AN ABSTRACT OF THE THESIS OF

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Title: Studies on Canker in European Filbert

Caused by Anisogramma anomala

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Anisogramma anomala causes a stem canker of filberts, Corylus avellana. This disease became a threat to the main U.S. filbert growing region when it was introduced into the Pacific Northwest around 1968. Various methods of artificially inoculating filbert trees and seeds were explored. Less than 1% of potted filbert trees inoculated with ascospores produced symptoms after 4 years. The disease was not transmitted following graft inoculation of orchard trees with infected tissue. Ascospores had no effect on germination or growth of Corylus seedlings. Spread of existing cankers was compared on the susceptible cultivar 'Daviana' and the more resistant cultivar 'Barcelona'. There was no significant difference ($t=.05$) in canker elongation between cultivars, or between spread up and down the stems. Vertical elongation of perennial cankers averaged 26.2 cm/year.

Methods for storing ascospores prior to germination, and conditions for germinating ascospores were explored. Spores exhibited

no loss of viability after 2 months when cankered twigs were stored below 0° C. Ascospores remained viable for less than 14 days following discharge in water. Drying was lethal to hydrated spores. Ascospores commenced germination 24 hours after hydration when placed on a suitable medium in a proper atmosphere. Germination occurred between 5° and 25° C, with an optimum of 20°. Optimum pH for germination was between 4.2 and 5.7. A direct relationship was detected between ascospore concentration and percent germination. Eight antibiotics were screened in an attempt to prevent bacterial contamination. 10 ppm Rifampicin or 100 ppm Kanamycin effectively inhibited bacterial contaminants without affecting ascospore germination. Germination was compared on many different complex and defined media, and with different amendments added to the media. Various Corylus extracts had no effect on spore germination. 1% ethanol and 5% sucrose increased germination. Casamino acids, cornmeal or peptone promoted growth beyond the initial germ tube. Several stages of ascospore germination and early hyphal growth are described.

Studies on Canker in European Filbert Caused by *Anisogramma anomala*

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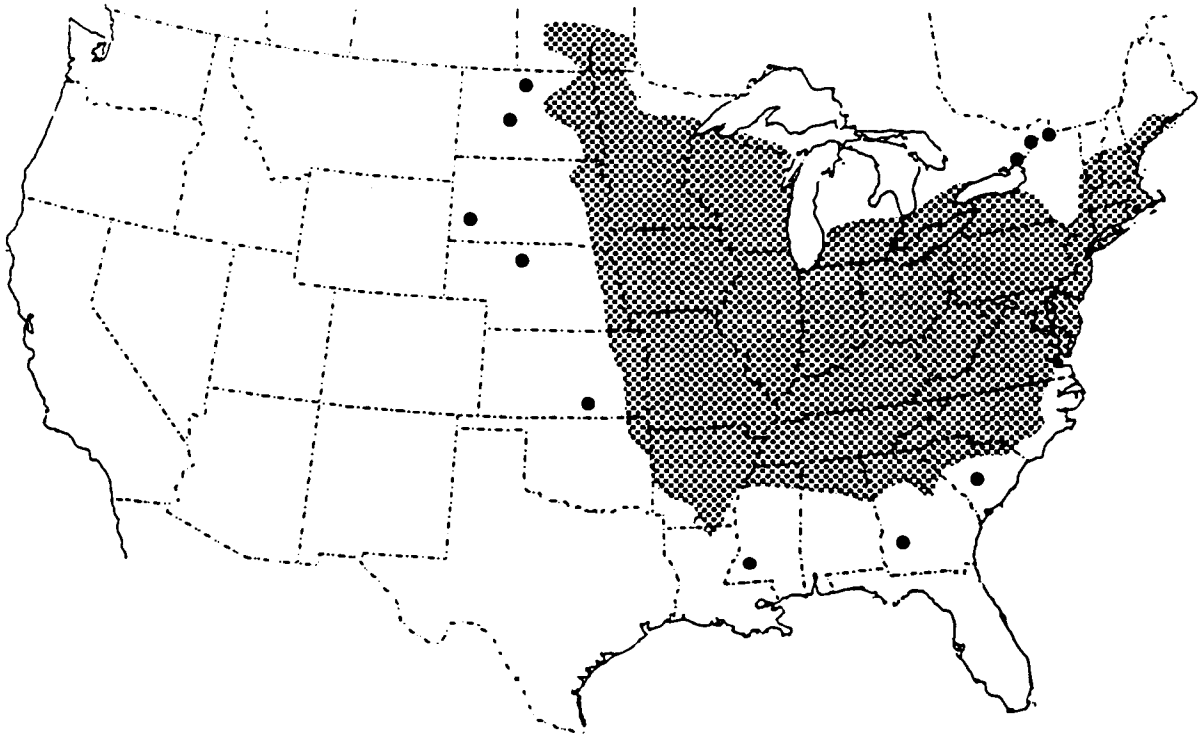
INTRODUCTION AND LITERATURE REVIEW

EARLY ATTEMPTS TO GROW FILBERTS

Since about 1850, attempts have been made to establish European filberts, *Corylus avellana* L., in the eastern United States (Barss 1921a; Barss 1930; Fuller 1896; Slate 1930). These attempts ended in failure primarily due to Anisogramma Canker, also called Eastern Filbert Blight, caused by the fungus *Anisogramma anomala* (Barss 1921a; Fuller 1896; Morris 1920). A test orchard at the New Jersey Experiment Station was reportedly destroyed by this "obscure enemy" in 1892 (Halsted 1892). Humphrey (1893) presented a description and illustrations of the fungus and the disease symptoms after a Massachusetts plantation owner reported that it was killing his European hazels. The disease had been known for years in nearly all the nurseries, parks and gardens of the older states (Fuller 1896), and was present throughout the natural range of *Corylus americana*, its natural host (Figure 0.1)(Barss 1921a; Barss 1930). It was not known outside of North America (Barss 1921a). Filberts which were being propagated by the Office of Plant Introduction in Washington D.C. for general distribution had to be destroyed in 1921 when they became infected (Barss 1921a). Fuller (1896) reports that "it is this blight, and nothing else, that has prevented the extensive cultivation of the...European filbert...and not the uncongenial soil and climate as...proclaimed by men whose theories are far greater than their practical knowledge of such subjects." Despite the difficulties of filbert blight and winter hardiness, the cultivation of filberts was still being promoted in New York in 1930 (Slate 1930).

Figure 0.1

Distribution of Corylus americana. Redrawn after Drumke (1964).



FILBERTS IN THE PACIFIC NORTHWEST

Anisogramma Canker was not known west of the Rocky Mountains, where C. americana does not occur, and filberts have been grown successfully in the Pacific Northwest since the region was settled by Europeans (Slate 1930). The only problem of consequence in the northwest was filbert bud mites, which were introduced from Europe on the original stocks (Barss 1921a). These eriophyid mites, Phytocoptella avellanae and Cecidophyopsis vermiformis, invade developing buds and cause a galling also known as "Big Bud" (Krantz 1974). A bacterial blight caused by Xanthomonas corylina has caused problems in young plantings (Miller et al 1949). Barss (1921a) proposed a quarantine on the shipment of Corylus material from the territory east of the Rocky Mountains to keep Anisogramma Canker out of western filbert orchards.

Many cultivars of C. avellana grown in the northwest today were introduced between 1885 and 1905, including the main commercial variety, 'Barcelona', and it's pollenizers, 'Daviana' and 'duChilly'. Today, Oregon's Willamette Valley grows about 97 percent of the United States filbert production, with the remainder grown in Washington. The filbert industry contributes 5 to 10 million dollars annually to the economy of the Pacific Northwest (Barron & Stebbins 1978). United States production is about 6 percent of the world filbert production, with Turkey, Italy and Spain being the main producers (Woodruff 1979).

Anisogramma anomala IN THE PACIFIC NORTHWEST

In 1968, cankers were observed on C. avellana by a filbert grower in western Washington, and in 1970 Anisogramma anomala was diagnosed as the causal organism (Davidson & Davidson 1973). Funds were

provided by the filbert industry and USDA to find out the extent of the infection and to learn more about the disease (Cameron 1976). A survey of filbert orchards in Clark and Cowlitz counties, Washington and adjacent Columbia county, Oregon, located 45 infected orchards in a 200 square mile area (Cameron 1976; Cameron & Gottwald 1977). The disease appears to have been introduced near Woodland, Washington (Gottwald 1980; Gottwald & Cameron 1980b; Lagerstedt 1979). In 1974 the Oregon Quarantine was expanded to prevent filbert material from moving from this infected area into Oregon (Cameron 1976). Additional orchards have since become infected, and several infected orchards have been removed. Spread of the disease between orchards is slow (Cameron & Gottwald 1977) and it is estimated to have been in the area for several years before the first report (Gottwald 1980; Gottwald & Cameron 1980a). The number of new infections within a susceptible tree and within an orchard approximately doubles each year (Gottwald 1980; Gottwald & Cameron 1980a). Inoculum dispersal over long distances appears to be infrequent, however the continuing advancement of the southern edge of the infected area poses a threat to the main filbert growing areas of Oregon (Gottwald & Cameron 1980b).

DISEASE SYMPTOMS

Young twigs are attacked first. Small areas of dead sunken bark appear which enlarge until the limb is girdled (Barss 1930). A brown stain is found in the cambial region beneath the bark where hyphae are rapidly colonizing (Gottwald & Cameron 1979). Spore producing pustules (stromata) break out in relatively straight lines through the dead bark, lengthwise on the branch (Barss 1930; Ellis & Everhart 1892; Fuller 1896; Humphrey 1893). Stromata occur in single or double

rows. Gottwald & Cameron (1979) observed that 3 - 5 rows may occur in older cankers. Fuller (1896) observed that stromata appeared on any wood not younger than two years, and more frequently on the upper side of the branch. When a larger branch or the trunk is infected the canker appears sunken, with an overhang of thick healthy bark around the margins (Morris 1920). The cankers are perennial and eventually grow into and girdle the main trunk. The roots appear not to be affected and may continue to send up suckers after the main tree has died (Barss 1930). Early literature from eastern U.S. reports that trees are killed 2 - 3 years after becoming infected (Barss 1930; Fuller 1896; Slate 1930). In the northwest a mature tree may be killed in 5 - 15 years, and younger trees become commercially worthless in 4 - 7 years (Gottwald & Cameron 1980b).

SUSCEPTIBILITY OF *CORYLUS* SPECIES AND CULTIVARS

Anisogramma anomala is indigenous to the common American hazel, *Corylus americana*. Fuller (1896) had "not been able to find any clump of these bushes of any considerable size that was entirely free from pustulous stems." The disease, though ubiquitous, is of little consequence on this host (Barss 1921a; Fuller 1896; Morris 1915; Slate 1930). It is reported that *A. anomala* does not occur on either *Corylus cornuta*, the beaked hazel, or its western form *C. cornuta var. californica* (Barss 1921a; Barss 1930; Fuller 1896; Morris 1920), however plant disease indexes for both the US (USDA 1960) and Canada (Conners 1967) cite reports of the fungus on *C. cornuta*, and Wehmeyer (Wehmeyer 1933) lists *C. cornuta* (*rostrata*) as a host. Other *Corylus* species which have been planted in this country and seem to be unaffected by this disease include the Turkish or Byzantine hazel, *C.*

colurna (Farris 1969; Morris 1915), and its close relatives the Chinese tree hazel, C. chinensis and the Indian tree hazel, C. jacquemontii (Farris 1969).

Cultivars of C. avellana, though more severely affected by the disease than C. americana, show different degrees of susceptibility (Barss 1921b, Cameron 1976, Morris 1920). Morris (1920) noted that 'Red Aveline' had a high degree of resistance. 'Barcelona', the main commercial cultivar, is more resistant than its pollenizer, 'Daviana', which is very susceptible (Cameron 1976). 'Gassoway' appears to be completely resistant (Cameron 1981). Several years after becoming infected, 'Daviana' trees will die, 'Barcelona' and 'duChilly' simply become unproductive (Cameron & Gottwald 1977). The new filbert cultivars 'Ennis', 'Butler', and 'Lansing' are all susceptible (Cameron & Gottwald 1978; Lagerstedt 1979). Susceptibility of C. avellana cultivars to Anisogramma Canker seems to be positively correlated with susceptibility to the eriophyid bud mites (Cameron 1976). Some cultivars, however, such as 'Montebello' and 'Jemtegaard-5' are resistant to the bud mites (Thompson 1977) and susceptible to Anisogramma Canker (Cameron, Sizemore & Gottwald 1980). C. cornuta is susceptible to bud mite and appears to be resistant to the disease.

THE CAUSAL ORGANISM

The fungus was first described by Peck in 1875, and given the name Diatrype anomala. Peck chose the species name because he thought the fungus anomalous "in its unusual spores and in its attacking living stems" (Peck 1875). Synonymy is as follows:

Diatrype anomala Peck (1875)

Cryptosporella anomala (Peck) Saccardo (1882)

Cryptospora anomala (Peck) Ellis & Everhart (1892)

Apiospora anomala (Peck) von Hohnel (1917)

Anisogramma anomala (Peck) Muller & von Arx (1962)

Anisogramma is a pyrenomycetous ascomycete in the order Diaporthales. In the most recent treatment of this group, Barr (1978) places the genus, along with 19 other genera, into the family Gnomoniaceae. Anisogramma anomala bears it's perithecia in a dark, pseudoparenchymatous stroma. Gottwald & Cameron (1979) describe the development and morphology of the stroma, perithecia, and asci. Stromata are 1.5-3.0 x 2-10 mm, aligned with their greatest length parallel to the long axis of the stem, and erumpent through the host's periderm.

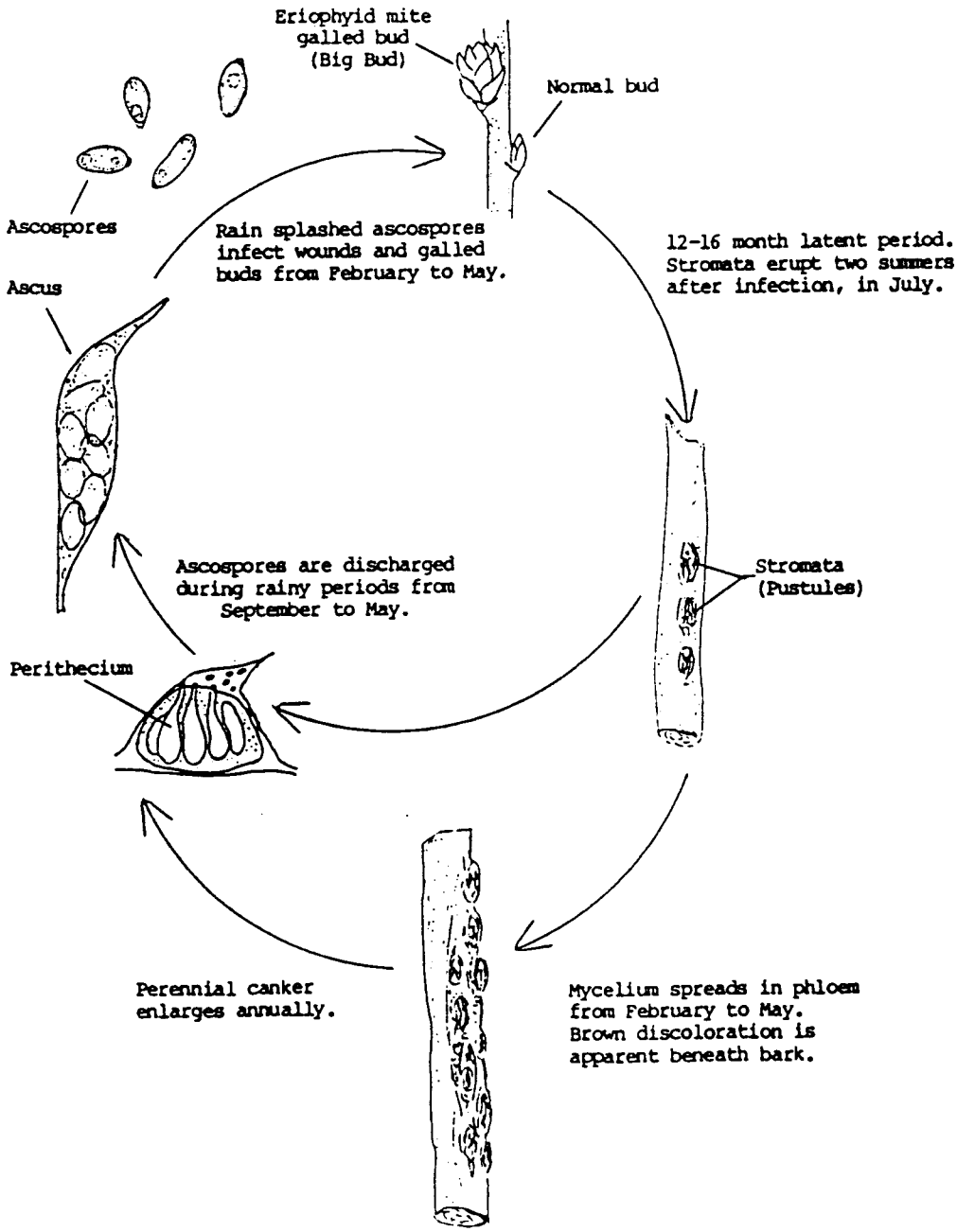
Perithecia are 250-830 um. diameter, 1000-2000 um. high, upright, and compressed laterally. Beaks are central, not converging, black against the stroma surface and protruding only slightly.

Asci are ellipsoid, 35-40 x 9-11 um. and contain 8 unequally 2 celled, hyaline ascospores. Mature ascospores measure 8-12 x 4-5 um. The lower cell is very small and caplike, 1-2 um. long and wide (Barr 1978; Gottwald & Cameron 1979; Muller & von Arx 1962; Wehmeyer 1933; Wehmeyer 1975).

Perithecia and asci in the Pacific Northwest are somewhat larger than those found in the northeastern U.S. (Gottwald & Cameron 1979). No other spore type or fruiting structure is known (Barr 1978; Gottwald & Cameron 1979). The only other species of Anisogramma, A. virgultorum, produces conidiophores and one-celled, hyaline conidia on the stroma (Barr 1978; Muller & von Arx 1962).

Figure 0.2

Lifecycle of Anisogramma anomala in the Pacific Northwest. Redrawn after Barr (1978), Barss (1921a), Humphrey (1893), Krantz (1974).



LIFECYCLE OF *Anisogramma anomala* IN THE NORTHWEST

The lifecycle of *Anisogramma anomala* is depicted in Figure 0.2. In the Pacific Northwest, ascospore discharge commences with the onset of the rainy season around October and continues until May. *C. avellana*, however, appears only to be susceptible from February to May (Gottwald & Cameron 1980a). During this period, *A. anomala* ascospores are able to infect the stems through wounds caused by freeze or mechanical damage or through mite galled buds (Cameron & Gottwald 1978; Gottwald & Cameron 1979). The susceptible period corresponds to the period when bud scales of mite infested buds are expanding (Gottwald & Cameron 1980a) and when the host is unable to form callus to protect wounds (Gottwald & Cameron 1979). Artificial inoculations produced a greater percent infection in mite infested than in healthy buds (Gottwald & Cameron 1980a).

The fungus enters exposed cells and colonizes the phloem from February to May (Gottwald & Cameron 1979). The cambium and xylem are invaded to a lesser extent (Cameron & Gottwald 1978). Mycelium spreads both up and down the stem at the same rate (Gottwald & Cameron 1980a). When cambial activity in the host resumes, a layer of callus is formed, walling off the developing canker (Gottwald & Cameron 1979). Hyphae which have penetrated the xylem are able to grow under the callus and reinvade the phloem during early spring the following year. No outward symptoms are visible the first summer following infection. There is a 12-16 month "latent period" before the stromata are visible in the orchard (Gottwald & Cameron 1980a). During March and April a year after infection, the mycelium begins actively spreading up and down the stem. A brown discoloration can be seen at

this time if the bark is peeled away. The invaded area soon appears sunken as the cambium is killed and surrounding tissues continue to grow (Gottwald & Cameron 1979). By July the perithecia, asci, and ascospores have formed and the stromata begin to erupt through the bark (Cameron & Gottwald 1978). During August the perithecia and stromata turn black and by autumn the ascospores are mature (Gottwald & Cameron 1979). When mature perithecia are exposed to free moisture, the asci and ascospores are released in a white viscous mass. Spores are dispersed by splashing rain and are carried through the orchard by wind blown water droplets (Gottwald & Cameron 1979; Cameron & Gottwald 1978). Long range dispersal is not as well understood, but appears to occur infrequently (Cameron & Gottwald 1977).

Cankers are perennial and continue to enlarge each year. Annual increase in length of individual cankers is highly variable and averaged 32 cm. in Washington (Gottwald & Cameron 1980b). Cankers over 200 cm. long have been found (Gottwald & Cameron 1980a). All attempts to grow the fungus in culture, either from ascospores, or by isolating from diseased tissue have been unsuccessful (Cameron & Gottwald 1977).

METHODS OF CONTROL

Quarantine: Barss warned in 1921 that "the present immunity from the eastern blight which we now enjoy will certainly be only temporary" unless a rigid quarantine is maintained against known hosts of the fungus (Barss 1921a). That quarantine was effective for almost 50 years at keeping the disease east of the Rocky Mountains. A quarantine is now being employed to prevent the movement of this disease a much shorter distance, from southern Washington into the

main filbert growing region in northern Oregon. Eradication in the northwest is not possible due to the presence of the disease on escaped C. avellana seedlings (Cameron 1976), and the reluctance of filbert growers to destroy their infected orchards (Cameron 1979).

Pruning: Many attempts have been made to control the disease through sanitation. Fuller (1896) and Slate (1930) recommended that infected branches be cut away well below the infection and burned. Morris (1931) considered Anisogramma Canker to be a minor concern. He reported that "if we go over the orchard once a year, cutting out blighted bark and protecting the exposed area, it is a comparatively simple matter to manage this pest." Cameron & Gottwald (1978) were not able to control the disease by pruning because the fungus frequently extends beyond the visible symptoms. Pruning cuts 2 feet below the last visible symptom often failed to halt the spread of a canker (Cameron Sizemore & Gottwald 1980), however pruning in December was more effective than pruning in March (Cameron 1981). Cameron (1981) suggests that during the summer the fungus does not extend much beyond the visible canker, and that pruning at this time may be more successful than winter pruning. Since infection occurs more than a year before symptoms are apparent, new infections will escape any attempt to prune out the disease, however pruning will reduce the inoculum level (Cameron 1979).

Chemical Control: Although fungicides had not been tried against this disease, Fuller (1896) suggested the use of Bordeaux or other copper solutions to protect trees from infection. Since infection preceeds symptom expression by a year, fungicides must be evaluated for at least two years to determine their effectiveness (Cameron 1979). Once this two year lifecycle was understood, and fungicide

tests were continued over several years, a significant reduction in the number of new infections resulted from the use of copper or triazole fungicides. Efforts were made to eliminate infection courts by controlling bud mites with insecticide, however this was not as effective as fungicides at preventing disease (Cameron Sizemore & Gottwald 1980).

Resistance breeding: It has been demonstrated that resistance to Anisogramma Canker is a heritable trait. 50% of genetic hybrids between the resistant cultivar 'Gassoway' and susceptible cultivars were resistant (Cameron 1981). Genetic resistance is probably the best long term solution to the Anisogramma Canker problem. Cameron (1981) suggests that the best control in existing plantings will be the combined use of sanitation, fungicides and insecticides.

RESEARCH OBJECTIVES

Filberts are presently evaluated for resistance by planting them in an infected orchard and waiting for natural infections to occur. The study that follows seeks to shorten this selection process by developing a reliable method of artificially inoculating a susceptible variety and producing disease symptoms. Both ascospores and infected filbert tissue are used as sources of inoculum.

A pure culture of the fungus would provide another inoculum source for resistance screening, and would remove many obstacles to the study of disease etiology. The objectives of the second study are to develop a method of germinating Anisogramma anomala ascospores, and to grow the fungus in culture.

Inoculation of *Corylus* Cultivars and Species With *Anisogramma anomala*, and Measurements of Annual Canker Growth

INTRODUCTION

Anisogramma canker, also known as Eastern Filbert Blight, was responsible for the failure of European filberts, *Corylus avellana*, to become established in the eastern United States (Fuller 1896; Barss 1930). This disease has now invaded the edge of the major U.S. filbert growing area in the Pacific Northwest (Cameron 1976). Efforts to control the disease have been largely unsuccessful (Cameron 1979; Cameron, Sizemore, Gottwald 1980), however cultivars differ in susceptibility and genetic resistance has been transferred from the cultivar 'Gassoway' in crosses with susceptible varieties (Cameron 1981). The main commercial cultivar, 'Barcelona', is considerably more resistant than its pollenizer, 'Daviana' (Cameron 1976). Resistance breeding may be a solution to this disease problem if progeny can be systematically evaluated. Evaluation is currently done by planting seedlings among diseased trees and waiting for natural infections to occur, however spread of the disease is slow (Cameron & Gottwald 1977) and it takes many years to evaluate the seedlings. Less than 50% of artificial inoculations have produced symptoms in the most susceptible varieties in their most susceptible condition. When an infection does occur, there is a period of 12 - 16 months before symptoms become visible (Gottwald & Cameron 1980a). This study seeks to develop a reliable method of producing infections in susceptible *Corylus* cultivars and explores the possibility of shortening the latent period between infection and symptom expression. Growth of existing cankers on 'Barcelona' and 'Daviana' are compared.

MATERIALS & METHODS

Filbert seedlings were grown from nuts that were collected in September 1980 from C. avellana cv. Daviana trees at the Oregon State University research farm in Corvallis. Kernels were soaked overnight in water containing 40 ppm Gibberellin A7 to break dormancy, and then grown in pots containing a peat, sand, and perlite mix.

Two year old C. avellana trees were obtained from a commercial nursery. C. cornuta var. californica trees were dug in MacDonald Forest, north of Corvallis, and transplanted into 2 gallon containers.

Ascospore suspensions for greenhouse inoculations were prepared by soaking cankered twigs overnight in a large beaker of water, allowing the ascospores to discharge, and then filtering the spore suspension through cheesecloth to remove plant debris. Spore concentration was determined with a Levy corpuscle counting chamber.

Hypodermic Inoculation of Seedlings I. 45 'Daviana' seedlings were inoculated in a factorial experiment with the following treatments:

1. 2 inoculum concentrations - 110 and 2200 spores/ml., and a distilled water check.
2. 3 inoculation locations - terminal bud, 2nd axillary bud, 4th axillary bud.
3. 5 incubation temperatures: 4, 7, 10, 18 and 24 degrees C.

Plants were placed in their respective growth chambers 1 week prior to inoculation. Lights were timed to provide a 15 hour daylength. Seedlings were inoculated by infiltrating 0.1 ml of spore suspension into dormant buds with a hypodermic syringe. After 4 weeks in the growth chambers plants were moved to a greenhouse bench.

Drench Inoculation of Seedlings. 5 month old 'Daviana' seedlings

were pruned to a single stem, 20 cm. tall, and all leaves and side shoots were removed. A 500 ml. graduated cylinder was filled to the brim with a spore suspension, and plants were inoculated by submerging the entire stem into the inoculum for 5 seconds. The following treatments were examined in this experiment:

1. Wounding of stems prior to inoculation.
2. Placing inoculated plants under intermittant mist for one week.
3. 4 incubation temperatures following inoculation: 7, 13, 18 and 24 degrees C.

Half of the plants were wounded by scraping the bark off one side of the stem for a distance of 20 cm. with a razor blade. Following inoculation, plants were incubated at the controlled temperatures for 8 weeks, then moved to a greenhouse bench.

Hypodermic Inoculation of Seedlings II. 44 Daviana seedlings were each hypodermically inoculated into 3 axillary buds with a spore suspension that had given positive germination in the laboratory. Following inoculation, plants were incubated at either 7, 13, 18 or 24 degrees C, with a 12 hour daylength. Trees were moved to the greenhouse after 8 weeks.

Spray Inoculation of 2 Year Old Trees. 60 'Daviana' trees and 40 'Barcelona' trees were wounded prior to inoculation by scraping the bark for 50 cm. with a knife. 20 unwounded 'Daviana' trees were inoculated as a check. Inoculation was with an ascospore suspension sprayed over the entire stem with an atomizer. Post inoculation treatments of wounded trees were as follows:

1. 20 'Barcelona' and 20 'Daviana' trees - kept dormant in a cold chamber (2° C) for 2 weeks and then moved outside for the summer.

2. 20 'Barcelona' trees and 20 'Daviana' trees - placed in greenhouse (24° C) to force out new growth and after 2 weeks moved outside.

3. 20 'Daviana' trees - grown for 8 weeks in greenhouse, moved to cold chamber to go dormant for 8 weeks, then placed outside.

4. The 20 unwounded 'Daviana' trees were placed directly outside after inoculation.

Inoculation of Corylus cornuta var. californica. Two C. cornuta var. californica trees were hypodermically inoculated into all buds on each of the following dates: 1 February, 15 February, 29 February, 14 March, and 29 March 1980. Plants were kept under mist for 1 week after inoculation.

Inoculation of Germinating Corylus Seeds. 480 'Daviana' seeds were stratified for 4 months (2° C) in moist sand to break dormancy. They were germinated in a factorial experiment with 8 replicates of 10 seeds in the following treatments:

1. Inoculated or uninoculated seeds.

2. 3 incubation temperatures: 4, 16 and 24 degrees C.

Seeds were inoculated by soaking them in either an ascospore suspension or in distilled water for 24 hours. They were then planted in sand and incubated at the 3 temperatures above for 5 weeks before being moved to a greenhouse bench. After 12 weeks in the greenhouse, percent germination was determined.

Field Inoculation. Inoculations were made in 10 year old trees in the orchard by removing a circle of bark with a 10 mm. cork borer and replacing the tissue with a piece the same size taken from a diseased tree, either immediately above or below an active canker.

Each inoculation was then wrapped well with plastic electrical tape. Inoculations were made once a month from 20 January, to 20 October, 1980. On each date, 10 inoculations were made on 'Daviana' trees and 20 inoculations were made on 'Barcelona' trees. During each of the first five months, 2 inoculations were made on potted C. cornuta var. californica trees.

Canker Measurements. During December 1980, 173 active cankers were measured on 2 - 5 year old suckers of both 'Barcelona' and 'Daviana' trees. Measurements were made of the stem diameter, the total length of the canker, and the distance that each canker spread both up and down during the previous growing season.

RESULTS and DISCUSSION

In these experiments, 344 trees were inoculated with Anisogramma anomala ascospores, many of these having multiple inoculations, and 310 graft inoculations were made using diseased Corylus tissue. 15 months after inoculation a single infection resulted from all of these treatments. This canker was on a 'Daviana' seedling which had been hypodermically inoculated in a single axillary bud with an ascospore suspension containing 2200 spores/ml. The tree was kept in a growth chamber at 4 degrees C. for 4 weeks and then placed in a greenhouse. A second tree had developed an infection 54 months after inoculation. This tree received the same treatment as the one above but was incubated at 16 degrees C.

Gottwald and Cameron (1980a) were able to achieve as much as 8.6% successful inoculations in greenhouse grown 'Daviana' seedlings similarly inoculated. The reasons for low infection rate in this

study are not known, however natural spread of this disease in the orchard is slow (Gottwald & Cameron 1980a) and the success of artificial inoculations are most likely limited by the same factors that are limiting natural spread. There are additional inoculation techniques that might be used if the pathogen could be grown in culture (Waterston 1968) however all attempts to grow Anisogramma anomala in culture have been unsuccessful (Cameron & Gottwald 1977, Gottwald & Cameron 1980a).

Failure of the graft inoculations to produce an infection may have been due to the difficulty of forming a graft union in Corylus. Filberts are notoriously difficult to graft successfully in the field (Lagerstedt 1971). Nearly all of the graft inoculation sites produced a callus ridge around the inoculum piece which may have prevented the mycelium from penetrating healthy tissue. Cameron (unpublished) has also been unable to transmit this disease by grafting.

Table 1.1

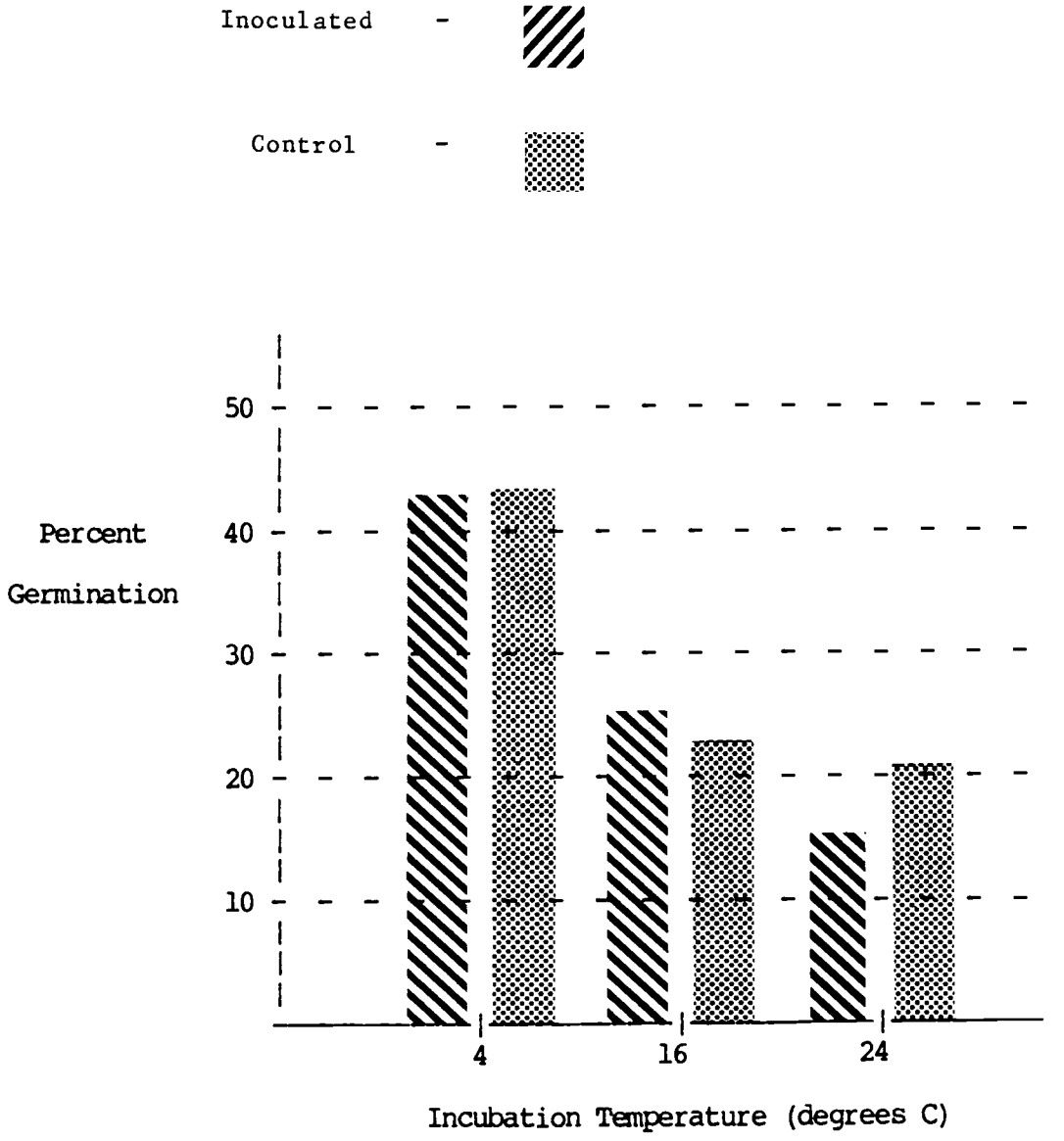
Analysis of Variance for Corylus avellana seed germination in Figure 1.1.

Source of Variation	df	SS	MS	F	
Treatments	5	59.250	11.850	4.08	**
Inoculation	1	.083	.083	.028	ns
Temperature	2	57.125	28.560	9.831	**
Inoc. x Temp.	2	2.042	.979	.337	
Error	42	122.000	2.905		
Total	47	181.250			

There was no significant difference in germination between Corylus avellana seeds that were inoculated with Anisogramma anomala ascospores and seeds that were not inoculated (Figure 1.1, Table 1.1). None of the resulting trees developed symptoms of Anisogramma Canker

FIGURE 1.1

Germination after 12 weeks of Corylus avellana seeds inoculated with Anisogramma anomala ascospores. Means of 8 replicates per treatment.



in two years.

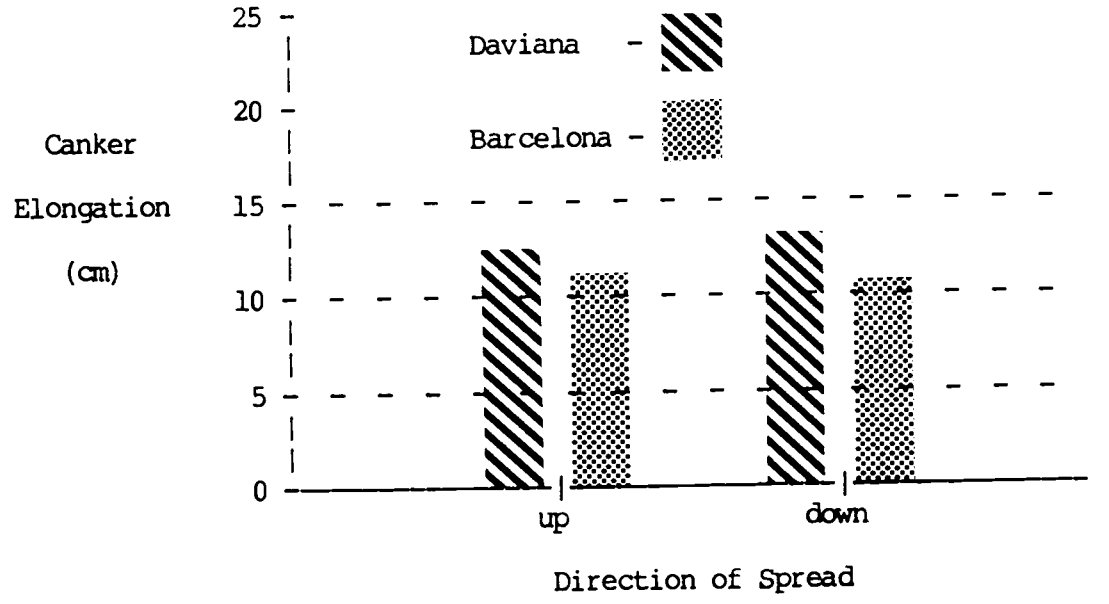
Vertical elongation of perennial cankers was found to average 26.2 cm. in 1980 (Figure 1.2). There was no significant difference in canker growth between the cultivars 'Daviana' and 'Barcelona', nor was there a significant difference between the spread up and spread down. There was a significant difference (t.01) in canker elongation between stems less than 2 cm. and stems greater than 2 cm. diameter. Average total growth of these cankers was 23.6 and 28.0 cm. respectively. Stem diameter is related to the age of a perennial canker since infections originate on small twigs (Barss 1930) and larger stems generally contain older cankers. These results are in agreement with Gottwald and Cameron (1980b) who calculated average annual spread to be 31.7 cm./year over a 4 year period. They found that yearly increase in canker length was about 18 cm. the first year and greater each subsequent year. Fungal mycelium has previously been found to spread up and down the stem at the same rate (Gottwald & Cameron 1980a).

It has been demonstrated that galled buds induced by the eriophyid mites Phytocoptella avellanae and Cecidophyopsis vermiformis act as infection courts for Anisogramma Canker, and that resistance to the disease is related to resistance to the mites (Cameron 1976; Gottwald & Cameron 1980a). This seems to be the case for the susceptible cultivar 'Daviana' and the resistant cultivar 'Barcelona'. Once infected, however, the rate of spread of a canker is the same in these two cultivars. The direct correlation between bud mite resistance and Anisogramma resistance does not hold for other Corylus species and cultivars. Corylus cornuta is susceptible to bud mite yet appears to be resistant to Anisogramma, and the filbert cultivars

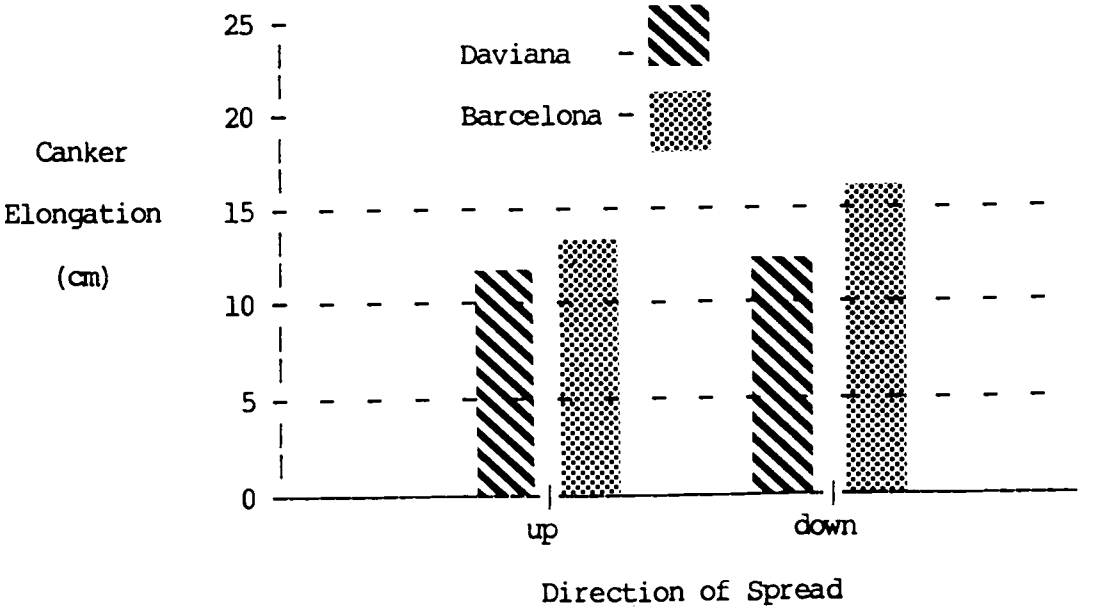
FIGURE 1.2

Annual increase in Anisogramma Canker lengths during 1981.

A - Stem diameter less than 2 cm.



B - Stem diameter greater than 2 cm.



'Montebello' and 'Jemtegaard-5' are resistant to bud mite, yet susceptible to Anisogramma (Thompson 1977; Cameron, Sizemore, Gottwald 1980). If other resistance mechanisms are to be discovered and breeding lines are to be fully evaluated, there first must be a reliable method of inoculation that by-passes the obvious mechanical barrier of a tightly sealed bud.

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Studies on Ascospore Germination in *Anisogramma anomala*

INTRODUCTION

Anisogramma anomala causes a stem canker in European filberts, *Corylus avellana*, that may render trees commercially worthless within a few years (Barss 1930, Fuller 1896, Gottwald & Cameron 1980b). Research on the biology of *A. anomala* and on disease dynamics have been limited by the inability to grow this fungus in culture, and by the difficulty of artificially inducing symptoms by inoculation. All attempts to culture the organism, either by germinating ascospores or isolating from diseased stems, have been unsuccessful (Cameron & Gottwald 1977, Gottwald & Cameron 1980a). Pretreatment with desiccation, washing, heating, freezing, and chemical treatments with furfural, acetic acid and alcohols have triggered germination of other ascospores (Gottlieb 1978, Sussman et al 1959, Sussman & Halvorson 1966), however all of these treatments failed to influence germination of *A. anomala* ascospores. The ability to grow the fungus in culture would remove many obstacles to the study of its morphology and disease epidemiology. Disease resistant germplasm is available and resistance is easily transferred by breeding (Cameron 1981), however, screening of the progeny involves waiting many years for natural infections to occur. A pure culture would enable the development of inoculation techniques to facilitate resistance screening. This study was undertaken to develop a method for storing *A. anomala* ascospores without losing viability, and to identify conditions that are suitable for ascospore germination *in vitro*.

MATERIALS & METHODS

Diseased twigs bearing current season's stromata were collected from an infected orchard in Clark County, Washington, beginning 25 August, 1980, and at monthly intervals through March, 1981. Twigs were sealed in plastic bags and stored either in a refrigerator (7°C), or in a freezer (0°C).

Preliminary experiments using triphenyl tetrazolium chloride (TTC) to determine spore viability indicated that ascospores will produce a short germ tube on the surface of agar media when covered with a glass coverslip. Germination proved to be a better indication of spore viability than the red color resulting from the metabolism of TTC.

Ascospores were prepared for the germination studies by dissecting perithecia out of the stromata under a dissecting microscope and crushing the perithecia in a small volume of distilled water. After the spores were mostly discharged (approximately 15 minutes) the perithecial remains were removed with a fine forceps.

Ascospores were germinated by aseptically placing a single droplet of spore suspension, either with a pasteur pipette or with the end of a glass rod, onto the surface of an agar medium in a petri plate. A 22 mm square, sterile glass coverslip was placed over each inoculation. Unless otherwise indicated, germination was on sterile 1% water agar.

Percent germination was determined by examining plates under a compound microscope at 160 X and evaluating the spores in the center of the microscope field while scanning across the plate. The criterion of germination was the formation of a germ tube whose length

was greater than or equal to its width. Areas of extremely high or low inoculum density, and bacterial colonies were avoided while making germination counts.

Time Required to Germinate. Plates of water agar were inoculated with an ascospore suspension and incubated at either 15, 20 or 25 degrees C. Percent germination of 500 spores was determined for each plate after 24, 48, 96, 144 and 264 hours.

Storage of Inoculum. To determine if viability decreases as twigs are stored, freshly collected cankered twigs were cut into 15 cm sections and placed in covered plastic boxes. Twigs from the susceptible variety 'Daviana' were kept separate from twigs of the more resistant variety 'Barcelona'. Boxes were placed either on the laboratory bench at room temperature (22°C), in a refrigerator (7°C) or in a freezer (0°C). At 3, 6, 10, 16, 25 and 56 days an ascospore suspension was prepared from a twig of each variety from each storage temperature. A plate of water agar was inoculated with each spore suspension and percent germination of 300 spores per plate was determined after 48 hours at 20 degrees C. A power outage after 56 days caused the thawing of freezer contents and the termination of this experiment.

To study the viability of ascospores stored as a suspension in distilled water, a spore suspension was prepared and divided between two vials. One vial was stored at 22 degrees C and the other was stored at 7 degrees C. At 0, 1, 2, 3, 4, 7 and 14 days, a plate of water agar was inoculated with each spore suspension. Percent germination was determined for each plate after 48 hours incubation at 22 degrees C.

To determine if ascospores are viable following drying, droplets

of spore suspension were dried onto sterile glass coverslips. Cover slips were stored either at 22 degrees C or at 7 degrees C for 2, 3 or 7 days. Viability was determined by inverting coverslips onto the surface of water agar with the spores against the medium, and incubating for 48 hours at 22 degrees C.

Effect of Temperature. Water agar was inoculated with a fresh ascospore suspension and plates were incubated at 5, 10, 15, 20, 25 and 30 degrees C. Percent germination was determined by counting 300 spores per plate at 48 hours and again at 72 hours.

Another spore suspension was prepared and stored at 7 degrees C. After 4 days, plates of water agar were inoculated and incubated at each of the 6 temperatures above. Percent germination of 500 spores per plate was determined at 48 hours and again at 8 days (192 hours).

Effect of Light. Inoculated plates were incubated in 2 growth chambers either under 24 hours continuous fluorescent light or under 12 hours light/12 hours dark. Additional plates were wrapped in 2 layers of aluminum foil to ensure total darkness and placed in each chamber. Percent germination of 300 spores per plate was determined after 48 hours at 20 degrees C. There were 2 replicates per treatment, each using a different spore suspension.

Effect of pH. The following buffer solutions were prepared according to Cruikshank et. al. (1975) using sterile distilled water:

Buffer	Range of pH Adjustment
Citrate Phosphate (citric acid + dibasic sodium phosphate)	2.6 - 7.0
Citrate (citric acid + sodium citrate)	3.0 - 6.2
Phosphate (monobasic sodium phosphate + dibasic sodium phosphate)	5.7 - 8.0

Buffers were prepared at twice the concentration desired. Water agar and potato dextrose agar (PDA) were prepared at twice the concentration desired and autoclaved at 121 degrees C for 15 minutes. Buffers were not autoclaved.

Six different citrate phosphate buffers spanning the pH range above were each mixed with an equal volume of water agar so that they diluted each other to the desired concentration. Five different citrate buffers and six different phosphate buffers were likewise mixed with equal volumes of PDA. The resulting pH of each buffered medium was determined with an Orion pH meter. Plates were inoculated with a spore suspension and percent germination of 300 spores per plate was determined after 72 hours at 15 degrees C.

Effect of Ascospore Concentration. Serial dilutions of fresh ascospore suspensions were prepared with sterile distilled water. Spore concentration of each dilution was determined with a Levi corpuscle counting chamber. Petri plates containing either water agar, 1/2 strength PDA, or 1/4 strength PDA were inoculated with the various spore suspensions. In some instances 10 ppm of Rifampicin was added to the concentrated spore suspensions.

Preventing Bacterial Contamination. In an attempt to decrease the bacterial contaminants which were always present when ascospores were removed from field collected stems, several antibiotics were screened both for their effect on ascospore germination and for their effectiveness against bacterial contamination. Stock solutions of 8 antibiotics were prepared and mixed with autoclaved water agar while the agar was still liquid, resulting in concentrations of 10 and 100 ppm for each antibiotic. A second series of plates was prepared

containing each antibiotic at 100 ppm with 10 ppm of Rifampicin added to each. Plates of 1/2 strength Bacto PDA were prepared with each of the three most effective antibiotics at the same two concentrations above. Control plates of both water agar and PDA were prepared with no antibiotic. Plates were inoculated with a fresh spore suspension having a concentration of 2×10^6 spores per ml. Percent germination of 200 spores per plate was determined after 5 days, and each plate was rated for bacterial contamination.

Effect of Host Extracts. Water extracts of various filbert tissues were prepared by grinding .5 g of either young stems, leaves, or mite galled buds in 20 ml distilled water with a mortar and pestle. Plant debris was removed by filtration. Extracts were mixed 1:1 (v:v) with water agar and either autoclaved at 121 degrees C for 15 minutes, or pasteurized at 90 degrees for 10 minutes. Plates were inoculated with an ascospore suspension and incubated either for 48 hours at 15 degrees C or for 5 days at 10 degrees C. Percent germination of 200 spores per plate was determined after incubation.

Stem extracts of both healthy and diseased trees, and of both a susceptible and a resistant variety were prepared as follows: 1.2 g of stem pieces were cut into small pieces with a razor blade, and soaked in 5 ml of either ethanol, acetone or xylene for 30 minutes. Plant debris was removed by filtration and samples were diluted to 100 ml with distilled water. 10 ml of each sample was mixed with 20 ml of autoclaved water agar and poured into a petri plate. The final solvent concentration in the media was 1.7%. Water extracts were similarly prepared as controls. Plates were inoculated with a fresh spore suspension and incubated at 15 degrees C. Percent

germination of 200 spores per plate was determined after 72 hours.

Ethanol extracts of healthy filbert stems and buds were prepared by soaking 1 g of chopped plant tissue in 5 ml of ethanol for 30 minutes, filtering out plant debris, and evaporating to 2 ml. Extracts were diluted to 50 ml with sterile distilled water and 1 ml of each solution was mixed with an equal volume of fresh ascospore suspension. The final ethanol concentration in the suspension was 2.0%. Distilled water and diluted ethanol were mixed with spores as controls. Water agar and PDA plates were inoculated and incubated at 15 degrees C. Percent germination of 200 spores per plate was determined after 48 hours.

Effect of Media and Atmosphere. Ascospore germination was compared on various natural agar media, and on defined media with varying concentrations of ethanol, sucrose, nitrate and agar. Atmosphere studies were done inside 12 liter glass bell jars sealed against a sheet of glass at the base with petroleum jelly. The atmosphere inside one jar was modified by placing a burning candle in the sealed jar with inoculated plates and allowing it to burn out. For the atmosphere studies plates were inoculated by streaking the ascospore suspension across the agar surface with the end of a glass rod and placing a coverslip over a portion of the inoculation.

In another series of tests, ascospore suspensions were mixed with still warm agar media, distributing the spores throughout the medium, which was then poured into petri plates and allowed to harden.

Analysis of variance and Tukey's multiple comparisons were performed on an IBM personal computer using Statistical Processing System (S.P.S.) version 4.0 (Kirk et. al. 1983).

RESULTS

No germination was observed with ascospores collected in August and germination was eratic, never exceeding 25% with spores collected in September. Satisfactory ascospore germination could be obtained from October to May. By the end of May, nearly all the spores in the orchard had been discharged and fresh inoculum was no longer available.

Cover slips were initially placed on the surface of inoculated media to facilitate making observations under the compound microscope. When it was observed that only spores under the coverslip germinated, this became the standard germination method. Water agar was the preferred medium for initial experiments since bacterial contaminants did not grow well on this medium.

Time to Germinate - Optimum Temperature. Ascospore germination commenced about 24 hours after spores were hydrated. Peak germination was achieved after 48 to 72 hours at 20 degrees C, and after 6 days at 15 degrees C (Figure 2.1). The optimum temperature for germination was approximately 20 degrees C (Figure 2.1, Figure 2.4), however by the end of 8 days there was no difference in germination between spores incubated at 5, 10, 15 and 20 degrees C (Figure 2.5). No ascospore was ever observed to germinate at 30 degrees C, even after being returned to a lower temperature.

Viability of Stored Inoculum. There was no difference in germination ($T = .05$) between ascospores collected from the susceptible cultivar 'Daviana' and spores collected from the less susceptible 'Barcelona'. These two spore sources were therefore treated as replicates for the data in Figure 2.2 and Table 2.1. No significant change in spore viability was observed during the 56 days that

cankered twigs were stored as a source of inoculum. Twigs stored at 0°C and at 22°C produced spores with a significantly higher percent germination than twigs stored at 7°C (Figure 2.2, Table 2.1). Frozen stromata were preferred as they were easier to dissect.

The viability of ascospores that had discharged into water declined quickly when the suspension was stored at 22 degrees C. However a suspension kept at 7 degrees increased in viability for 4 days before gradually declining. At 22 degrees, germination dropped below 10% after 3 days, but at 7 degrees, germination did not drop below 10% until after 14 days (Figure 2.3). This increase in viability for a few days after hydration was observed on several occasions. Increasing bacterial populations appeared to correlate with decline in spore germination for both of these treatments. Spores did not germinate in the water prior to being placed onto media. Germination of discharged spores that were dried and then rehydrated never exceeded 3 percent (Table 2.2).

Effect of Light. There was no significant difference between 0, 12 and 24 hours daily fluorescent light on germination (Figure 2.6).

Optimum pH. Spore germination on water agar with citrate-phosphate buffer was optimum at pH 4.2. The optimun pH on PDA with phosphate buffer was 5.7, however this was the lowest pH tested using this media-buffer combination, and a lower pH may have resulted in better germination (Figure 2.7). When the pH of PDA was adjusted with citrate buffer over the range 2.6 to 7.0, no ascospores germinated on any of the plates.

FIGURE 2.1

Time required for Anisogramma anomala ascospores to germinate on water agar at 3 incubation temperatures. Percent germination of 500 spores.

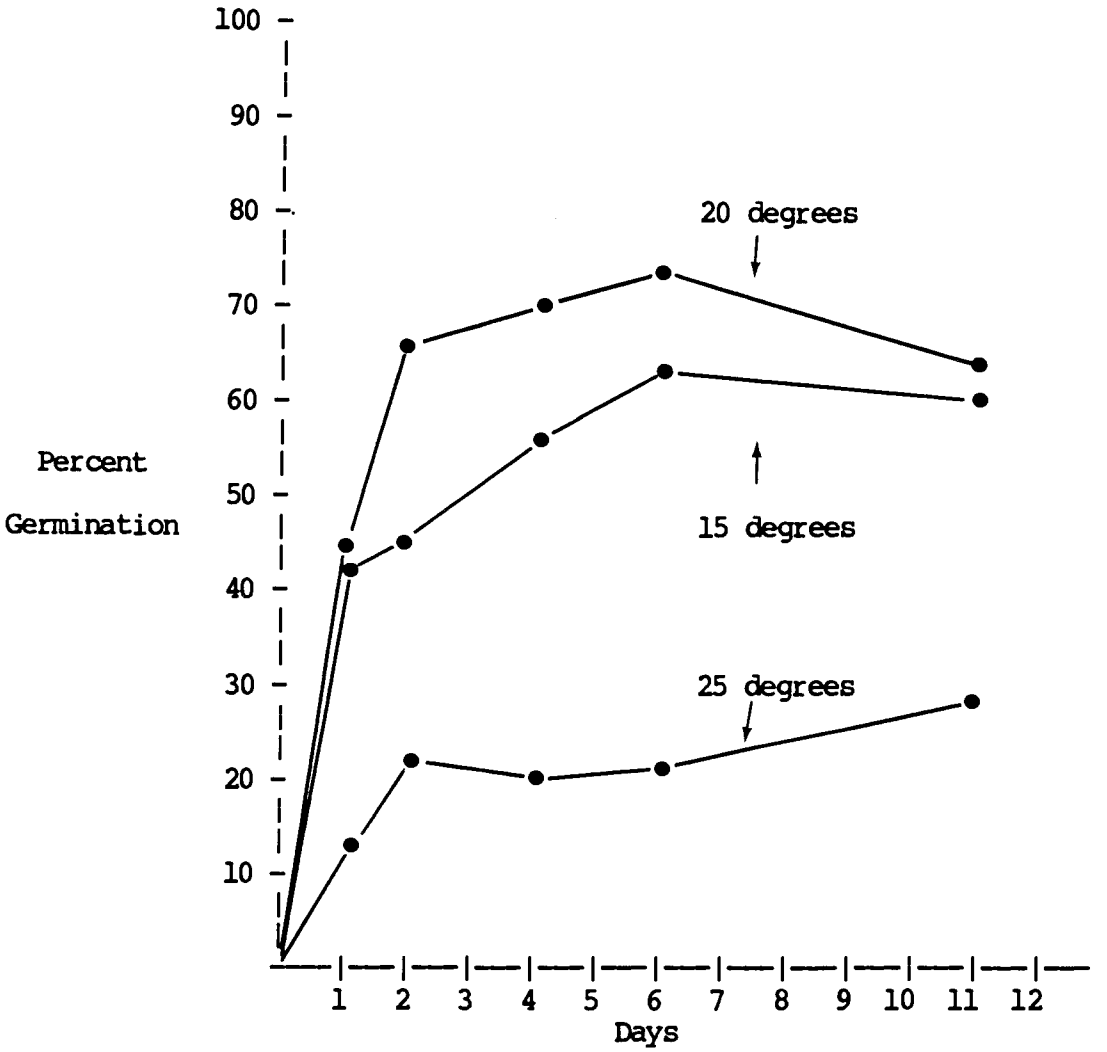


FIGURE 2.2

Viability of Anisogramma anomala ascospores in infected twigs stored at 3 temperatures. Percent germination of 300 spores. Means of 2 replicates.

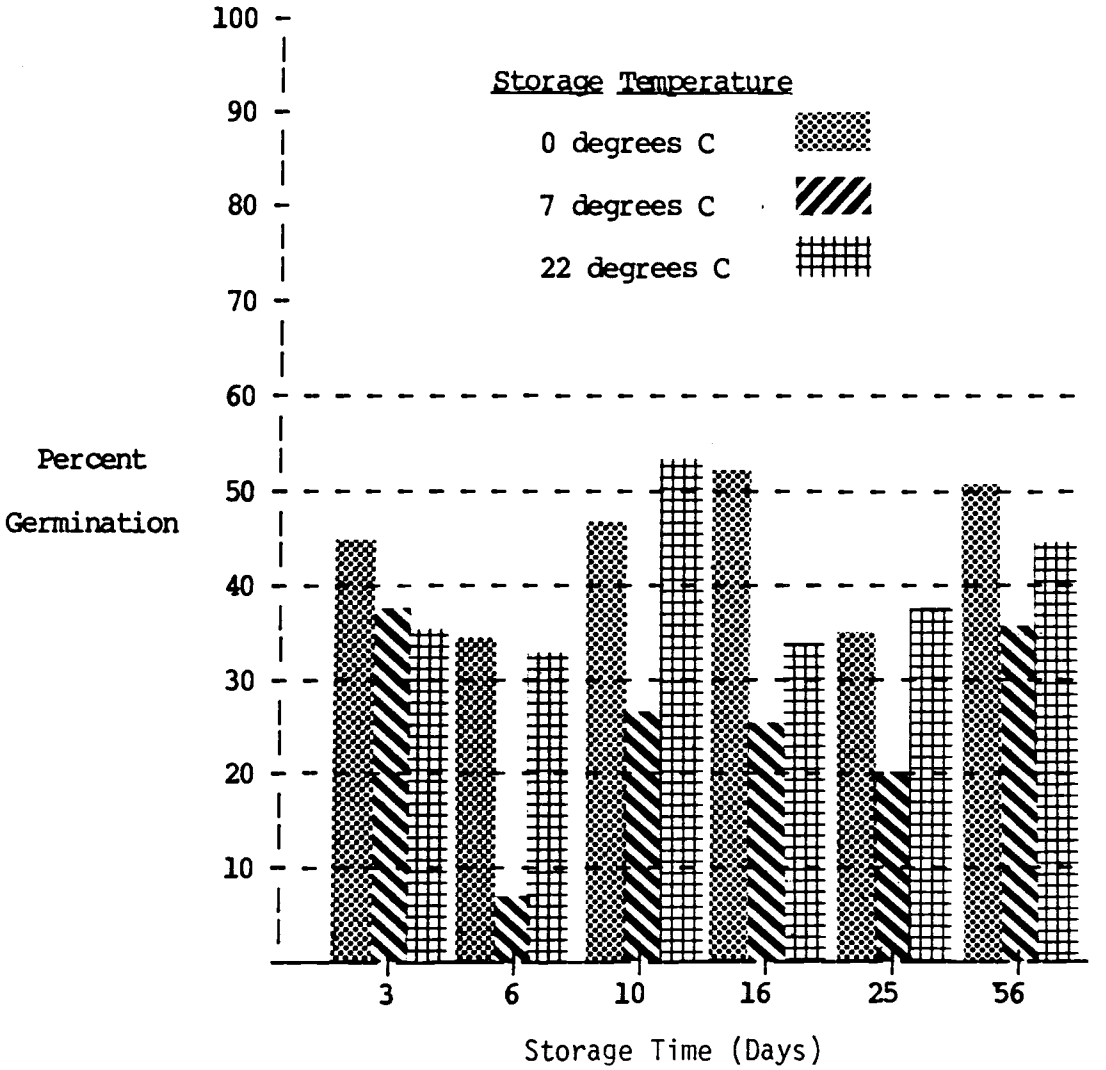


FIGURE 2.3

Viability of Anisogramma anomala ascospores stored as a suspension in distilled water at two temperatures. Percent germination of 300 spores after plating out on water agar.

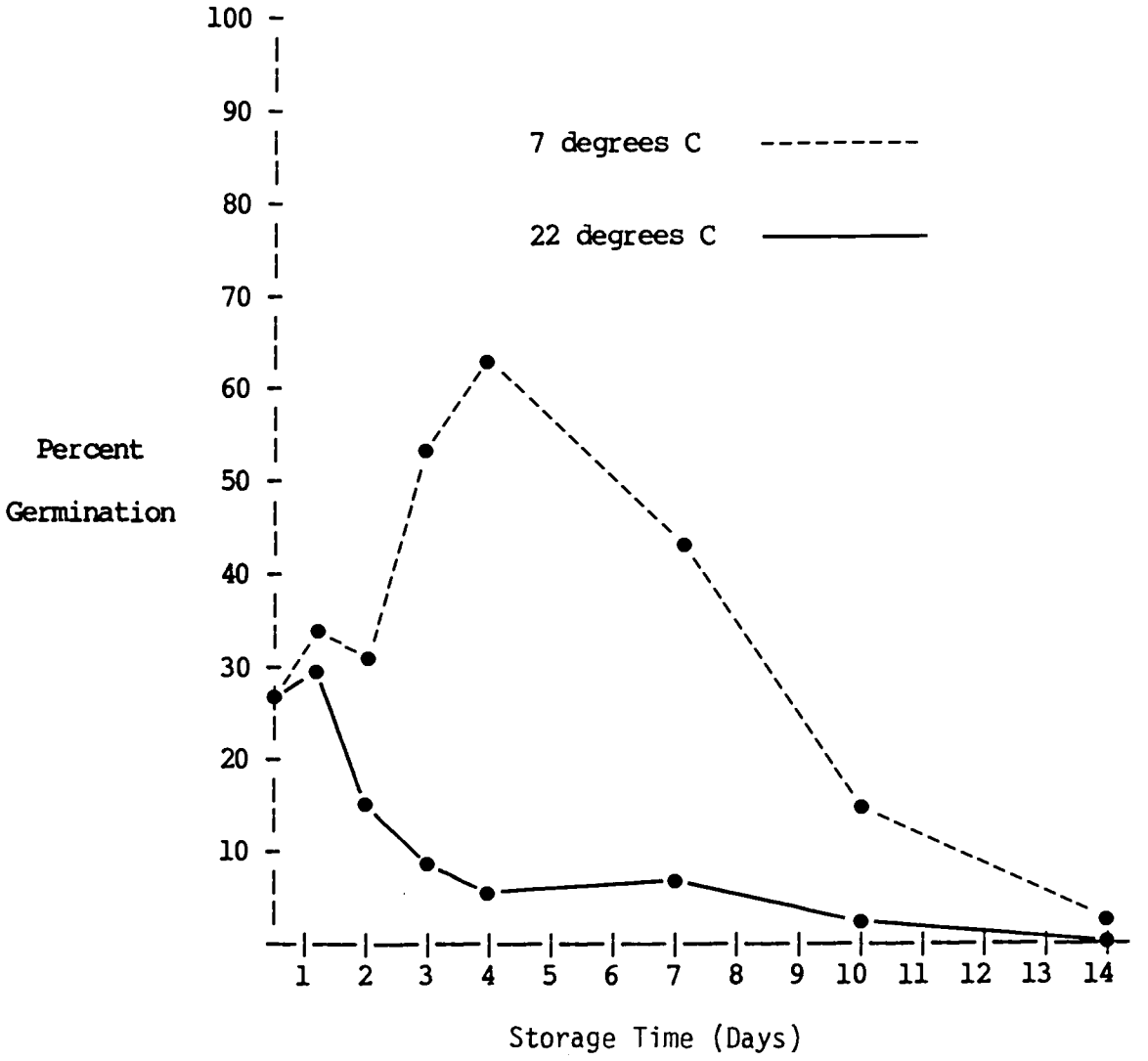


FIGURE 2.4

Optimum temperature for germination of Anisogramma anomala ascospores. Percent germination of 300 spores after 48 hours and after 72 hours. Means of 2 replicates.

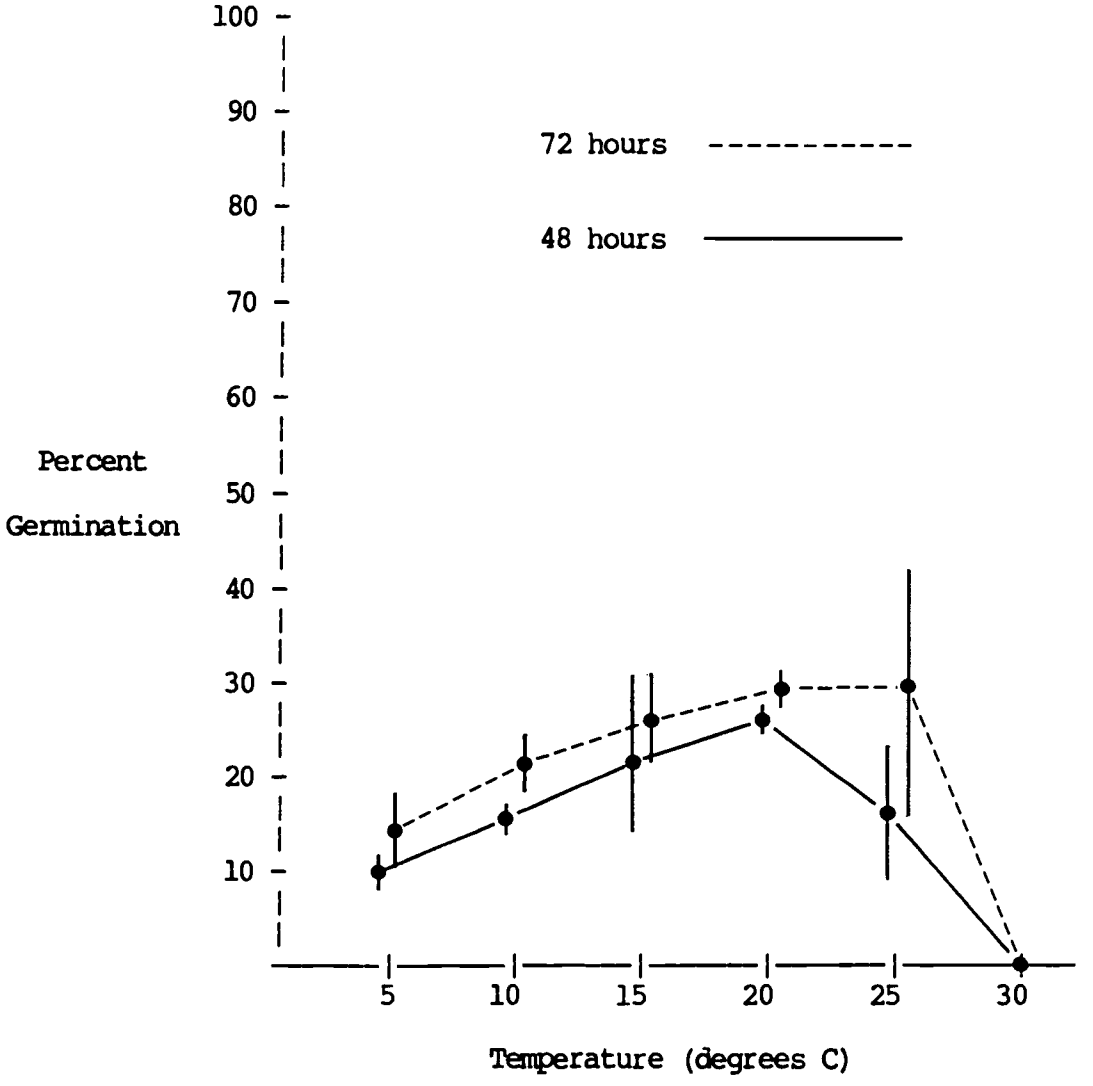


FIGURE 2.5

Optimum tempreature for germination of Anisogramma anomala ascospores. Percent germination of 300 spores after 48 hours and after 8 days. Spores stored in suspension for 4 days prior to plating out.

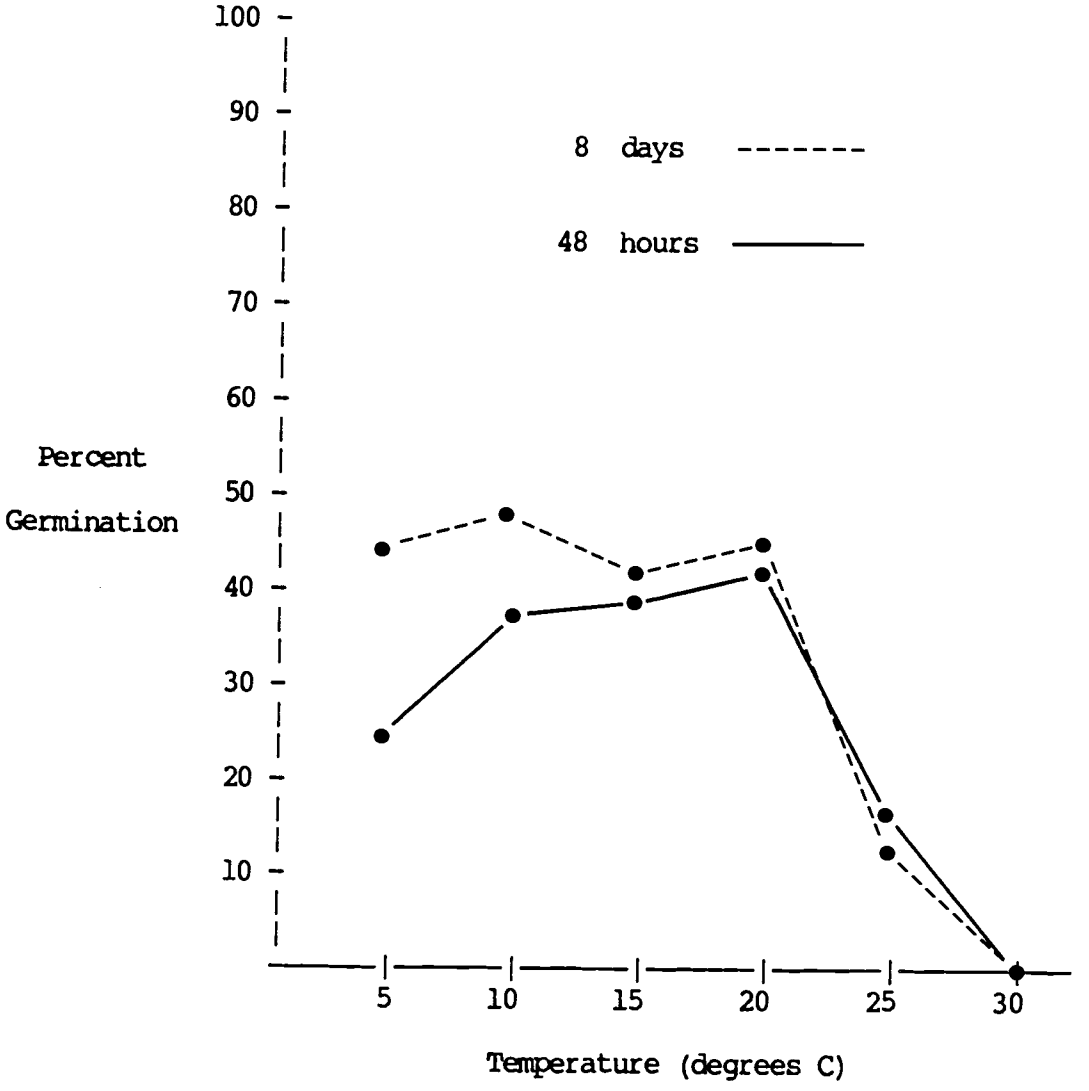


FIGURE 2.6

Effect of fluorescent light on germination of Anisogramma anomala ascospores. Percent germination of 300 spores after 48 hours. Means of 2 replicates.

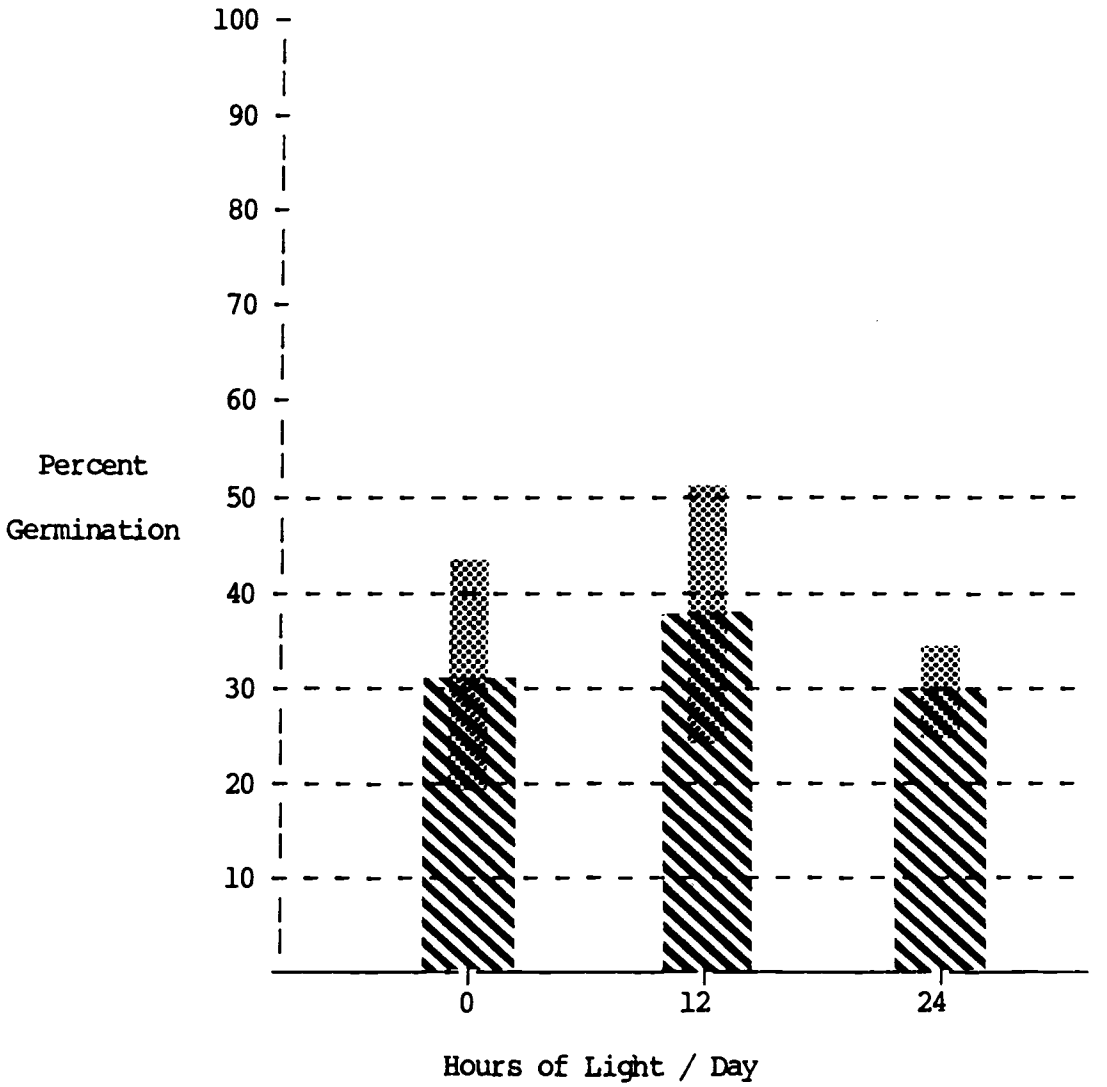


TABLE 2.1

Viability of Anisogramma anomala ascospores from stromata stored at 3 temperatures. Percent germination of 300 spores after 48 hours at 20 degrees C. Means of 2 replicates.

Storage Temperature (degrees C)	Storage Time (days)						
	3	6	10	16	25	56	$\bar{X}$
0	45.2	35.0	48.0	49.0	35.8	59.3	45.4 a
7	37.0	7.7	26.5	25.4	20.4	36.9	25.7 b
22	35.3	32.5	53.3	34.1	38.0	45.3	39.8 a

Means followed by the same letter are not significantly different. Tukey's LSD (.01) = 10.4

TABLE 2.2

Viability of Anisogramma anomala ascospores following drying. Percent germination of 300 spores after 48 hours at 22 degrees C. Means of 2 replicates.

Time Stored (days)	Storage Temperature (degrees C)	
	7	22
0	32.0	35.3
1	0.5	2.7
3	0	1.0
7	0	1.3

Effect of Spore Concentration. A direct relationship was found between ascospore concentration and percent germination. When percent germination was plotted against $\log_{10}$ spore concentration, the relationship is nearly linear between 10^4 and 10^6 spores per ml. (Figure 2.8, Figure 2.9). The data from figures 2.8 and 2.9 are summarized in figure 2.10. Addition of 10 ppm Rifampicin to the undiluted spore suspension significantly improved germination at the higher spore concentrations (Figure 2.8). The storing of concentrated ascospore suspensions for 24 hours prior to dilution did not improve germination of the dilute samples. When various media were compared using two spore concentrations (Table 2.3), germination was significantly higher ($P=.01$) at 3×10^6 than at 5×10^5 spores/ml.

Preventing Bacterial Contamination. When incorporated into water agar, Ampicillin and Penicillin did not inhibit bacterial growth. Chloramphenicol, Cyclohexamide and Gentamicin inhibited ascospore germination. Kanamycin, Rifampicin and Streptomycin inhibited bacterial contamination without affecting ascospore germination. By combining Rifampicin with each of the test antibiotics, it was shown that any inhibition of germination was caused by a direct effect of the antibiotic, and not by an inability to control the bacteria (Table 2.4). At 100 ppm, Rifampicin, Kanamycin and Streptomycin were equally effective at eliminating bacterial contamination on PDA with no inhibition of spore germination. Only Rifampicin was effective at 10 ppm (Table 2.5).

Surface sterilization of perithecia in 50% ethanol or 2.5% sodium hypochlorite had no adverse effect on spore germination, as long as perithecia were well rinsed following the sodium hypochlorite treatment. The effect on bacterial contamination in the resulting

FIGURE 2.8

Effect of spore concentration on germination of Anisogramma anomala ascospores. Percent germination of 200 spores on 1/2 strength PDA.

A - Fresh spore suspension diluted and germinated immediately - no antibiotic.

B - Fresh spore suspension + 10 ppm Rifampicin diluted and germinated immediately.

C - Spore suspension stored 24 hours prior to dilution - no antibiotic

D - Spore suspension + 10 ppm Rifampicin stored 24 hours prior to dilution

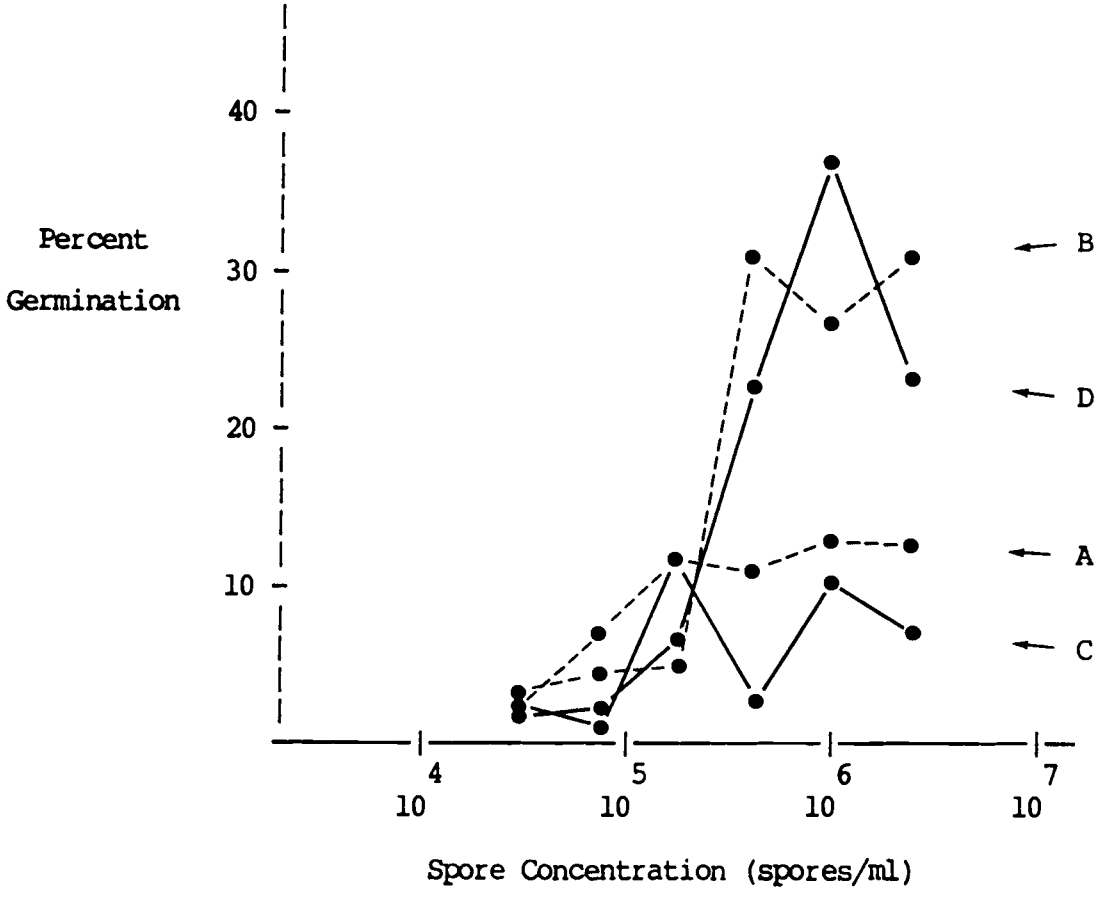


FIGURE 2.9

Effect of spore concentration on germination of Anisogramma anomala ascospores. Percent germination of 200 spores on either water agar or PDA.

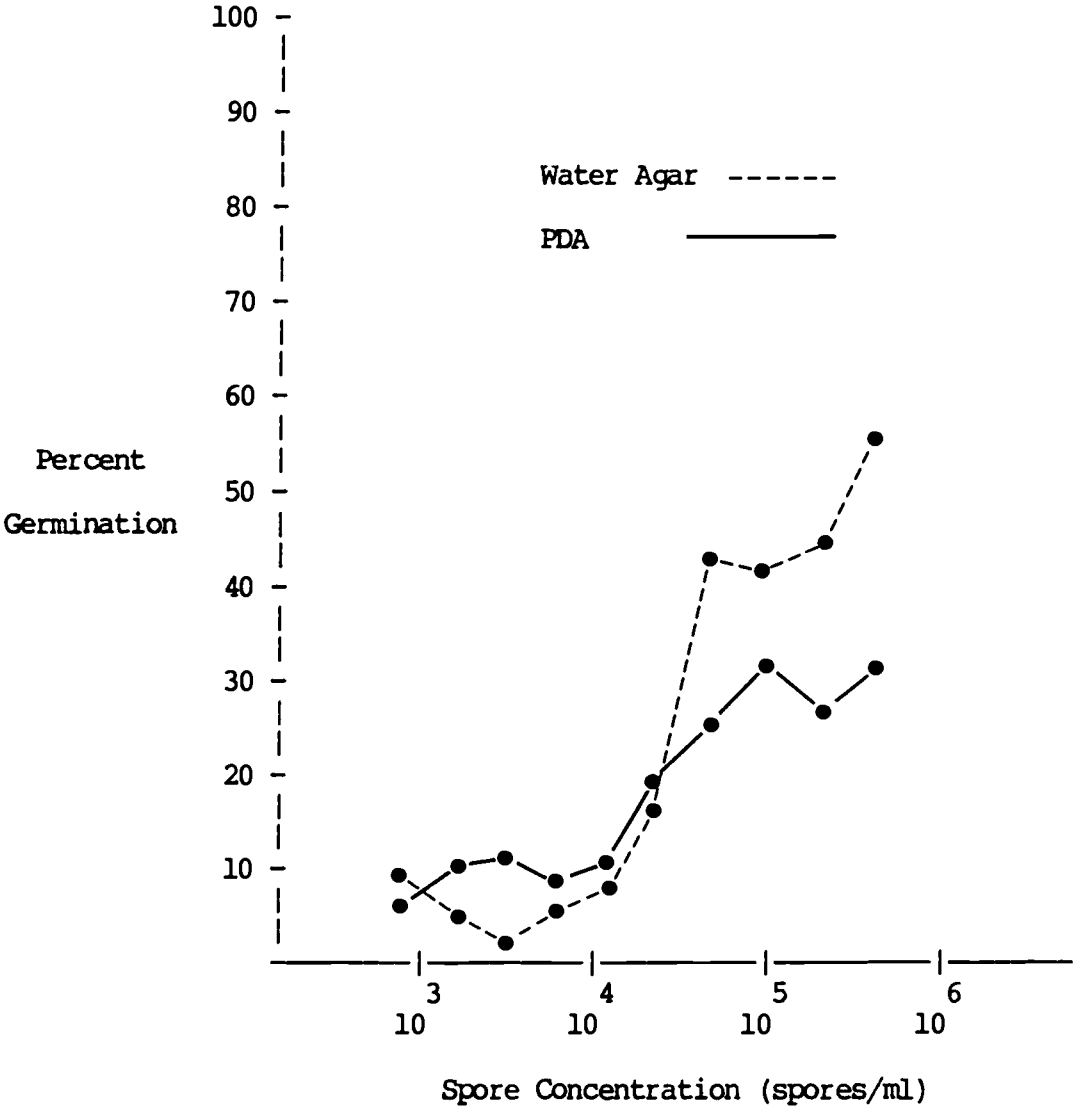


FIGURE 2.10

Effect of spore concentration on germination of Anisogramma anomala ascospores. Means of data from Figure 8 and Figure 9 converted to percent of highest germination for each experiment. Only replicates B and D were used from Figure 8.

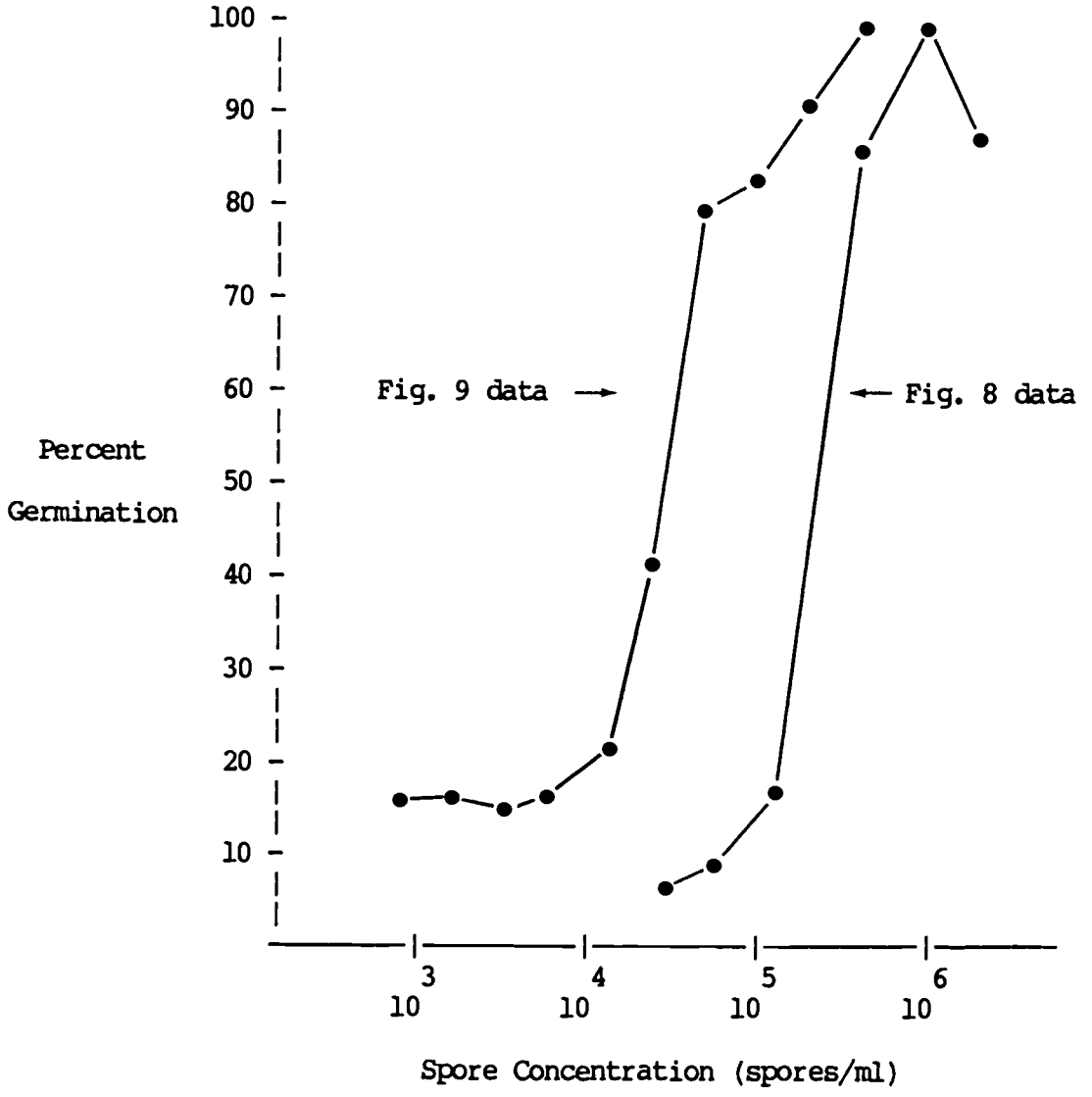


TABLE 2.3

Germination of Anisogramma anomala ascospores on various media. Percent germination of 200 ascospores at two spore concentrations. Media contains 10 ppm Rifampicin unless indicated.

Media	5×10^5 spores/ml (mean of 3 reps)	3×10^6 spores/ml (mean of 3 reps)	$\bar{X}$	
Water + 1% agar (no antibiotic)	22.0	22.3	22.2	bd
Water + 1% agar	12.8	39.5	26.2	d
Water + 1% agarose (no antibiotic)	0	0.2	0.1	ac
Czapek Dox agar	9.5	13.5	11.5	ab
Czapek Dox agar + 1 g/l L-cystein	8.3	23.2	15.7	bd
Czapek Dox agar + 1.0 g/l sodium thioglycolate	9.3	17.2	13.3	bc
Mueller Hinton agar	18.5	24.2	21.3	bd
$\bar{X}$	11.5 **	20.0 **		

Means followed by the same letter do not differ significantly.
Tukey's LSD (.05) = 12.12 for media means.
Tukey's LSD (.01) = 6.83 for concentration means.

TABLE 2.4

Effect of antibiotics on Anisogramma anomala ascospore germination and on bacterial contamination. Percent germination of 200 spores. Antibiotics in water agar.

Antibiotic	10 ppm		100 ppm		100 ppm (+ 10 ppm Rifampicin)	
		a				
Ampicillin	54.0	+	52.0	+	73.0	-
Chloramphenicol	0	-	0	-	0	-
Cyclohexamide	0	+	0	-	0	-
Gentamicin	13.5	-	10.0	-	9.0	-
Kanamycin	44.5	-	56.5	-	84.0	-
Penicillin	18.5	+	26.5	+	26.5	-
Rifampicin	71.5	-	43.5	-		
Streptomycin	66.5	+	24.0	-	41.5	-
Control 1	36.5	-				
Control 2	54.0	+				

a. '-' = no bacteria
'+' = bacteria present

TABLE 2.5

Effect of antibiotics on Anisogramma anomala ascospore germination and on bacterial contamination. Percent germination of 200 spores. Antibiotics in PDA.

Antibiotic	10 ppm		100 ppm	
		a		
Kanamycin	61.5	++	56.5	-
Rifampicin	77.0	-	76.0	-
Streptomycin	59.0	++	60.5	-
Control 1	63.0	+		
Control 2	71.0	++		

a. '-' = no bacteria
'+' = bacteria present
'++' = extensive bacterial contamination

spore suspension, however, was negligible. The ethanol treatmant appeared to enhance spore germination (Table 2.6).

Effect of Host Extracts. Water extracts of various Corylus tissues, when incorportated in water agar, had no significant effect on germination (Table 2.7). Ethanol extracts of stems caused a significant increase in germination when incorporated in the medium. Acetone extracts of stems were no different from water controls, and there was no germination at all on medium containing xylene extracts (Table 2.8). When they were combined with the inoculum, ethanol extracts of various Corylus tissues had no effect on germination (Table 2.9).

Effect of Media. When different concentrations of ethanol were incorporated in the media, a slight increase in germination was observed between 0 and 1 percent. At 5 percent, ethanol was nearly lethal (Figure 2.11). A preliminary experiment with various concentrations of sucrose as the only carbon source produced no significant difference in germination between 0 and 5 percent sucrose, and total inhibition of germination at 25 percent. When no antibiotic was added to the media, spore germination was reduced at 1 and 5 percent sucrose, however this was likely the result of luxurious bacterial growth when sugar was present, and not a direct effect of the sucrose (Table 2.10). In a better replicated experiment using the same defined medium, but varying both the carbon and nitrogen sources, a significant increase in germination was observed at 5 percent sucrose over lower levels. The bacterial problem was eliminated with the addition of 10 ppm rifampicin to the media. No difference in germination was found between 0.1% and 0.5% nitrate (Table 2.11). By increasing the carbon dioxide and decreasing the oxygen in a sealed

FIGURE 2.11

Influence of ethanol in water agar on germination of Anisogramma anomala ascospores. Percent germination of 200 spores. Spore concentration = 5 x 10E5 spores/ml.

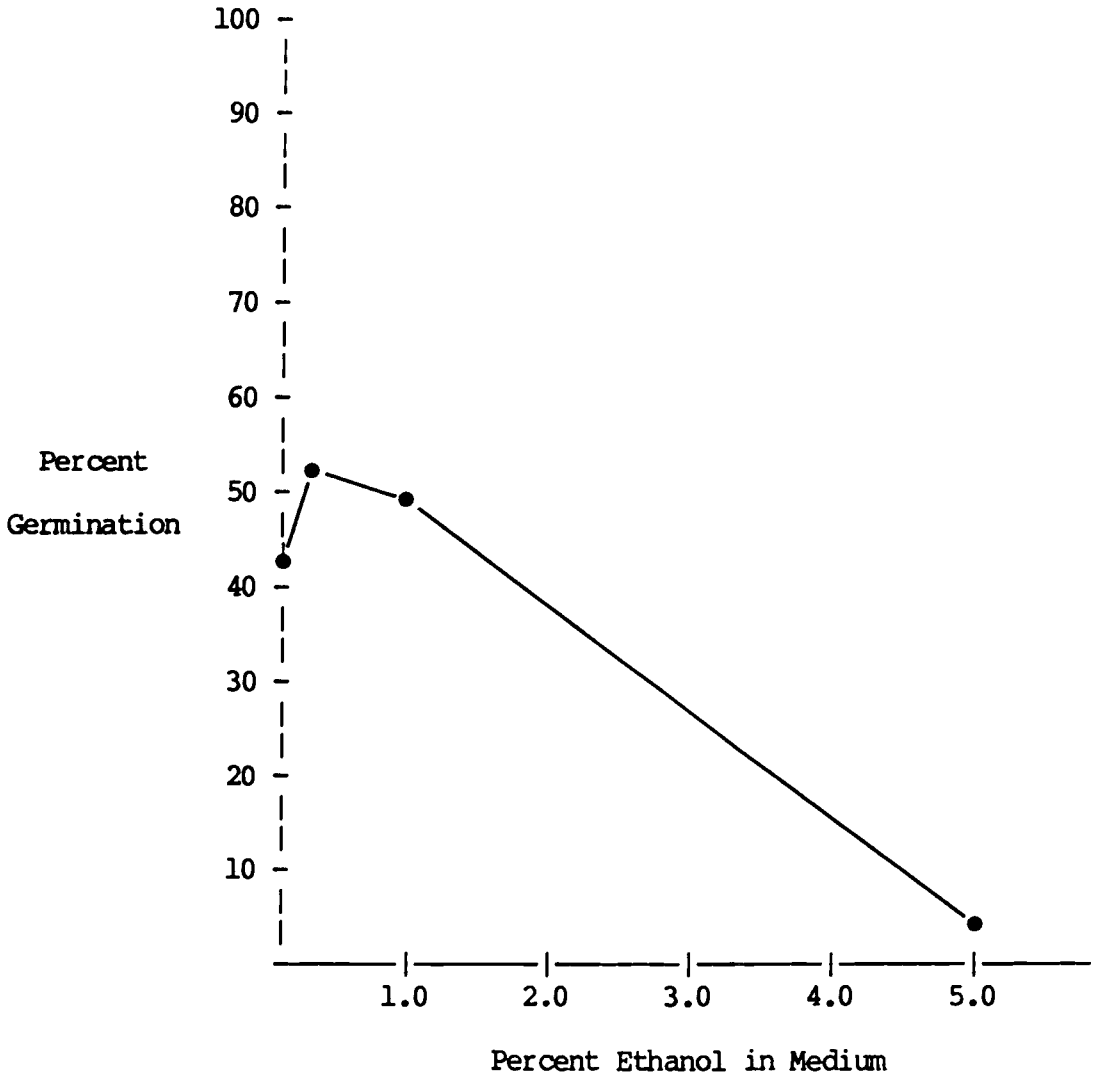


TABLE 2.6

Effect of surface sterilization for 30 seconds on germination of Anisogramma anomala ascospores. Perithecia surface sterilized in 50% ethanol (EtOH) or 2.5% sodium hypochlorite (NaHClO). Percent germination of 200 spores on water agar + 3 ppm streptomycin (A) or on water agar + 40 ppm streptomycin (B).

Disinfectant	Media A	Media B
50% Ethanol	70.5	88.5
2.5% Sodium Hypochlorite	58.0	63.5

TABLE 2.7

Effect of water extracts of filbert tissues on germination of Anisogramma anomala ascospores. Percent germination of 200 spores after either 48 hours at 15 degrees C, or 5 days at 10 degrees C. Means of 4 replicates. Extracts in media.

Extract	15°	10°	$\bar{X}$	(sd)
Stem	34.8	28.0	31.4 ns[a]	(10.6)
Leaf	32.8	22.5	27.6 ns	(10.9)
Big Bud	16.0	29.75	22.9 ns	(12.7)
Control	22.5	27.5	25.0	(7.2)
$\bar{X}$	27.1	26.9		

a - not signigicant Tukey's LSD (.05) = 14.99 for means

TABLE 2.8

Effect of filbert stem extracts on germination of Anisogramma anomala ascospores. Percent germination of 200 spores. Extracts in media.

Extract	Source of Stem Tissue: Infected Daviana	Healthy Daviana	Infected Barcelona	Healthy Barcelona	$\bar{X}$	
Water extract	39.5	38.0	22.5	35.5	33.8	a
Xylene extract	0	0	0	0	0	b
Acetone extract	30.0	21.5	25.5	36.5	28.4	a
Ethanol extract	60.5	77.0	49.0	66.0	63.1	c
$\bar{X}$	32.5	34.1	24.3	34.5		

Means followed by the same letter do not differ significantly.
Tukey's LSD (.01) = 20.71 for extract means.
Tukey's LSD (.05) = 15.4 for source means.

TABLE 2.9

Effect of ethanol extracts of filbert tissues on germination of Anisogramma anomala ascospores. Percent germination of 200 spores on either water agar or PDA. Extracts in inoculum.

Treatment	Water Agar	PDA
Stem Extract	61.0	54.0
Bud Extract	60.0	62.0
Ethanol Control	27.0	58.5
Water Control	55.0	63.5

TABLE 2.10

Effect of sucrose on Anisogramma anomala ascospore germination. Germination on Czapek media (Appendix 1) with varying concentrations of sucrose as the only carbon source. Percent germination of 200 spores.

Percent Sucrose	Antibiotic in Media	
	10 ppm Rifampicin	None
0	12.5	16.5
1.0	18.5	7.5
5.0	17.0	9.5
25.0	0	0

TABLE 2.11

Effect of sucrose and nitrate concentration on Anisogramma anomala ascospore germination. Germination on Czapek defined medium (Appendix 1) with varying sucrose and nitrate concentrations. Percent germination of 200 spores.

Percent Sucrose	Percent Germination (mean of 3 reps.) .1% Nitrate	.5% Nitrate	$\bar{X}$		(sd)
0.1	46.2	40.2	43.2	a	(5.90)
0.5	41.5	46.2	43.8	a	(8.18)
1.0	43.8	39.3	41.6	a	(5.76)
5.0	57.8	52.7	55.3	b	(9.23)
$\bar{X}$	47.3	44.6			

Means followed by the same letter do not differ significantly.
Tukey's LSD (.05) = 10.18 for sucrose means.
Nitrate means do not differ significantly (P=.05).

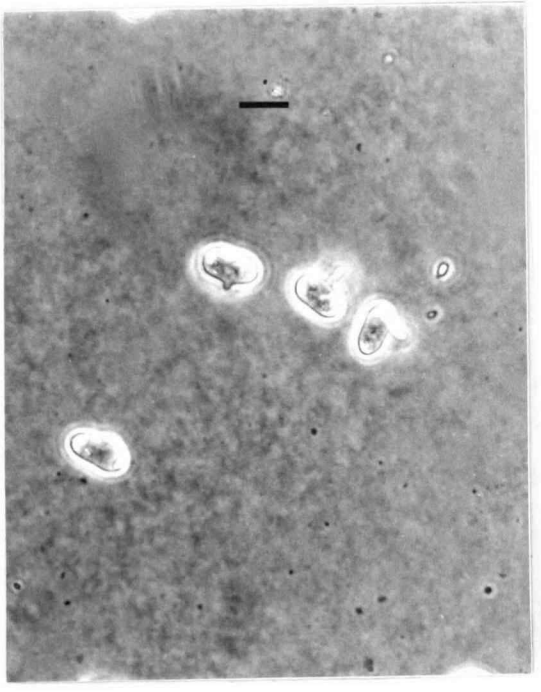
bell-jar, it was found that spores would germinate even when they were not covered by a glass coverslip. In a normal atmosphere, only spores sealed between a coverslip and the agar medium would germinate (Table 2.12).

Media containing cysteine or thioglycollate to provide reducing conditions had no effect on spores outside the coverslip. When agarose was substituted for agar, producing a medium almost totally lacking in nutrients, germination approached zero (Table 2.3). Percent germination on various complex prepared media was not significantly different from the water agar controls, however germ tubes were more likely to develop large vesicles when peptone, cornmeal, or casamino acids were incorporated into the media (Table 2.13).

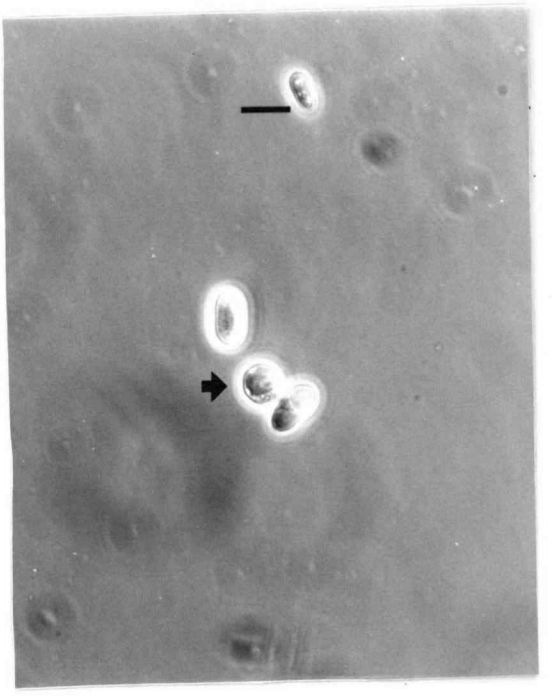
Description of Germinating Ascospore. The ascospore of Anisogramma anomala is hyaline and ellipsoid with a single septation dividing a small caplike cell from the rest of the spore. A single germ tube was typically observed to originate from the long side of the spore, at a right angle to the spore wall (Figure 2.12-A). Occasionally 2 germ tubes emerged from a single ascospore and rarely a germ tube emerged from the small end of a spore. It was not determined whether these apical germ tubes emerged from the "cap" cell or from the large cell, however it has been assumed that the small cell is sterile (Gottwald & Cameron 1979). About 24 hours after germination commenced, germ tubes were 2 - 3 um wide and 3 - 9 um long. At the end of 2 to 3 days germ tube elongation ceased. Germ tubes never exceeded 15 um in length on water agar and averaged less than 6 um. Germ tube length on PDA never exceeded 8 um. Between day 5 and day 14, the tips of many germ tubes began to swell, eventually forming a hyaline, globose vesicle, 5 - 10 um diameter (Figure

FIGURE 2.12

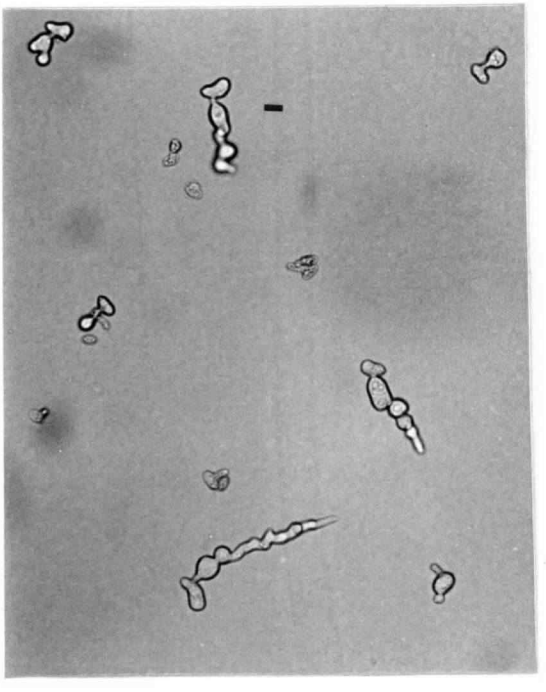
A. Typical germ tube emergence. Bar = 10 um.



B. Germ tube has swollen to form a vesicle. Bar = 10 um.



C. Budding of secondary vesicles and emergence of secondary germ tube. Bar = 10 um.



D. Cluster of swollen vesicles and a secondary hypha. Bar = 10 um.

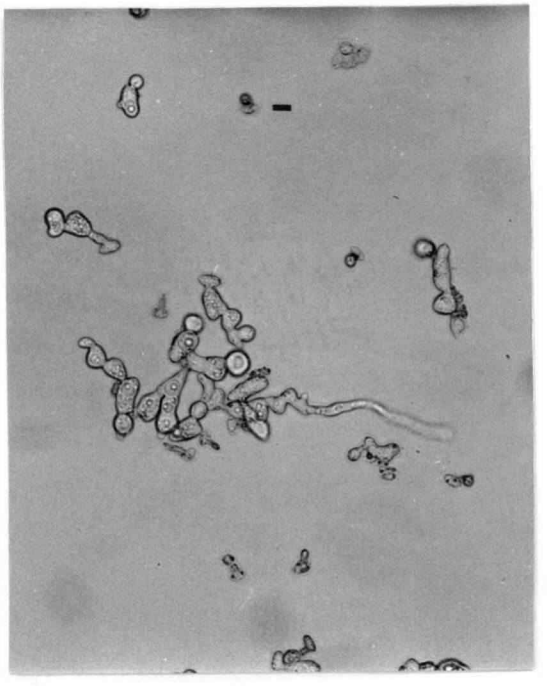


TABLE 2.12

Germination of Anisogramma anomala ascospores either under a cover slip or exposed on the surface of the media. Percent germination of 200 spores on Czapek medium, either in a candle jar or in a normal atmosphere.

	Percent Germination (mean of 8 reps.)		(sd)
Normal Atmosphere			
Under coverslip	42.8	a	(11.0)
Exposed	2.5	b	(1.2)
Candle Jar			
Under coverslip	45.2	a	(7.9)
Exposed	49.9	a	(6.7)

Means followed by the same letter do not differ significantly.
Tukey's LSD (.01) = 9.47

TABLE 2.13

Germination of Anisogramma anomala ascospores on various complex media. Percent germination of 300 spores after 24 hours and after 96 hours.

Media	24 hours	96 hours
Bacto prune agar (a)	54.7	64.7
Peptone agar (b,e)	51.7	83.6
Bacto cornmeal agar (a,e)	33.3	58.0
Bacto malt agar (a)	46.7	75.3
Casamino acids agar (c,e)	42.7	71.0
All of the above (d)	55.3	62.7
0.5% water agar	32.7	89.3
1.0% water agar	44.3	56.7
2.0% water agar	41.7	44.7

a - media prepared 3/4 strength
b - 7 g agar + 7 g Bacto Peptone #3/liter water
c - 7 g agar + 7 g Bacto Casamino acids/liter water
d - a mixture of equal amounts of the 5 previous media
e - spores formed more distinct vesicles on these media

2.12-B). Vesicle formation was not as pronounced on water agar as on more enriched media (Table 2.13), and growth beyond this stage on water agar was never observed. In some instances when spores were germinated on PDA or on media containing casamino acids or peptone, the vesicles and their attached spores swelled into large irregular masses. At about 14 days, either secondary germ tubes emerged from this amorphous mass, or secondary vesicles budded out one on top of the other. Finally a secondary germ tube would emerge from the outermost vesicle (Figure 2.12-C). These secondary germ tubes continued to elongate for several days, forming actual hyphae (Figure 2.12-D), however this growth also would cease, and a living in-vitro colony was never achieved. The formation of large vesicles and continued secondary growth as described above, was observed more frequently when spores were embedded by mixing with the media prior to hardening. It was difficult, however, to make germination counts with this technique as few spores were visible in a microscope field, and they did not lie in a single plane.

DISCUSSION

Many of the early experiments had tremendous variability in spore germination, especially between different spore suspensions. Two factors appear to be responsible for most of that variability:

1) Growth of bacterial contaminants.

2) Differences in ascospore concentration.

Using a single spore concentration, and given the same conditions, germination was relatively consistent.

Prior to antibiotic evaluations, low incubation temperatures and low nutrient media, such as water agar, were used to retard bacterial contamination. Presence of bacteria prior to germination caused a decrease in percent germination. The development of bacterial populations often caused the cessation of germination, and in some instances caused the deterioration of germ tubes that had already formed. No attempt was made to identify the different types of bacteria observed. The lower germination of inoculum from twigs stored in a refrigerator was likely a result of antagonistic bacteria that thrived in the cool, moist conditions. Had the ascospores in stored stromata lost viability, one would expect a gradual decrease in percent germination over time. This was not the case (Figure 2.2, Table 2.1).

Spores of most fungi germinate best between pH 4.5 and 6.5, and are not generally affected by visible light (Cochrane 1960). The optimum pH for Anisogramma anomala spores was within this range, and they were not affected by light.

The increased germination of concentrated spore suspensions was not the result of a germination-triggering substance released by the

spores. Increase in germination of dilute spores was not observed when spores were stored for 24 hours as a concentrated suspension prior to diluting (Figure 2.8). The concentration effect was only observed after spores were plated out in a suitable environment, and could have been a result of respiration products. This was likely the same phenomenon that allowed germination in the environment between a coverslip and the medium, or when embedded in the medium, but not on the exposed surface of the medium. Spores of many fungi germinate poorly or not at all in dense concentrations (Allen & Dunkle 1971, Cochrane 1960, Gottlieb 1978, Macko 1981). The necessity for high spore concentration precluded the use of the dilution method of eliminating contaminants.

A normal atmosphere contains about 20.9% oxygen and 0.03% carbon dioxide (Weast 1968). Improved growth of certain facultative anaerobes has been reported at 5% carbon dioxide (Mitruka 1976). Basidiospores of Schizophyllum commune required carbon dioxide for germination, and high spore concentrations were also advantageous (Hafiz & Niederpruem 1963). Allowing a candle to burn out in a closed container will give about a 10% carbon dioxide atmosphere, and was a recommended method for culturing the bacteria Neisseria gonorrhoeae (Difco 1953). The atmosphere in a candle jar was able to overcome the coverslip requirement (Table 2.12) for A. anomala ascospores. Germination did not occur, however, in the totally anaerobic environment of the liquid spore suspension, although spores remained viable for 14 days (Figure 2.3). Cysteine or thioglycolate are sometimes able to provide a reducing condition that is favorable to these facultative anaerobes (Difco 1953). The quantities of these substances added to Czapek-Dox agar (Table 2.3) were not able to

overcome the coverslip requirement.

Very good germination, and continued growth did occur when spores were embedded in the media. An added advantage of embedding spores in the media was that spores were isolated from bacterial contaminants as well as from other ascospores. Spore concentration appeared to be less critical in this situation.

When ethanol extracts of filbert stems or buds were added to inoculum and plated out, there was no significant effect (Table 2.9). However when ethanol stem extracts were added to water agar, there was a significant increase in germination over water extracts (Table 2.8). This increase in germination may have been due to the ethanol itself rather than to any substance extracted from the filbert stem. Alcohols have been reported to enhance germination in other fungi (Allen 1965, Sussman & Halvorson 1966). A slight increase in germination was observed when a small amount of ethanol was added to the media (Figure 2.11). When perithecia were surface disinfected with 50% ethanol, the improved germination may have been due in part to a small residue that was carried into the inoculum (Table 2.6). It is also possible that the differences observed in Table 2.6 were due to the inhibitory effect of a sodium hypochlorite residue.

Ascospores of Anisogramma anomala have no dormancy requirements, thus no pretreatment or chemical trigger is necessary to initiate germination. Some nutrients, however, are required for germination. When agarose was substituted for Bacto-agar producing a medium almost totally lacking in nutrients, germination was drastically reduced (Table 2.3). The increase in germination for the first few days after wetting (Figure 2.3) suggests that a germination inhibitor is being

leached out. Most fungi have some means of preventing germination of spores prior to discharge (Allen 1965, Allen & Dunkle 1971, Gottlieb 1978) and it is likely that such an inhibitor exists either within A. anomala ascospores, or in the mucous that surrounds them.

Several substances were able to prevent spore germination under otherwise favorable conditions. Chloramphenicol or cyclohexamide at 10 ppm, xylene at less than 2%, and an unknown component of the citrate buffer completely inhibited germination. 25% sucrose, levels of ethanol greater than 5%, and residues of sodium hypochlorite following surface sterilization also had an adverse effect on ascospore germination.

Significance of results in the orchard. Viable spores are available in the orchard beginning in September, whenever free moisture is available to discharge and disseminate them. Rain splashed ascospores are the only known inoculum source for both short and long distance spread of the disease (Cameron & Gottwald 1978, Gottwald & Cameron 1979). In the Pacific Northwest, the rainy season begins in early autumn and continues through winter and spring. Spores are being dispersed around the orchard during this entire period, and the ambient temperatures are usually within the range of 5 to 20 degrees C that favors germination. Conditions that allow dispersal would also leach any water soluble inhibitors that may be present. Ascospores must reach an infection court quickly. If they are allowed to become dry, the spores will not be viable upon rehydration, and prolonged exposure to other microorganisms in the environment may also erode their ability to germinate. Water is therefore necessary for 1) spore discharge, 2) washing of possible germination inhibitors, 3) dissemination of the inoculum and 4)

maintaining a moist environment until the fungus has penetrated the host.

The spread of Anisogramma Canker over long distances is reported to be slow. Within an infected orchard or within a tree, the number of new cankers was found to approximately double each year in a very susceptible cultivar (Gottwald & Cameron 1980b). Gottwald and Cameron (1980a) determined that the natural infection period occurs between February and May. This period corresponds to the period when bud scales of mite infested buds are expanding (Gottwald & Cameron 1980a) and when the host is unable to form callus to protect wounds (Gottwald & Cameron 1979). Since spores are able to germinate beginning in September, the factor limiting natural infection must reside in the host and not in the pathogen. In the Pacific Northwest, conditions are ideal for spore discharge and germination for 3 - 5 months prior to the time of maximum host susceptibility. Depletion of the inoculum supply prior to this susceptible period would be a limiting factor in spread of the disease.

This fungus was introduced from the eastern United States. In the native range of Anisogramma anomala, cankers are more likely to remain dry or frozen during the fall and winter months. Spring rains then release and disseminate ascospores during the period when the host is most susceptible. A. anomala has been an important pathogen in places like Massachusetts (Humphry 1893) New York (Slate 1930) New Jersey (Halsted 1892) and Washington D.C. (Barss 1921), making it impossible to grow European filberts in those locations.

In the laboratory, ascospore germination is facilitated by high spore concentrations, and by the oxygen stress imposed under a

coverslip, in a candle jar or beneath the surface of the medium. High ascospore concentrations are not likely to occur once spores have been splashed around in an orchard. It is very likely that the factors influencing germination are the same for all of the situations above. When trapped beneath a coverslip or embedded in the media, the respiration of the spores, especially when their concentration is high, increases the carbon dioxide and reduces the oxygen concentration. This is also what happens when a candle is allowed to burn out in a closed container. The natural infection sites in the filbert host are eriophyid mite galled buds or "big buds" (Gottwald & Cameron 1979, Gottwald & Cameron 1980a). The respiration of these mites as well as that of the Corylus tissue they inhabit could provide the same oxygen stress that has influenced spore germination in vitro.

Given the limitations imposed by early depletion of inoculum, the lethal consequences of desiccation and the deleterious effect of native bacteria, it is surprising that this disease is able to spread as well as it does.

SUMMARY OF RESULTS

1. Ascospores can be collected in the orchard between October and May. Cankered twigs can be stored frozen for use at other times of the year.
2. Ascospores begin germinating 24 hours after hydration, and at low temperatures remain viable for several weeks as long as they do not dry out.
3. Optimum temperature for germination is 20 degrees C, but good germination can occur between 5 and 20 degrees.
4. Fluorescent light has no effect on ascospore germination.

5. The optimum pH for germination is between 4.2 and 5.7, and spores will germinate in the range 3.0 to 7.5.

6. The optimum spore concentration for *in vitro* germination is 10^6 spores/ml.

7. 10 ppm Rifampicin in the media discourages growth of accompanying bacteria without inhibiting ascospore germination.

8. *Corylus* extracts in the medium failed to improve spore germination or induce growth beyond the initial germ tube.

9. Percent germination and amount of growth are affected by nutrients in the media.

10. Either a lowered oxygen level or an elevated carbon dioxide level is required for germination. Germination will not occur under normal aerobic conditions, or under total anaerobic conditions.

11. The process of germination has several stages:

    a. Swelling of the ascospore and production of a short primary germ tube.

    b. Enlargement of the germ tube tip into a vesicle.

    c. Swelling of both the original spore and the attached vesicle to several times their original size. Sometimes accompanied by the budding off of additional cells.

    d. Growth of a secondary germ tube and hyphae.

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GENERAL CONCLUSION

In his plant pathology textbook, Agrios (1978) writes, "for fungi that we do not know in the laboratory from spore to spore, there is only one solution: Learn how to grow them." These studies bring closer, the ability to grow Anisogramma anomala in the laboratory. Conditions were identified whereby infected tissues could be stored for long periods while maintaining ascospore viability. Methods were developed for initiating spore germination and discouraging bacterial contamination of culture media. Environmental conditions that are optimum for germination were identified.

Several obstacles still prevent the screening of Corylus varieties for resistance to this disease. We are still not able to grow the fungus in culture and there is no reliable method of artificially producing infections in susceptible trees. There are other lines of research that may lead to a better understanding of this disease. It may be possible to identify physiological differences between known resistant and susceptible varieties. Thus an index of susceptibility could be developed to screen trees for disease resistance. Defoliation might be used to reduce the latent period between inoculation and symptom development. This method is used in the tropics to substitute for a cold dormant period in deciduous fruit production. By defoliating a tree, two or more crops may be produced in a single year on one plant. Defoliation is also used to reduce the latent period following inoculation of woody virus indicators (Fridlund 1980).

Now that the environmental conditions favorable for ascospore germination have been identified, it should be easier to examine the

nutritional requirements for fungal growth, especially different carbon and nitrogen sources. Relative humidity may also be important. If the bacteria associated with deterioration of ascospores were identified and cultured, they may have potential as a biological control. Spread of the disease in the orchard is first to the sensitive 'Daviana' pollenizers, and only after several years to the more resistant 'Barcelona' variety. It would be interesting to remove all the 'Daviana' trees from a portion of a newly infected orchard, or look at the effect of different pollenizer spacings on spread of the disease. 'Barcelona' trees may escape infection if the inoculum reservoir in the pollenizers is removed.

Many gaps remain in our understanding of the disease caused by Anisogramma anomala. These studies have closed some of the gaps that stand in the way of culturing this fungus, and have shed light on some environmental conditions that may effect disease spread.

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APPENDIX

Appendix 1

Recipe for Standard Czapek Medium

$MgSO_4\ 7H_2O$	0.5	g.
KH_2PO_4	1.0	g.
KCl	0.5	g.
$FeSO_4\ 7H_2O$	.01	g.
$NaNO_3$	3.0	g.
Sucrose	30.0	g.
Agar	15.0	g.
H_2O	1.0	liter