture the fungus on artificial medium have failed.

Axenic culture of A. anomala is desirable for a number of reasons: (1) Screening for compounds toxic to the fungus would be greatly simplified, (2) Environmental factors most conducive to fungal growth could be delineated, (3) Nutritional requirements and other basic physiological factors could be manipulated with greater ease, and (4) Ability to grow the fungus and inculate filberts would allow

resistance screening to be carried out more quickly and easily. One of the aims of the research reported here was to develop an artificial growth medium for A. anomala.

The effects of hydration, pH, washing, and temperature upon the germination and growth of A. anomala ascospores are reported here in Chapter 1. Events following germination and leading to establishment of ascospore-derived colonies of A. anomala in agar medium were also examined and are reported in Chapter 2.

Peck originally described A. anomala in 1876 (Peck 1876) from specimens on Corylus americana. Saccardo recorded its pathogenicity to C. avellana in 1882 (Saccardo, 1882). More recently the fungal life cycle and development in the host was described (Gottwald and Cameron 1979). A. anomala colonizes host vascular tissue, primarily phloem parenchyma, and invades xylem after hyphae begin to penetrate the cambium. Upon removal of the outer bark of the infected wood, diseased tissues may be recognized by brown discoloration of cambium and adjacent cell layers. In perennial infections (cankers) there are sunken areas on the bark in which perithecia are embedded in a mass of fungal hyphae (stroma). Individual cankers may elongate at rates of up to 30 cm per year.

As the host cambium becomes more active in the late spring and callus develops around the infected area, the rapid fungal colonization of tissue declines. However, hyphae extending into the xylem may act as primary colonizers of new tissues the following spring, allowing

perennial infections to perpetuate until the top of the host tree is killed. Cankers have not been observed to spread into the filbert root system; thus the roots of infected trees with dead tops may continue to produce suckers which may later become infected.

Invading hyphae are appressed tightly to host cell walls, and apparently spread by enzymatic degradation of cell walls in contact with the fungal cell. Once a host cell is pierced, it is filled with fungal branch hyphae. The hyphae then invade adjacent cells. No evidence of cell necrosis in advance of the infection has been observed, though later in the growing season tannins are sometimes produced in advance of infection as cortical parenchyma is exposed to the fungus.

Throughout the summer and early autumn the fungal mass beneath the bark expands, using colonized vascular tissues as its nutrient base. At this time linear expansion of the infected area is nearly over, and growth of the fungus is primarily in the dimension perpendicular to the long axis of the infected stem. A pad of expanding fungal tissue (stroma) develops, eventually breaking through the cambial and bark layers to become exposed. As the stroma expands, perithecia bearing asci and ascospores develop embedded within it. Within the same perithecium, some ascospores may mature as early as August and others as late as November. Asci develop in waves, with mature asci delinquescing at the base and floating free as a sticky mass in the centrum while new asci develop from ascigerous hyphae at the perimeter of

the locule.

As the jelly-like mass within a perithecium imbibes water and swells, the perithecial contents become slightly pressurized. Ascospores are liberated when the masses of loose spores, asci, and cell contents are ejected through the ostiole during periods of wet weather. Ascospores are disseminated over short distances primarily through rain splash and over greater distances by wind-blown rain.

From 5 to 100 stroma may be formed within the margins of a single seasons growth, depending on the length and girth of the canker. Each stromatic pustule may contain from 20-60 perithecia. Perithecia retaining their contents may be collected from August until May of the following year.

Ascospores are the only known propagules of A. anomala. An anamorphic state was described for the only other species in the genus Anisogramma, A. virgultorum, a pathogen of birch trees in England and northern Europe (Massee 1914). This report is an isolated one, however, as no mention is made of a conidial state of A. virgultorum in numerous other references (Dennis 1968, Rabenhorst 1887, Fuckel 1869).

Ascospores enter the host through two avenues: (1) wounds made by the physical forces of wind, abrasion, orchard equipment, pruning, and ice breakage, and (2) buds colonized by the eriophyid mite Phytocoptella avellana. These distorted mite-galled buds are composed of masses of disorganized tissue engendered by the feeding of the mites. These "big buds" grow to be many times larger than

uninfected buds. The distorted bud scales fail to enclose the bud, thereby allowing water-borne ascospores of A. anomala to be washed into the interior of the afflicted bud. By either means of entry, the fungus gains access to exposed vascular elements. Disorganized vascular elements are present in the spongy tissue composing the big bud (Larew 1979). It is thought that mite-galled buds act as the primary infection court. Gottwald (1980) attained high infection rates in container-grown filberts by injecting ascospore suspensions with a syringe into both galled and healthy buds. Cameron (unpublished) compared the number of cankers associated with healthy versus mite infested buds; 36 cankers resulted from 100 mite infested buds while only one resulted from 100 healthy buds. The critical factor regarding initial penetration of the fungus into the host appears to be a wound allowing access to host vasculature, rather than any particular physiological state which may be present in mite-galled versus wounded buds free of mite-induced damage. It is not known whether wounding by any of the factors mentioned is absolutely required for infection, because it is difficult to place ascospores nondestructively within the very tightly closed scales characteristic of dormant Corylus avellana buds.

CHAPTER 1

STUDIES ON ASCOSPORE GERMINATION OF *ANISOGRAMMA ANOMALA*

ABSTRACT

This study examined the effects of pH, temperature, washing, and post-hydration drying on germination of *Anisogramma anomala* ascospores. Spores germinated at similar rates on media of pH 4, 5, and 6. pH effects were most pronounced during the post-germination growth stages, with best growth occurring in the pH range of 5 to 6. Germination rates were highest at 10 degrees C <1 s d =0.05), though spores germinated readily from 5 to 15 degrees C. Washed ascospores germinated at significantly higher rates than unwashed ascospores (P=0.05, 0.01, and 0.06 for three experiments). Germination rate was inversely related to length of the post-hydration drying period. 5 minutes of drying resulted in a 45% reduction in germination, and a one hour drying period decreased germination by 98.5%. Those environmental factors which were best for germination in vitro reflected field conditions prevailing during the natural host infection period.

STUDIES ON ASCOSPORE GERMINATION OF *ANISOGRAMMA ANOMALA*

INTRODUCTION

Anisogramma anomala Peck (E. Muller) causes a canker disease on *Corylus avellana*, the European filbert. *A. anomala* is endemic to northeast-central North America as a pathogen of the native hazelnut (*Corylus americana*), but it is much more virulent on the european filbert varieties used in filbert production in the Pacific Northwest. The epidemiology of Eastern Filbert Blight and the life cycle of the causal organism have been described by Gottwald and Cameron (1980a, 1980b).

There is presently no effective control measure for this disease other than the removal of infected wood or of entire infected trees. Development of chemical control methods as well as resistance breeding program would be aided by the ability to grow the fungus in vitro.

This study examined some of the factors which influence the germination of *A. anomala* ascospores in vitro as a fist step in the development of a cultivation method for this fungus. The effects of medium pH, temperature, ascospore washing, and post-hydration drying were investigated.

MATERIALS AND METHODS

Preparation of ascospore suspensions

Unless otherwise noted, ascospore suspensions used in all experiments were prepared in the following manner. Stems of C. avellana bearing A. anomala perithecia were collected from an infected orchard near the town of La Center in Clark County, Washington. After airing to rid the stems of surface moisture, they were stored at -20 degrees C until needed. Freezing the stems eradicates some fungal contaminants in the stroma and preserves A. anomala ascospores from degradation by bacteria and other fungi.

Following thawing at room temperature, stroma-bearing stem tissue was surface sterilized in 0.5% NaOCl for 2 minutes and rinsed in distilled water. The upper portion of the stroma containing perithecial necks was excised with a sterile scalpel. Ascospores were collected by plunging the tip of a sterile needle into the exposed spore mass within each perithecium. The spore slime was then suspended in sterile distilled water and spores were allowed to be liberated from asci for approximately one hour. Occasional agitation of the suspension was performed by repeated suction and expulsion of the liquid through a Pasteur pipette.

In some experiments, ascospores were washed by several rounds of centrifugation (10 minutes at 2000 rpm in a Sorvall SS-34 rotor) and resuspended in sterile distilled

water or dilute nutrient solution.

Application of spore suspension to agar medium

Drops of ascospore suspensions were applied to the surface of solid medium with a Pasteur pipette; approximately 0.03 ml per plate was applied in all experiments. The density of ascospores in the suspension was determined by with a Levy haematocytometer. Density was adjusted to 4-6 x 10(6) spores/ml prior to inoculation by dilution with sterile distilled water.

Media

The culture medium employed in most of the investigations reported here was modification of Barnett's Oak Wilt Agar (Barnett 1953). OWA was originally developed as a sporulation medium for Ceratocystis fagacearum, a fungus which colonizes tissues similar to those attacked by A. anomala (Struckmeyer et al., 1958). Medium constituents are listed in Table 1. Rifamycin (Rifampicin, Sigma Chem. Co.) was incorporated into the medium to prevent growth of bacterial contaminants. Rifamycin was first dissolved in 10 ml 50% ethanol per liter of medium and then added to autoclaved medium cooled to approximately 50 degrees C. The final concentration of rifamycin in the culture medium was 10ppm (10mg/l) in all experiments.

Evaluation of germination

Ascospore germination was assessed by observation of inoculated media with a Zeiss microscope equipped with an ocular mounted grid. Percent germination was determined by counting the number of germinated and ungerminated ascospores lying within the boundaries of the grid. In most cases several groups of 100 ascospores per grid field were counted. If the grid field did not cover a sufficient number of ascospores, those spores immediately adjacent to the grid boundaries were included to bring the total number up to 100.

Fields of view in the microscope were selected at random by bringing them into position while the microscope was in a relatively unfocussed state. This procedure avoided selection of local areas on the agar surface with unusually high or low germination rates. If the randomly selected field contained contaminating material, the field was not counted and another was selected.

Effect of post-hydration drying

Spore suspensions prepared as described were deposited upon sterile glass microscope slides and allowed to dry in the draft of a laminar-flow transfer hood at room temperature. This process was judged to be completed when the suspension lost the reflective qualities of aqueous solutions and when scratching the spore-laden area with a

steel needle resulted in the removal of the fried spore layer only in the region where the disturbance occurred. These evaluations were carried out while viewing the dried magnifcation of 25X. Approximately fifteen minutes was required for dehydration of spore suspensions. The 0 minute treatment slide was not allowed to dry and was slides of 1, 5, 15, and 30 minutes and of 1, 2, 4, 8, 24, and 48 hours of drying time were stored at room temperature (1, 5, and 15 minutes) or 7 degrees C (30 or more minutes of drying).

After the allotted drying period had elapsed, ascospores were resuspended in distilled water, adjusted to a density of 4x10(6) spores/ml, and deposited onto the surface of OWA2YE medium. Inoculated plates were incubated at 15+-1 degrees C.

Effects of washing

Ascospores were collected as described and suspended in one-half strength OW2YE liquid medium. A portion of this suspension was washed in 2 volumes of the dilute medium and then centrifuged for 10 minutes at 2000 rpm in a Sorvall SS-34 rotor. Pelleted ascospores were then resuspended in medium and the process repeated. Final resuspension was done using the dilute nutrient medium. Both washed and unwashed spore suspensions were adjusted to a density of 5x10(6) spores/ml and plated out on OWA2YE solid medium. Spores were assessed for germination from 6 to 11 days after plating.

Effects of temperature

Ascospores collected as described were suspended in half strength OW2YE liquid medium. Spore density was adjusted to 5x10(6) spores/ml. Agar medium was inoculated with drops of spore suspension; inoculated plates were incubated in the dark at 5+-1, 10+-1, 15+-1, and 20+-2 degrees C.

Effect of medium pH

Unbuffered OWA2YE medium was adjusted after autoclaving to pH 3 through 8 with 1N HCl and 1N NaOH. Drops of ascospores suspension with a spore density of 5x10(6) spores/ml were applied to the agar surface.

Citrate-phosphate buffered OWA2YE medium was prepared in 0.15M citrate-phosphate buffer (McIlvaine 1921) as follows. An equal volume of double-strength filter sterilized buffer solution was added to double strength OWA2YE medium. This brought both medium and buffer concentrations to single strength levels. The final pH of the media treatments were adjusted to pH 3 through 7 with 1N HCl and 1N NaOH. Ascospore suspensions prepared to a spore density of 5x10(6) spores/ml were applied as drops to the agar surface. For all buffered and unbuffered treatements, inoculated Petri plates were incubated in the dark at 20+-2 degrees C.

RESULTS

Effect of post-hydration drying

A. anomala ascospores are very susceptible to damage by dehydration. Ascospores imbibe water readily at the beginning of the germination process. If, following the initial hydration, they are then subjected to drying, the percentage germination decreases quickly with increasing drying time (Figure 1).

After a drying period of one minute, germination decreased to 86% of the control treatment (no drying period), and after 5 minutes of drying to 55% of control. This trend of increased spore mortality proceeded until approximately one hour of drying time. At this point only 1.5% of the ascospores germinated relative to the control treatment.

After one hour of drying, spore germination was essentially zero, though an occasional spore began to germinate after the 4-hour treatment of post-hydration drying. In no case, however, did the long-treatment spores develop past the germ tube stage of growth. Those spores which survived the drying treatment were incapacitated to degree sufficient to prevent normal germination and growth. In contrast, spores dried for one hour were observed to germinate normally in some cases, though the majority of them did not proceed past the vesicle stage.

Effect of washing

Ascospores washed twice in dilute nutrient medium germinated at higher rates than unwashed spores. In three experiments, with three replicates of 100 spores each, washed ascospores of A. anomala germinated at rates significantly higher than those attained by unwashed ascospores (Table 2).

Effect of temperature

Significant differences in germination rate between temperature treatments were observed in two experiments, with three replicates each, in the temperature range of 5 to 20 degrees C (Table 3). ANOVA detected differences in each experiment with $P<<.025$.

Fisher's protected lsd test detected treatment differences in both experiments (1 s d=.05). In experiment 1, the lowest rate of ascospore germination was found at 20 degrees C; temperatures of 5, 10, and 15 degrees C did not produce significantly different germination rates. In experiment 2 the highest germination value was obtained at 10 degree C, followed by values for 5 and 15 degrees, with 20 degrees again being least conducive to germination.

Effect of medium pH

The effect of medium pH on ascospore germination was

determined by qualitative evaluation of germination and subsequent growth. Quantitative data are lacking because of problems encountered during the experiments, and because it is difficult to quantify hyphal morphology or radial colony expansion with such a slow growing organism.

There were marked differences in both spore germination and subsequent fungal growth between treatments in the pH range 3 through 8. No differences were noted between unbuffered and citrate-phosphate buffered treatments. Few ascospores germinated at pH's 3, 7, and 8; those which did germinate did not proceed past the primary vesicle stage of growth. Ascospores germinated at approximately equal rates on media of pH 4, 5, and 6. The most striking differences between treatments in this range were the growth rates of the fungus, the rapidity with which each growth stage was attained, and the morphology of the resultant hyphae and colonies.

Spore germination proceeded at the same rate at pH 4 and 5, while spores at pH 6 were somewhat delayed in their development. At pH 4 many aerial hyphae were produced, with few being appressed to the medium or immersed. These hyphae were generally short and densely branched. This condition led to the establishment of the largest colonies after a period of many weeks. Hyphae produced at pH 5 were longer, less branched, and generally more appressed to the surface of the medium than those produced at pH 4. Colonies at pH 5 did not develop as quickly as those on pH 4 medium, but they ultimately attained the same size.

The development of germination structures was slower at pH 6. Resultant hyphae were most often immersed in the culture medium, exhibited a helical growth pattern, and were somewhat knotted and convoluted compared to those produced on pH 5 medium. Aerial growth was very limited until many weeks had elapsed and a considerable mass of immersed hyphae had been produced. Colonies on pH 6 medium were smaller, sparser, and less frequent than those growing at pH 4 and 5.

DISCUSSION

Media

A. anomala ascospores apparently contain sufficient endogenous nutrient reserves to support the formation of a very short germination tube. Spores washed repeatedly in distilled water will begin to germinate on purified water agar, as well as in glass-distilled deionized water (unpublished observation). The percentage germination under these conditions was very low, however, and it is possible that the spore washing procedure did not entirely free the suspensions of empty asci, mycelial fragments, or other perithecial contents which may have contributed small amounts of nutrients to the suspension. These contaminating compounds may have allowed a limited number of spores to begin germination. The germination process inevitably ceased at a very early phase, with germination tubes rarely attaining the vesicle stage of growth.

In the course of preliminary experiments with Anisogramma, many formulations of common mycological culture media, both defined and undefined, were tested for their ability to sustain fungal growth. For example, Corn Meal Agar (Difco) promoted germination compared to water agar but did not allow growth to proceed beyond the short hyphal stage (unpublished). A complex, undefined medium rich in plant and animal extracts such as yeast extract and casein hydrolysate, supplemented with a carbohydrate and some

mineral salts not only increased germination rates but also allowed the fungus to attain slow but relatively substantial growth when compared to earlier formulations (unpublished). OWA2YE medium is the best medium yet developed for extended growth of A. anomala.

Effect of post-hydration drying

Following liberation from the ascus, ascospores are sensitive to dehydration. Once washed inside a bud of other wound site by rain water percolation, spores are protected from dessication by the water trapped in the host tissues. This protection from drying is very important in the establishment of the infection, as a drying period of 5 minutes may decrease germination rates by 50%.

Ascospores of A. anomala do not germinate quickly; the vesicle stage of growth is not attained until at least 48 hours after their introduction to nutrient medium at 20 degrees C (unpublished). The sensitivity of the hydrated spores to dessication may indicate why mite-galled buds are the primary infection court. Such buds are efficient collectors of rain (and ascospores) because of the vase-like arrangement of the bud scales and the spongy nature of the enclosed tissue. Episodic drying periods may kill ascospores which are not protected by a film of water. Spores which become lodged in the buds or other wounds would be amply hydrated for a considerable period following the cessation of rainfall.

Effect of washing

There have been many reports of inhibition of fungal spore germination at high inoculation rates (Gottlieb 1978). This phenomenon has been proposed to be the result of the action of endogenous germination inhibitors upon the mass of spores. These inhibitors are thought to be active only when a sufficient number of spores are present, as the effect of the inhibitor is additive, with increasing inhibition correlated with increasing spore density.

High inoculation densities (5x10(6)) were used in these experiments, and results show that washed ascospores germinate significantly better than unwashed ascospores germinate significantly better than unwashed spores. Germination of unwashed A. anomala ascospores is apparently inhibited by high spore density, since at the same density washed spores germinate more readily than unwashed spores. If the spores are washed repeatedly, germination rates are significantly increased. Presumably any inhibitors initially present in the spore suspension are removed by repeated washing.

The inhibitor may be present in the ascospores, or it may be a component of the slimy matrix in which the spores are embedded. The manner by which the ascospores were collected for these experiments ensured that an appreciable mass of empty asci, paraphyses, mycelial fragments, and cell contents were present in the unwashed spore suspensions. It is possible that inhibitors present in the crude preparation

were removed by the washing steps, resulting in the increased germination noted in the washed treatments.

In nature, perithecial contents are exuded through the ostiole in response to hydration of the enclosed spore mass. As the spore slime is exposed, it is dispersed by rainwater as the ascospores are freed from the ascus. The infection process is initiated when spores are lodged in host wounds by rainfall and rain splash. It is most likely that individual ascospores or spores yet enclosed in asci are separated from the main body of spores and perithecial debris and amply washed by rain. These effects may have been mimicked by the centrifugation and resuspension steps of the washing procedure.

Effect of temperature

A. anomala ascospores germinate most readily at temperatures in the range of 5 to 15 degrees C, with an optimal temperature near 10 degrees. In both experiment 1 and 2 incubation of inoculated plates at 10 degrees resulted in the highest germination rate, though in experiment 1 this rate was not significantly different from those of 5 and 15 degrees. In experiment 2, germination was significantly higher at 10 degrees than at either 5 or 15 degrees C.

The experimental temperature optimum is consistent with field temperatures prevailing during the infection period of February through May (Gottwald and Cameron 1980a). Cool, wet weather predominates during this period in western

Oregon and Washington. It is not surprising that optimal germination conditions in vitro should reflect the field environment during the periods of spore release and host infection. In both experiments, ascospores germination was significantly reduced at 20 degrees C, which is above the environmental temperature range during the infection period.

Effect of medium pH

The ability of A. anomala to grow in the pH range 4 through 6 is not unusual. Most rapid growth occurred at pH 4 and 5; growth at pH 6 was delayed but eventually equivalent to that on lower pH media. The most noticeable effect of pH was on growth habit.

Angiosperm sap is in the pH range of 5 to 7. Hyphal morphologies of A. anomala grown in this range are most like those exhibited in the host, while the short, stubby pattern seen on media of pH 4 is different. It is possible that the good growth attained at lower pH is due to better availability or utilization of medium constituents at lower than at higher pH. The tendancy for the fungus to grow aerially may indicate the general unsuitability of the low pH medium. Similarly, the tendency toward increasing immersion of hyphae as pH 6 is approached may indicate a more amenable environment for the fungus, and one which more closely simulates the conditions within the host.

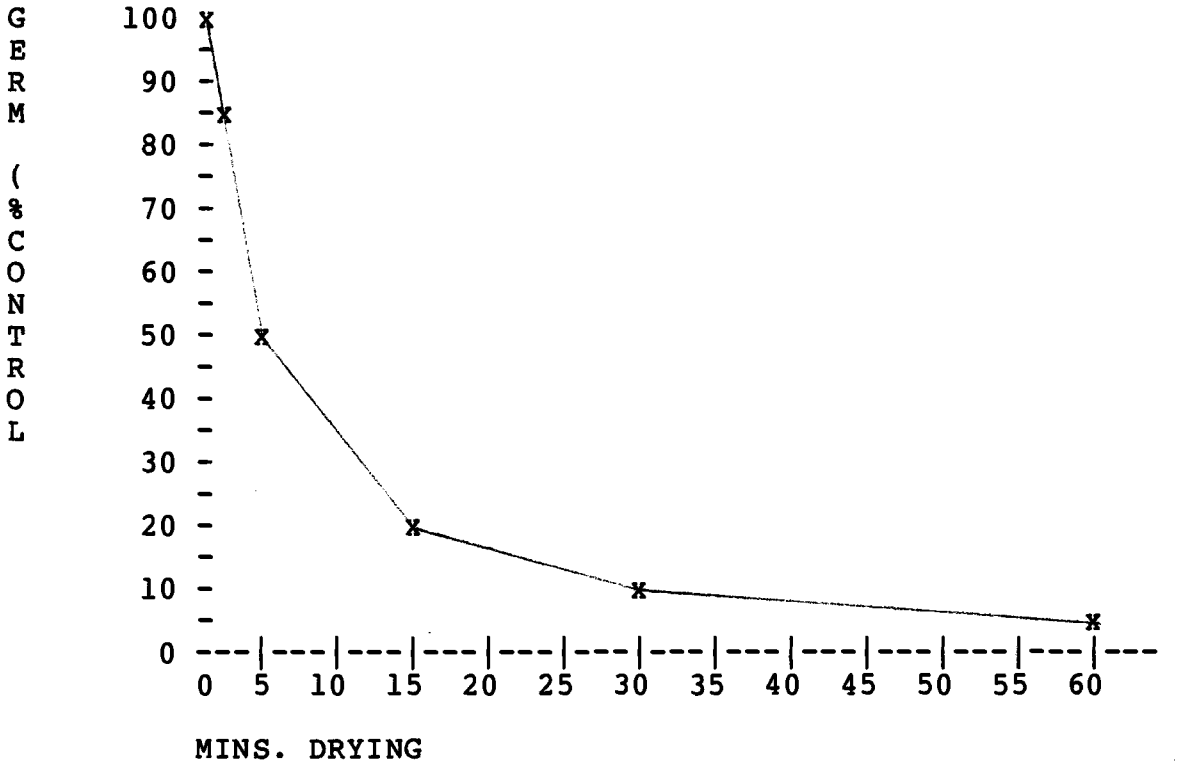

Fig. 1 - Effect of post-hydration drying on subsequent germination of A. anomala ascospores.

TABLE 1 - MEDIUM CONSTITUENTS

Maltose	5.0 g
Case in hydrolysate (Casamino acids, Difco, Bacto)	1.0 g
Yeast extract (Difco, Bacto)	1.0 g
KH_2PO_4	1.0 g
$MgSO_4*7H_2O$	0.5 g
$ZnSO_4*7H_2O$	0.2 mg
$MnSO_4$	0.1 mg
Agar (Difco, Bacto)	20.0 g
Distilled water	to 1 l

TABLE 2 - EFFECT OF WASHING ON ASCOSPORE GERMINATION

exp #	treatment	germ%(1)	P
	washed	18.66%	
1			.05
	unwashed	9.66%	
	washed	22.33%	
2			.01
	unwashed	12.66%	
	washed	12.66%	
3			.06
	unwashed	.900%	

(1) means of 3 replicates per treatment

P = significance level, Student´s t-test

TABLE 3 - EFFECT OF TEMPERATURE ON ASCOSPORE GERMINATION

temp C	germ%(1)	
	exp 1	exp 2
5	19.66% a(2)	15.33% b
10	22.33% a	25.33% a
15	17.00% a	15.66% b
20	10.33% b	4.66% c

(1) means of three replicates

(2) means in the same column followed by the same letter are not significantly different (Fisher's protected lsd test, lsd=(0.05))

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CHAPTER II

THE DEVELOPMENT OF *ANISOGRAMMA ANOMALA* IN VITRO

ABSTRACT

The growth of *Anisogramma anomala* on artificial medium is described. The ascospore germination process follows this sequence: (1) spore hydration and swelling, (2) emergence of the germination tube, (3) vesicle formation, (4) production of hyphal initials, and (5) hyphal growth. Hyphal morphology is similar to that exhibited in the host *Corylus avellana*. Attempts to maintain fungal growth for periods longer then several months were unsuccessful.

CHAPTER II

THE DEVELOPMENT OF *ANISOGRAMMA ANOMALA* IN VITRO

INTRODUCTION

The ascomycete *Anisogramma anomala* has not previously been culturable on artificial medium. While ascospores of this species germinate readily on many culture media, fungal growth past the germination stage has been difficult to maintain. The lack of an effective method for culturing this fungus has proven to be a hindrance in attempts to develop control methods for eastern filbert blight. Both chemical control and resistance breeding programs could benefit greatly from a source of the pathogen which is both accessible and easily manipulated. Further, axenic culture of A. anomala would allow physiological studies to be carried out on this fungus. The results described here represent the first successful maintenance of this fungus on artificial medium.

MATERIALS AND METHODS

Ascospore source and spore suspension preparation

Filbert (Corylus avellana) stems bearing perithecia of A. anomala were collected near the town of La Center in Clark County, Washington. After air drying to elimiinate surface moisture, the stems were sealed in plastic bags and stored at -20 degrees C to eliminate fungal contaminants and preserve the Anisogramma ascospores. Prior to preparation of spore suspensions, the stems were thawed at room temperature, surface sterilized for 2 minutes in 0.5% sodium hypochlorite, and then rinsed in distilled water.

Ascospores were removed aseptically from perithecia by excising the upper portion of the fungal stroma and plunging the tip of a sterile needle into the exposed spore mass within individual perithecia. For each suspension, ascospores were harvested from many perithecia and from 2 to 4 cankers; each suspension then contained ascospores from a number of field isolates.

Collected spores were immediately placed in sterile distilled water. Spores were allowed to be liberated from asci for approximately one hour. Occasional agitation of the suspensions was performed by repeated suction and expulsion of the liquid through a Pasteur pipette. Spore suspensions were adjusted to a spore density of 5x10(6) spores/ml by dilution with sterile distilled water, and then applied to agar medium with a Pasteur pipette.

Media and culture conditions

The OWA2YE medium used in this study was a modification of Barnett´s Oak Wilt Agar (Barnett, 1953). Medium constituents are listed in Table 1. Medium pH was adjusted after autoclaving to pH 4 through 6 with 1M NaOh and 1M HCl. Inoculated Petri dishes were incubated in the dark at 15+-1, 20+-2, and 25+-2 degrees C.

RESULTS

Pre-germination

Ascospores of A. anomala hydrate rapidly following contact with water, swelling from the dormant size of 8-12 x 4-5 m to the hydrated size of 10-14 x 6.5-8 m. After hydration but prior to germ tube emergence the polar areas of the spore are filled with numerous refractile bodies (Fig. 2) which are not seen in moribund spores.

Germination

The germination tube emerges from the transverse wall of the spore. Germ tubes may form at any position relative to the surface of a solid substrate. No germ pores have been observed in the spore coat of the ascospore. In most cases germ tube formation is followed by the formation of a large vesicle which is generally elongate and swollen toward to apex, but which may be spherical or irregularly shaped (Fig. 3). These vesicles are variable in size, ranging from 10-40 m in diameter and from 10-100 m in length. Vesicles are often filled with granular inclusions similar in appearance to the refractile bodies visible in hydrated, ungerminated ascospores. A septum is usually formed between the spore and the vesicle.

At incubation temperatures in excess of 22 degrees C the normal germination process is disrupted. A large

percentage of ascospores germinating at high temperature do not from a germ tube in the usual manner but rather swell to a remarkable degree and become oblong or spherical structures with a diameter of up to 100 m (Fig. 4). Aberrant germination of fungal spores at high temperatures has been well documented (Anderson and Smith, 1972). Most of the cell volume is taken up by a single or several large vacuoles. Cytoplasm is distributed as a thin area adjacent to the cell wall, and a thickened area, possibly containing the cell nucleus, is often seen. These grossly swollen cells remain viable, as structures analagous to germ tubes or hyphal initials are borne on them. Normal vesicles are often produced from these giant cells (Fig. 5). The small aborted cell at the apex of ascospores is often observed to be little changed by this process of unusual swelling. These aborted cells remain visible as small protrusions on the surface of the giant cells.

In some cases during the course of normal development, additional vesicles are evolved from the primary vesicle, resulting in a beaded appearance of the germinating ascospore (Fig. 6). These vesicles may act as assimilatory structures though this cannot be confirmed due to the fact that in planta infectio structures have never been observed.

During the course of these investigations, glass coverslips were often applied to the agar surface as soon as the inoculation solution had become absorbed into the medium. Under the coverslip, ascospore germination typically proceeded only as far as the vesicle or short

hyphal stage. This may indicate that A. anomala is inhibited by reduced oxygen supply. Ascospores on the same agar surface but not covered by the coverslip often produced macroscopic colonies.

Following development of the vesicle, one or more small protrusions appear adjacent to the vesicle apex. These protrusions elongate, giving rise to hypae which at first are often irregularly shaped and somewhat vesicle-like in character (Fig. 7). These structures develop into smooth, straight-walled hyphae from 4-6 m in diameter. Hyphae may grow to a length of several hundred micrometers before branches are initiated. Hyphae are at first irregularly septate, with septation becoming more regular as growth progresses.

Hyphal branching patterns in A. anomala cultures were extremely variable. Branch initials may halt their development and grow no further, continue to grow linearly, or develop numberous short, stubby branches (Fig. 8). A hypha may terminate in a densely branched luster, then send out a single hypha which elongates. This linear growth may continue or soon terminate in another whorl of short branches. This pattern of fungal development has been observed in the host, with a single hypha penetrating a cell, filling it with branches, then moving on to another more or less adjacent cell (Gottwald and Cameron, 1979) (Fig. 9).

As the hyphae elongate, the original germination structures (spore, vesicle) begin to accumulate a brown

pigment which diffuses into the surrounding culture medium to some degree (Fig. 10). Pigments eventually appear in the older hyphae, accompanied by a marked thickening of the hyphal walls. To this point only very small colonies (approximately 5.0 mm diameter) have been produced on agar medium. These colonies appear brown-black, with the growing margins remaining hyaline (Fig. 11)

Hyphae of A. anomala grow aerially, appressed to the agar surface, and immersed in the medium. Densely branched structures are most often found immersed in the agar, though occasionally they are formed aerially or on the agar surface. No structures which could be construed as conidia or other asexual spore types were identified in this study.

Fungal colonies were impossible to maintain for longer than several months. Repeated transfers of germinating spores and growing colonies to both liquid and solid media failed to appreciably prolong growth or colony expansion.

DISCUSSION

Repeated transfers of germinating spores and growing colonies to both liquid and solid media had no effect in increasing fungal growth. If nutrient depleiton was the limiting factor, repeated transfers to fresh medium should have prolonged vigorous growth, as it would if growth was limited by the accumulation of toxic compounds or metabolites in the medium. Only very few ascospores germinate in distilled water or purified water agar (unpublished data), and fairly extensive though slow growth is sustained over a period of many weeks on the OWA2YE medium used in this study. The culture medium used must supply essential nutrients for germination and subsequent growth. However, critical nutritional or environmental factors must be missing from the present cultivation protocal, as growth proceeds steadily up to a certain point, then invariably stops.

The degree to which hyphae of A. anomala grow into the agar is apparently affected by the pH of the culture medium. At pH 4, hyphal growth is primarily above the surface of the agar, and colony diameter is limited. As the pH of the medium increases to the range of pH 5-6, increasing immersion of hyphae in the agar and appression of hyphae to the agar surface is observed, along with an increased incidence of the densely branched structures described earlier. Immersed hyphae tend to be more convoluted in their growth habit than hyphae growing appressed to or above

the agar surface. Immersed hyphae also often grow in a helical manner while this characteristic is lacking in non-immersed hyphae.

A. anomala grows very slowly on artificial medium. In the filbert host the fungus is capable of rapid growth, as cankers greater than two years of age extend an average of 30cm in length from the period before filbert bloom, budswell and increasing activity of host cambium to the time when the leaves of the host are fully expanded. The largest canker recorded for a 4 year old infection was 189cm in length (Gottwald and Cameron 1980a). After leaf expansion is completed linear canker growth slows dramatically bas the fungus enters the reproductive phase and the host lays down callus tissues around the infected area.

A. anomala exhibits two distinct growth rates in the host: a rapid phase in which the fungus may colonize appreciable amounts of tissue followed by a slow phase during which colonized tissue acts as a nutrient base to support sexual development.

Through pruning experiments, the time of most rapid canker expansion has been shown to be that period in the host when cambial activity is at a minimum and the ability to form callus is poor. Pruning branches two feet below the visible margin of the canker (indicated by discoloration of the vascular tissues beneath the bark) in December 1979 failed to completely remove the fungus from the pruned branch, and the infections were observed to spread in the following season. Pruning cuts made two feet below the

canker margin in late March of 1979 removed the fungus completely (Cameron 1980). It has not been conclusively shown that the fungus grows most rapidly when the host is dormant but temperatures are high enough for fungal growth to proceed. It is unclear at this time whether maximal rates of fungal growth occur while the filbert host is fully dormant or just starting to become more active in response to rising temperature and increasing photoperiod. The time of rapid canker extension overlaps the time in late winter and early spring when the host tree is becoming more active physiologically. Development and fertilization of the filbert flowers occurs during this time, as does increased production of plant hormones accompanying early bud development. Increasing translocation of nutrients from the root system toward the crown of the tree also occurs during this period. It is possible that the physiological state of the host tissue more favorable to fungal growth at this time and that a particular combination of nutrients triggers and/or sustains rapid growth fungal development.

Alternatively, it is equally possible that rapid fungal growth is due to the absence of a host-produced inhibitor. This hypothesized inhibitor may be produced only after the cambial cells become fully active, and thus the fungus is allowed a head start on the tree early in the growing season. Once the inhibitor is produced, fungal growth slows, accompanied by an increasing ablility of the host to limit expansion of the canker by the formation of callus tissue. It is also conceivable that no chemical triggers or

inhibitors exist, and that the fungus simply expands while conditions allow growth of the fungus but prevent callus formation by the host. Laboratory experiments investigating the growth of A. anomala in culture at temperatures of 5 to 10 degrees C indicate that the fungus is capable of growth at these temperatures.

The growth rate attained by A. anomala in culture corresponds to some degree to the slow growth phase exibited in the host. It is most likely that this is slow growth is caused by the absence of a sufficient supply of specific nutrient(s) required for rapid growth. The fungus growing on OWA2YE is supplied with the nutrients necessary to maintain slow growth for a period of several months, a critical factor is still missing.

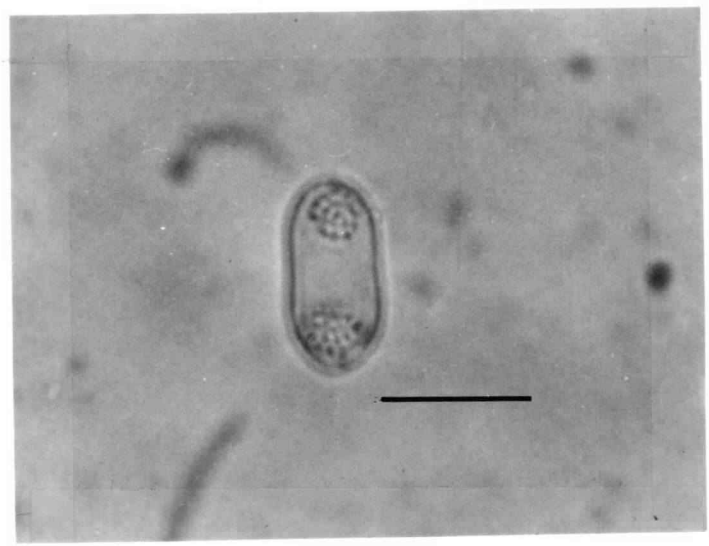

Fig. 2 - Hydrated ascospore with refractile bodies. Bar = 10 μm

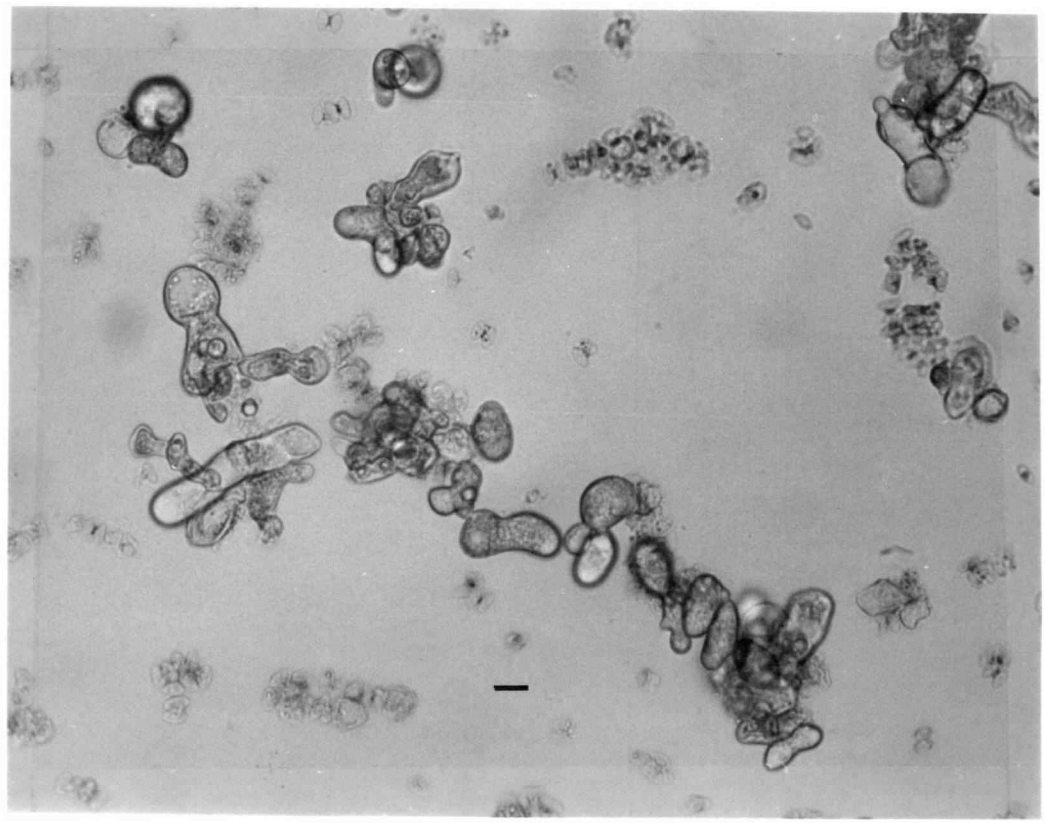

Fig. 3 - Vesicles, spherical and irregular. Bar = 10 μm

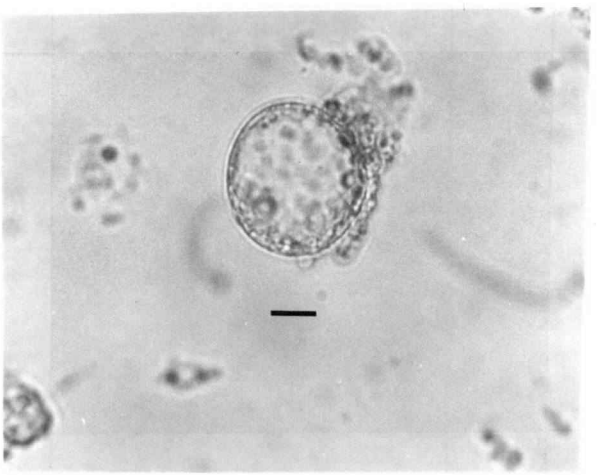

Fig. 4 - Giant cell resulting from incubation at 25 degrees C. Bar = 10 μm

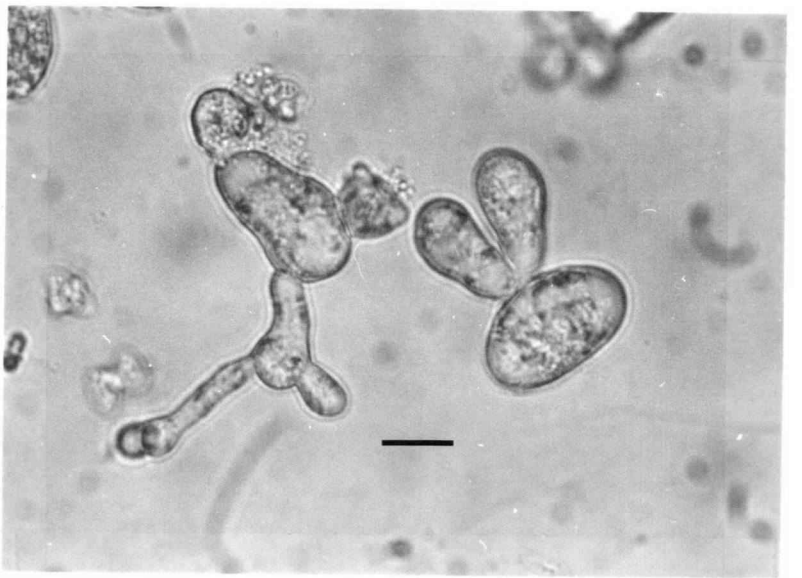

Fig. 5 - Giant cells with vesicles and hyphal initials. Bar = 10 µm

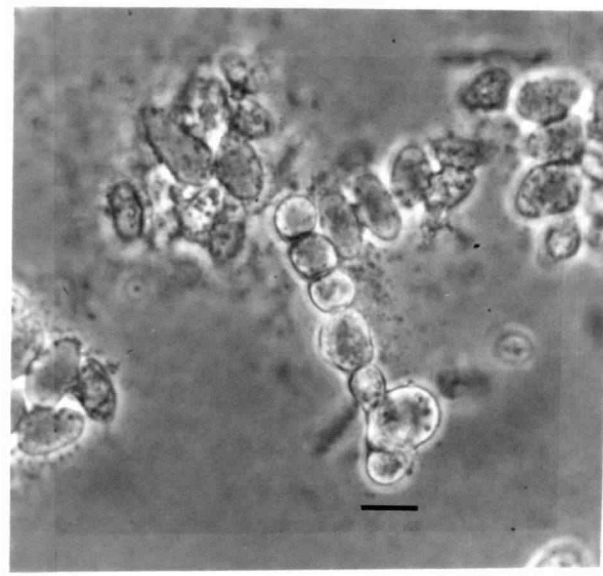

Fig. 6 - Secondary and tertiary vesicles. Spore remnant at bottom of vesicle chain. Bar = 10 μ m

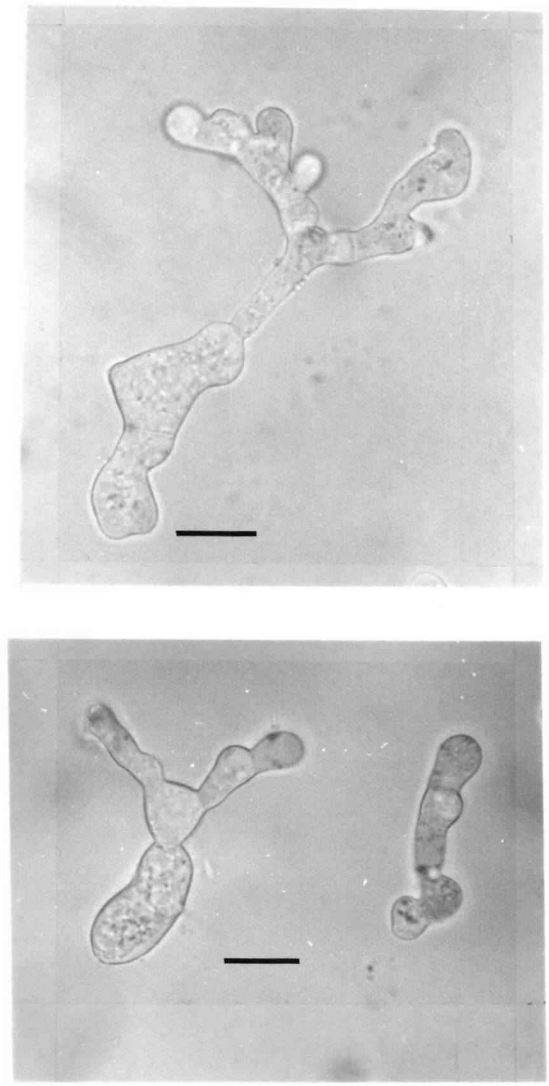

Fig. 7 - Hyphal initials. Bar = 10 µm

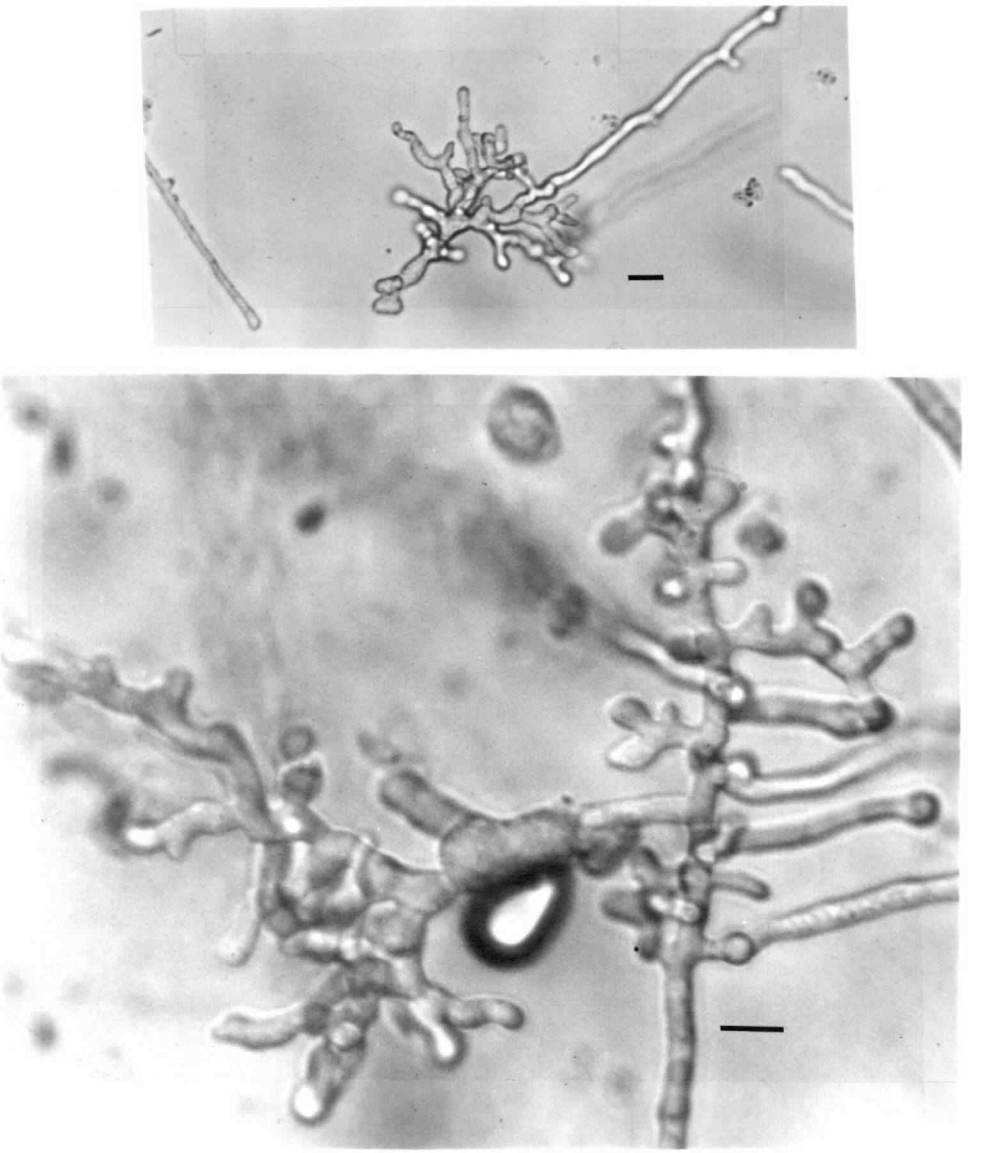

Fig. 8 - Whorl of branches. Bar = 10 µm

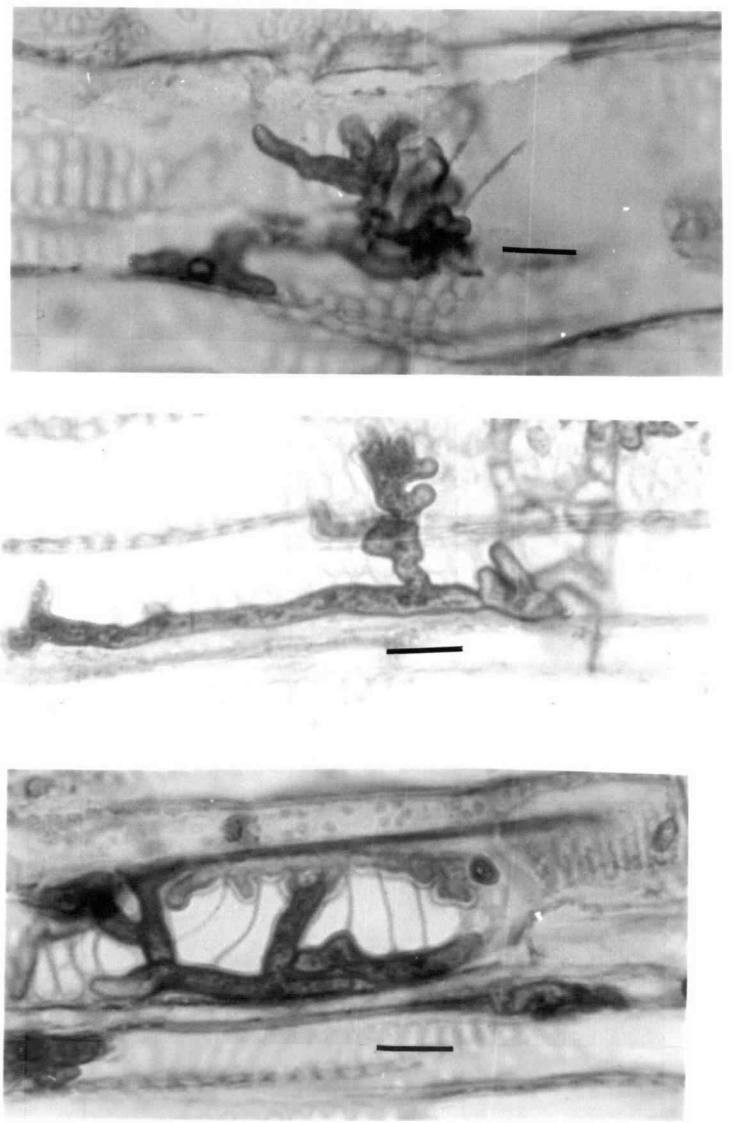

Fig. 9 - Hyphae in host phloem. Bar = 10 μm. (Slides courtesy of T.R. Gottwald)

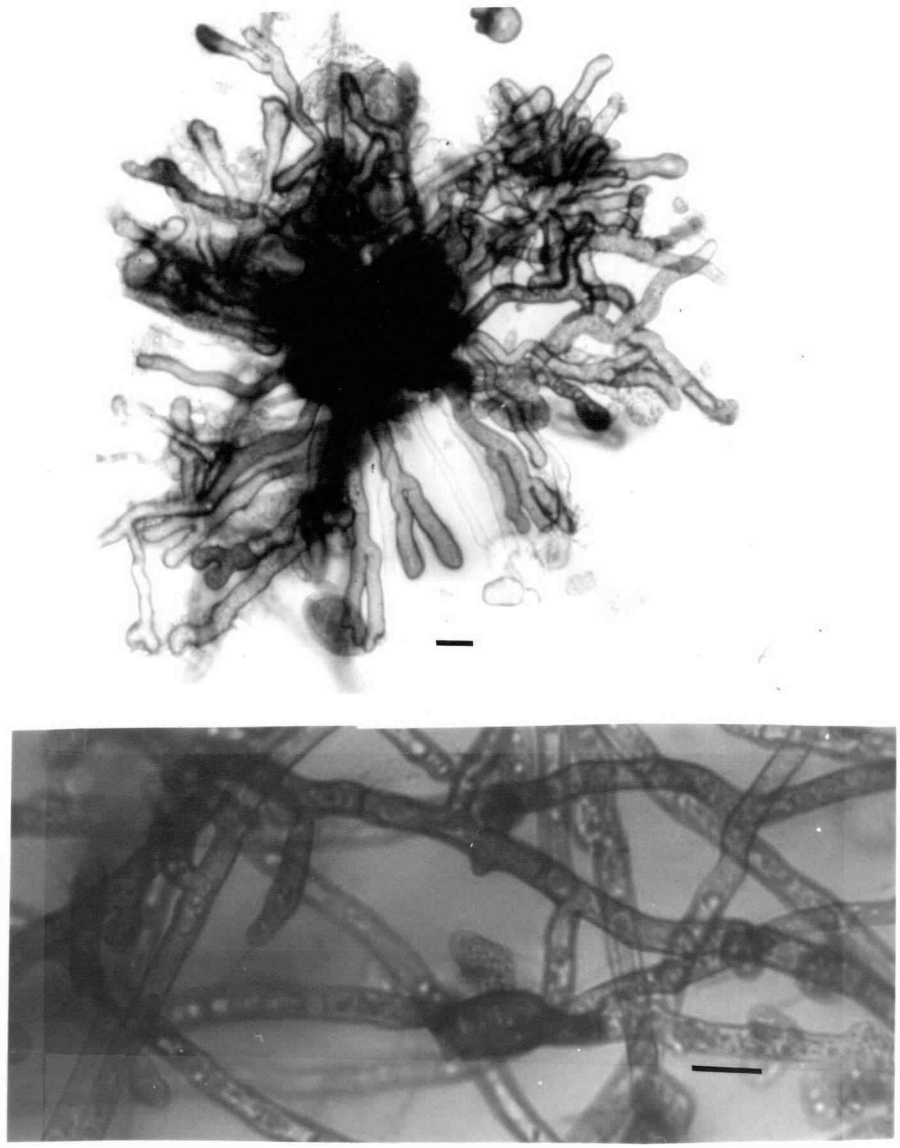

Fig. 10 - Pigmented older hyphae. Bar = 10 µm

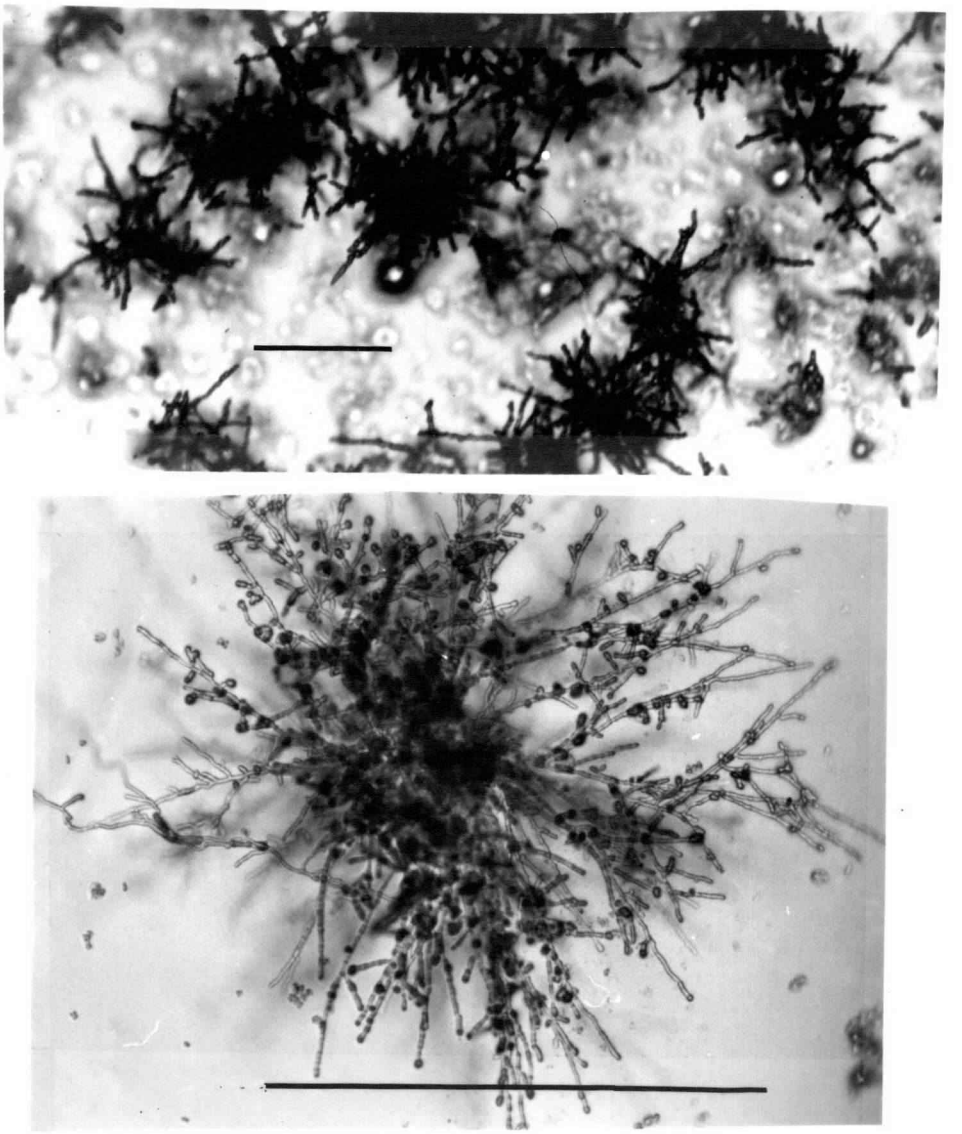

Fig. 11 - Colonies of A. anomala. Bar = 100 μm

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SUMMARY

Environmental factors most conducive to the germination and growth of A. anomala in vitro have been identified, and small colonies of the fungus are now maintainable for a period of several months in the laboratory. These studies have laid the groundwork for further research into the cultivation of A. anomala on artificial medium and may assist future researchers in the development of methods for prolonging the active growth phase in the laboratory.

In vitro cultivation of A. anomala would be of great utility in the development of control methods for eastern filbert blight. Better understanding of the ecology and physiology of A. anomala would permit the fungus to be manipulated with greater ease, and would provide valuable information on the fungus which is a threat to the filbert industry in the Pacific Northwest.

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APPENDIX

APPENDIX

Inoculum storage

During these investigations it became apparent that prolonged storage of A. anomala ascospores resulted in decreased viability. the effect was most noticeable when the stroma-bearing host tissue was stored at temperatures above freezing. The stromatic cushions of fungal tissue support a wide variety of microorganisms, some of which are capable of degrading A. anomala ascospores. Furthermore, stem pieces were quickly overgrown by contaminating fungi which rendered strerile collection of ascospores difficult, even when the stems were surface sterilized both before being placed in storage as well as immediately preceding inoculum preparation.

Freezing the tissue at -20 degrees C was of great utility in preserving the ascospores from the ravages of contaminating microbes. Unfortuneately, extended storage at low temperatures appears to kill some fraction of A. anomala ascospores in the perithecia. Due to the difficulty in long-term storage, it has not been possible to investigate the relationships between different field isolates of A. anomala regarding spore germination rates and subsequent colony growth on different media.

A consequence of this storage problem was the relatively low germination rate of ascospores used in these investigations. Many nonviable spores were observed in all

experiments, often making distinctions between controls and treatments very difficult to assess. It was common for germination rates to be indistinguishable between treatments (most being very low, between 1 and 20 percent), making quantitative evaluation difficult. At the same time noticeable differences in growth habit were observed among the small percentage of germinated ascospores. These qualitative differences in both germination performance and subsequent growth are difficult to evaluate and express quantitatively.