

Infection of European Hazelnut by *Anisogramma anomala*: Ascospore Adhesion, Mode of Penetration of Immature Shoots, and Host Response

J. N. Pinkerton, J. K. Stone, S. J. Nelson, and K. B. Johnson

First author: USDA-ARS, Horticultural Crops Research Laboratory, 3340 NW Orchard Ave., Corvallis, OR 97330; second, third, and fourth authors: Department of Botany and Plant Pathology, Oregon State University, Corvallis, 97330-2902.

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ABSTRACT

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Ascospores of *Anisogramma anomala* adhered irreversibly to vegetative internodes of European hazelnut shoots (*Corylus avellana*) within 5 min of initial contact. The concentration of spores retained was greatest on and at the base of trichomes of the stem internode nearest the apical bud. Reported differences in susceptibility of internodes to infection corresponded to relative differences in spore adhesion and histological maturation in the cuticle and epidermal cells. The infection process was studied with light and transmission electron microscopy on susceptible cv.

Ennis. After contact with the host, an adhesive pad developed on the lateral wall of ascospores. Germ hypha penetrated through the pad and host epidermis directly. After breaching the wall, penetration hyphae expanded into vesicles that were similar in morphology to those observed in vitro. However, in all specimens observed, further colonization apparently was restricted by a hypersensitive-like response of the host. Apposition of callose, cell necrosis, and formation of a cicatricial layer isolated the fungus and halted colonization of the adjacent cells. Observations suggest that most attempts by *A. anomala* to infect *C. avellana* are prevented by host response and that successful infections are rare.

Additional keywords: eastern filbert blight, histopathology.

Eastern filbert blight, caused by *Anisogramma anomala* (Peck) E. Müller (Diaporthales, Gnomoniaceae), was introduced into European hazelnut (*Corylus avellana* L.) orchards of the Pacific Northwest from the eastern United States where it is endemic on *C. americana*. Since its discovery in southwest Washington in 1972 (5), the disease has spread into 50% of the hazelnut production areas in Oregon and Washington (13,33). The disease has caused increased production costs for many orchardists, and in areas where the pathogen was first detected, the orchards have been destroyed (33).

The biology of *A. anomala* and epidemiology of eastern filbert blight has been elucidated only recently. Ascospores are released from perithecia borne in stromata on diseased hazelnut branches during periods of precipitation from early fall through late spring (14,34). Hazelnut is susceptible, however, only after vegetative buds break dormancy during early March. Vegetative shoots are susceptible until the apical buds stop growing in midsummer (21, 38). Young shoot tissues also are differentially susceptible to *A. anomala*—the first leaf and internode nearest the apical bud are highly susceptible and the older leaves and internodes are more resistant (21). After the fungus infects a young shoot, it systemically colonizes the xylem, cambium, and phloem. Cankers are produced in the phloem after 12 months or more of incubation (12,38). During late May of the second year, stromata develop within cankers, and perithecia mature in stromata by early fall (12).

As the perennial cankers expand, infected branches are girdled, fruit-bearing wood is reduced, and ultimately, mature trees die in 5 to 15 years.

The objective of this research was to characterize the infection process of *A. anomala* on juvenile European hazelnut shoots. Our research proceeded in three phases: (i) observation and quantification of ascospore adhesion and germination on plant surfaces, (ii) examination of ascospore germination and germ hypha morphology in vitro, and (iii) documentation of the infection process and host response by histopathological studies.

MATERIALS AND METHODS

Inoculum preparation and plant materials. Each November from 1991 to 1993, hazelnut branches bearing stromata of *A. anomala* were collected in diseased orchards, sealed in polyethylene bags, and frozen at -10°C until needed. Ascospore suspensions of *A. anomala* used in adhesion and infection studies were prepared by thawing stored branches, cutting the tops off 10 to 30 stromata, and dissecting out whole perithecia. Excised perithecia were crushed with forceps, placed in a Syracuse glass of deionized water, and allowed to hydrate for 1 h. The spore suspension was drawn off with a fine pipette, after which the suspension was adjusted to the appropriate spore concentration with the aid of a hemacytometer. For in vitro studies, two additional steps were taken to reduce potential microbial contamination of the ascospore suspension. Single perithecia were collected, and the beaks were removed with a sterile scalpel. Perithecia were dipped in 95% ethanol (EtOH) for 5 to 10 s and transferred to a dish containing rifampicin (10 mg/liter) in sterile deionized water, where they were hydrated and crushed.

Corresponding author: J. N. Pinkerton; e-mail address: pinkertj@bcc.orst.edu

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Hazelnut seedlings or clonal trees of cv. Ennis were used in the experiments. Seedlings were propagated from nuts collected from open-pollinated trees of cv. Ennis in commercial orchards. To produce seedlings, collected nuts were stratified for 10 weeks at 4°C. After shelling, the kernels were soaked for 8 h in 0.004% gibberellic acid (ProGibb, Abbott Laboratories, North Chicago) and planted in moist vermiculite and allowed to germinate. Seedlings were transplanted into pots containing a commercial growth medium (33.3% peat, 33.3% pumice, and 33.3% bark) and grown in a greenhouse at 20 ± 4°C. Clonal Ennis trees were obtained in a dormant condition from a commercial nursery as 2-year-old bare-root plants. Trees were potted in 4-liter plastic pots that contained the commercial growth medium and grown in a greenhouse (20 ± 4°C, 14-h photoperiod).

In vitro germination and hyphal growth. Ascospore suspensions (1×10^4 ascospores per ml) were placed in sterile, 50-ml petri plates. Cellulose nitrate membranes (Whatman CM, 47 mm diameter, 0.2 µm pore size, Whatman, Clifton, NJ) were cut into wedge-shaped pieces, autoclaved in deionized water, and air-dried on sterile paper towels. Membranes were submerged in the spore suspension for 1 min, removed with forceps such that the membrane surface remained horizontal, and blotted on a sterile paper towel. After excess moisture was removed, four membranes were immediately transferred, blotted side down, to modified Murashige and Skoog medium (MMS) amended with 0.05% activated charcoal and thickened with 0.15% agar (40). Membrane pieces were incubated at 20°C in the dark for periods of 1, 3, 6, 12, 18, 24, 48, 72, 96, or 168 h. After each incubation period, four membranes were removed from the MMS medium, blotted on dry paper towel, stained in a drop of 0.05% trypan blue in lactoglycerol on a microscope slide, and sealed under a cover glass. The membranes were scanned at 160× by bright field microscopy, and spore germination and hyphal morphology were recorded for 50 to 100 spores per membrane. Photomicrographs were made of specimens that represent typical spore and hyphal morphology for each incubation time.

Spore adhesion and germination on plants. Adhesion and germination of ascospores on young hazelnut shoots were investigated in four studies. These studies were done with seedlings of cv. Ennis that had developed to the 6 to 8 node stage. Prior to inoculation, the seedlings were rinsed in tap water for 5 min to remove dirt, insect honeydew, and insecticide residues and allowed to dry for 2 to 3 h. In each study, the inoculum was amended with the stain Calcofluor white M2R (Sigma Chemical Co., St. Louis) to facilitate observation of spores on the plant surface. Calcofluor (0.05%) was added to the ascospore suspension, and after 10 min, the spores were rinsed twice, centrifuged at $300 \times g$ for 3 min, and the pellet was resuspended in deionized water. The final inoculum concentration was 1×10^6 spores per ml. The shoots were inoculated by immersing the five most distal internodes and leaves in the spore suspension three times for 15 s each, after which they were shaken gently to remove excess inoculum. Plants were placed immediately in a 100% relative humidity (RH) enclosure and incubated in a growth chamber at 20 ± 1°C with a 14-h photoperiod until shoot samples were collected (38). After incubation, the shoots were observed microscopically, and based on the amount of water observed on their surfaces, a qualitative estimate of the hydrophobicity was made. The effect of Calcofluor on spore germination was evaluated by inoculating subsamples of stained spores (1×10^5 spores per ml) onto cellulose nitrate membranes as previously described, culturing at 20°C on MMS for 5 days, counting germinated spores, and comparing the percentage of germination with that of non-stained ascospores.

In the first study, four inoculated shoots were collected 24 h after inoculation to ascertain the location of ascospores and whether they had germinated. The four most distal leaves were cut from each shoot, and two 4-mm² subsamples were cut from each leaf. Two whole bracts also were sampled at the second node and cut

in half. One-half of the leaf and bract specimens was mounted on a microscope slide adaxially and the other half abaxially, flooded with 0.05% fluorescein diacetate (FDA), and sealed under a cover glass. Two epidermal strips, 5 to 10 × 2 to 3 mm, were removed from each of the four inoculated internodes and mounted in FDA. Epidermal strips from the first two internodes also were sampled at 96, 120, 192 and 288 h after inoculation. Mounted tissues were observed at 480× with a Zeiss epifluorescence microscope (Zeiss, Oberkochen, Germany). A blue filter set (485-nm excitation and 520-nm emission) was used to observe fluorescence from Calcofluor-stained spores, and a 440-nm excitation/470-nm emission filter was used to observe hyphae stained with FDA. The number and location of spores on the plant surface in four fields of view (2.4 mm²) were recorded for each subsample. Ascospore germination was evaluated first with the blue filter set by examining spores for distended cell walls. If germinated spores were suggested under these conditions, the specimen also was examined for germ hyphae stained with FDA. The density of trichomes on the first four internodes was determined by counting the number of trichomes in four microscope fields at 400×. The experiment was replicated three times.

In a second study to determine the requisite time for ascospore adhesion, two shoots were collected 1, 3, 6, 12, and 24 h after inoculation, and two epidermal strips were removed from the two youngest internodes. One epidermal strip from each internode was immediately mounted on a microscope slide in deionized water, while the other strip was placed in a beaker of deionized water and stirred at 150 rpm for 5 min before mounting. The density of spores per square millimeter was determined in four microscope fields on each epidermal piece. The experiment was replicated three times. Because there was no significant effect of replication, data for each incubation time and treatment were pooled for analysis by paired *t* tests. The effect of incubation time on spore adhesion was analyzed with analysis of variance (ANOVA) (SPSS, Chicago).

A third study was designed to ascertain the mechanism of spore adhesion by comparing adhesion of live and killed ascospores on hazelnut shoots. A suspension of stained ascospores (1×10^6 spores per ml) in deionized water was split into four fractions and treated in the following manner: nontreated, boiling for 5 min, addition of sodium azide (10 µl/ml), or addition of cyclohexamide (50 µl/ml). One hour after treating the spores, detached shoots with leaves removed were dipped three times into one of the treated suspensions and shaken to remove excess inoculum. Shoots were incubated in the light at 20°C and 100% RH until samples were collected. Four shoots from each treatment were collected 5, 15, 30, 60, and 240 min after inoculation and washed by stirring for 5 min at 500 rpm in a beaker of deionized water. Two epidermal strips were removed from opposite sides of the second internode of each shoot, mounted on microscope slides, and the density of spores was determined by counting ascospores in four fields of view on each strip. The experiment was replicated four times. The effects of treatment and time on ascospore adhesion were analyzed with ANOVA.

A final study was conducted to determine the specificity of adhesion of ascospores of *A. anomala*. Four detached shoots of *C. avellana* (cv. Ennis seedlings), *Acer palmatum* Thunb., *Betula pendula* Roth., *Crataegus douglasii* Lindl., and *Prunus serrulata* Lindl. were each dipped three times in a suspension of Calcofluor-stained ascospores (1×10^6 spores per ml) and incubated at 20°C and 100% RH. After 4 h, four shoots were removed from the chamber, placed in a beaker of deionized water, and stirred at 500 rpm for 5 min. Two epidermal strips were removed from internodes of each shoot, and spore density was quantified as in other experiments. The experiment was replicated three times, and the effect of host on ascospore adhesion was compared by ANOVA.

Spore adhesion on glass, polystyrene, and Parafilm also was evaluated. Materials were prepared by cutting 25-mm² pieces from microscope slides cleaned with 95% EtOH and polystyrene petri

dish lids; pieces of Parafilm (American National Can, Greenwich, CT) were placed on glass pieces, which then were warmed on a hot plate to bond the two materials. Four pieces of each material were attached to the bottom of a 50-mm-diameter polystyrene petri dish with small drops of epoxy glue (Loctite Corp., Cleveland) and flooded with 15 ml of Calcofluor-stained spore suspensions (1×10^5 spores per ml). Ascospores were allowed to settle onto pieces of the experimental substances for 4 h, after which the pieces were removed from the spore suspension and rinsed for 10 s with a directed stream of deionized water from a wash bottle. Treated materials were mounted on a microscope slide, and the spores were counted in four microscope fields. As a control, the aqueous suspension of ascospores was allowed to evaporate from the petri dish that contained pieces of each material. The percentage of spore adhesion was calculated as the number of spores per square millimeter after incubation and washing divided by the density of ascospores on the surface of the control material after evaporation.

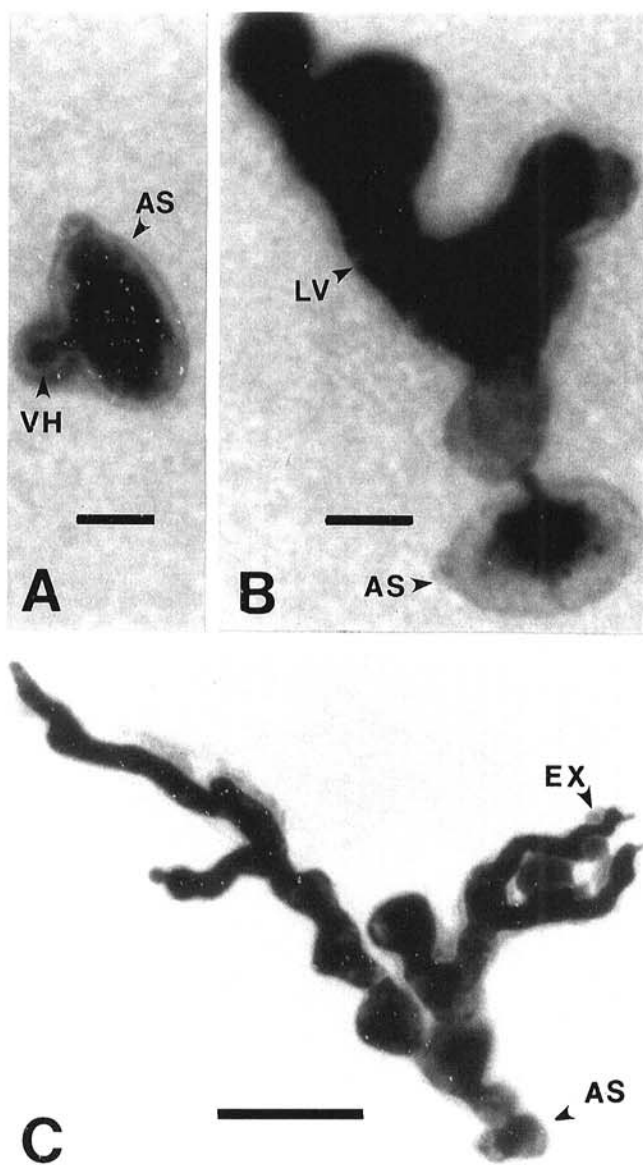


Fig. 1. In vitro germination of ascospores (AS) and early hyphal morphology of *Anisogramma anomala* cultured on cellulose nitrate membranes on modified Murashige and Skoog medium, stained with trypan blue lactoglycerol, and observed with bright field microscopy. **A**, Vesiculate germ hypha (VH) after incubation for 24 h at 20°C. Bar = 5 μ m. **B**, Lobate vesicle (LV) after 96 h. Bar = 5 μ m. **C**, Hyphal development and branching after 120 h. Deposits of extracellular material (EX) were observed on hyphae. Bar = 20 μ m.

Mode of infection. Actively growing shoots of clonal Ennis trees were sprayed to runoff with an aqueous suspension of 1×10^7 ascospores per ml, enclosed in polyethylene bags, and incubated in a growth chamber at 20°C (14-h photoperiod) (21). The bags were removed after 5 days. At 4-day intervals after inoculation, the five internodes that were nearest the apical bud at the time of inoculation were sampled and prepared for histopathological studies. Five pieces of stem tissue, 1 to 1.5 mm long, were cut from the proximal end of individual internodes. The stem pieces were fixed under vacuum for 5 h in 1.5% glutaraldehyde-1% formaldehyde buffered with 0.2 M cacodylate buffer (pH 7.2) (17) and rinsed overnight in buffer. Samples were postfixed for 4 h in 2% OsO₄ and dehydrated in the following series: 20 min in 25% acetone, 20 min in 50% acetone saturated with uranyl acetate, and three 20-min exchanges of 100% acetone. Samples were embedded in Spurr's resin by vacuum infiltration (37). Fixation, postfixation, dehydration, and embedding were done at 4°C. For preliminary observation, embedded stems were cut into 10- μ m-thick sections with a glass knife and examined with phase contrast microscopy at 480 \times for adhered and germinated spores on the epidermis. Thick sections bearing germinating spores were reembedded in resin blocks, and 0.15- μ m-thick sections were cut with a diamond knife. The thin sections were collected on formvar-coated grids, stained with 0.2% lead citrate (41), and examined for spore germination and penetration of germ hyphae with a transmission electron microscope (TEM) (Phillips Analytical, Eindhoven, the Netherlands).

Maturation of stem epidermis. While stripping the epidermis from the stems, it was observed that this layer on the internode nearest the apical bud (first internode), and to a lesser extent on the second stem internode, was firmly attached to the underlying parenchyma. On the more infection-resistant third and older internodes (21), however, the epidermis was easily separated from the underlying tissue. Therefore, the structure of the epidermis on internodes of various ages was examined by TEM to observe developmental changes that may affect resistance in the third and fourth internodes. Tissue was prepared for TEM as described above but only 0.15- μ m-thick sections were made.

Colonization. Branches, 1 cm in diameter, with 1-year-old cankers of eastern filbert blight were collected from naturally infected Ennis hazelnut trees during February and April 1995. Five branches collected at each date were trimmed to leave 10 to 20 cm of asymptomatic wood on either side of the canker. Blocks of wood were cut radially at 5-cm intervals from the edge of the canker to the end of each branch. Sections were cut longitudinally by hand through each block, stained with trypan blue in lactoglycerol (39), and examined for the presence and distribution of characteristic

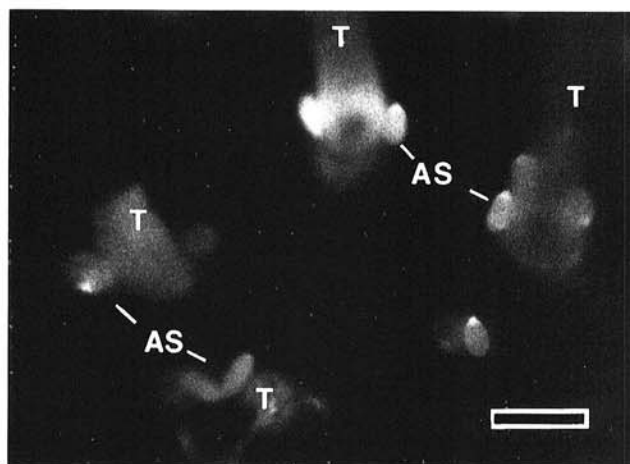


Fig. 2. Epifluorescence micrograph of Calcofluor-stained ascospores (AS) of *Anisogramma anomala* adhered at the base of trichomes (T) on the surface of the second internode stem tissue of hazelnut seedlings. Bar = 25 μ m.

hyphae and haustoria of *A. anomala* (38) in the xylem, cambium, and phloem tissue. The presence of necrosis and other wound responses in proximity to *A. anomala* hyphae also was recorded.

RESULTS

In vitro germination and growth. Ascospores became swollen to 2 to 3 times their original size after inoculation onto MMS. Distention of the side wall began 6 h after inoculation. After 12 h, germ hyphae, 1 μ m in length, were observed in the distended region, and by 18 h, germ hyphae were 2 to 3 μ m in length. The distal ends of the hyphae expanded to form spherical (5 to 8 μ m diameter) vesicles that were commonly surrounded by a thin sheath of extracellular material after 24 h (Fig. 1A). The maximum germination incidence of 20 to 32% occurred at 24 h, after which time most nongerminated spores lysed. By 96 h, vesicles were 15 to 20 μ m in diameter and lobate, and ascospores became vacuolated (Fig. 1B). A fine hypha connecting the ascospore to the vesicle could be seen at this time. After 96 h and by 168 h, one to four coarse, secondary hyphae, often surrounded by thick extracellular sheaths, had grown from the vesicles (Fig. 1C).

Site of attachment and spore adhesion. Calcofluor-stained ascospores were easily observed on plant surfaces and were lodged

TABLE 1. Differential adhesion of ascospores^a of *Anisogramma anomala* on the stem^b, leaves, and bracts of 8-week-old shoots of *Corylus avellana* seedlings and the density of trichomes^c

Hazelnut tissue ^d	Ascospores/mm ²	Trichomes/mm ²
Internode 1	58.1 \pm 2.3 ^e	1,018.8 \pm 68.2 ^f
Internode 2	15.5 \pm 2.9	483.4 \pm 93.8
Internode 3	14.2 \pm 4.1	429.7 \pm 24.5
Internode 4	8.5 \pm 7.1	289.7 \pm 69.5
Expanded leaf 1	12.2 \pm 4.3	
Expanded leaves 2-4	3.8 \pm 3.6	
Bracts at node 2	11.3 \pm 1.8	

^a Ascospores were stained in 0.05% Calcofluor white M2R for 10 min and rinsed twice in deionized water before they were inoculated on shoots. After inoculation, shoots were incubated at 20°C and 100% relative humidity for 24 h.

^b Epidermal strips were removed from shoots after incubation, mounted on microscope slides, and ascospores were counted.

^c Trichomes on each internode were counted in four microscope fields at 400 \times with epifluorescent illumination. The first four internodes were examined on five shoots.

^d Internode 1 was defined as the stem tissue from the apical meristem to the youngest expanding leaf, internode 2 was between the youngest and second youngest leaves, and so on to internode 4.

^e Standard deviation of the mean of four replications.

^f Standard deviation of the mean of trichome density on five shoots.

TABLE 2. Adhesion of ascospores^a of *Anisogramma anomala* on the epidermis of 8-week-old shoots of *Corylus avellana* seedlings

Incubation ^b time (h)	Ascospores/mm ²		
	Unwashed ^c	Washed ^d	P ^e
1	140 \pm 21 ^f	156 \pm 18 ^f	0.727
3	148 \pm 15	120 \pm 12	0.158
6	105 \pm 16	123 \pm 26	0.547
12	101 \pm 13	111 \pm 12	0.584
24	138 \pm 18	131 \pm 19	0.813

^a Ascospores were stained in 0.05% Calcofluor white M2R for 10 min and rinsed twice in deionized water before they were inoculated on shoots.

^b After inoculation, shoots were incubated at 20°C and 100% relative humidity for 24 h.

^c Epidermal strips were removed from shoots after incubation and immediately viewed microscopically.

^d Epidermal strips were removed from the first two internodes of shoots after incubation and washed vigorously in deionized water for 5 min before mounting on microscope slides.

^e P value obtained from *t* test comparison of three replications of 24 washed and nonwashed epidermal strips.

^f Standard deviation of the mean.

most commonly near the bases of trichomes (Fig. 2). Densities of spores were greatest on the first internode stem tissue (Table 1), which also had the greatest density of trichomes and retained the thickest film of water on its surface after inoculation. Moreover, ascospore densities on the first internode and the youngest, nonexpanded leaves were probably underestimated because focusing through closely packed trichomes to observe spores was difficult. The number of ascospores that adhered on the second through fourth internodes was less than on the first internode (Table 1). Similarly, adhesion of ascospores was greater on the first expanded leaf than on older leaves (Table 1). The density of trichomes was greater on the first internode than on the older tissue (Table 1).

Approximately 2,000 Calcofluor-stained ascospores on stem surfaces were examined for germination during the study. Even 192 h after inoculation, neither germ hyphae nor vesicles were observed on the plant surface. After 192 h, the fluorescence of the Calcofluor-stained spores became faint, and the study was terminated.

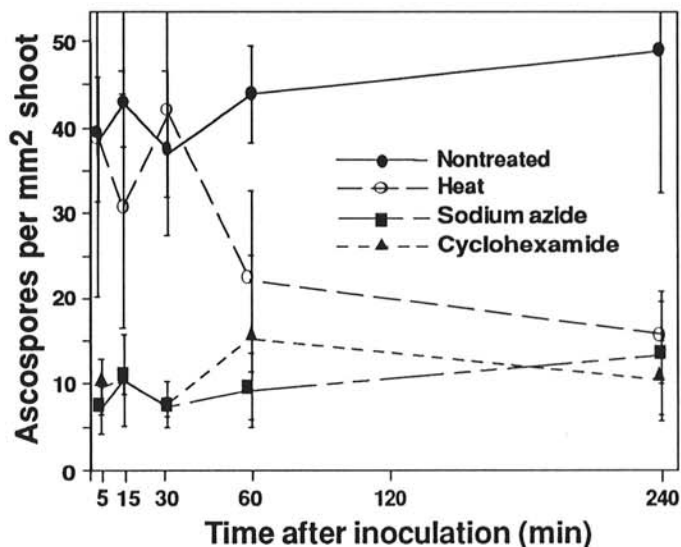


Fig. 3. Adhesion of treated and nontreated ascospores of *Anisogramma anomala* on the surface of young hazelnut shoots. Ascospores in aqueous suspension were treated by boiling for 5 min or by incubating in 100 ppm of sodium azide or 50 ppm of cyclohexamide. The adhesion of ascospores on the epidermis was determined at 5, 15, 30, 60, and 240 min after inoculation.

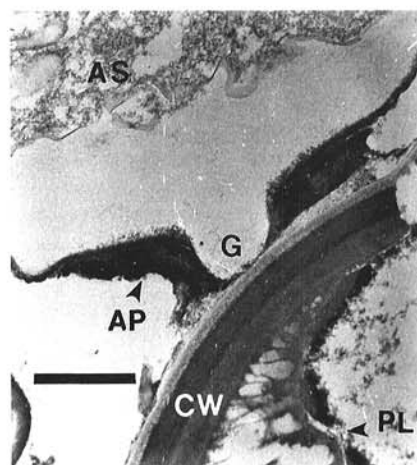


Fig. 4. Transmission electron micrograph of a germinated ascospore (AS) of *Anisogramma anomala* on the epidermis of the first stem internode of hazelnut 96 h after inoculation. Depicted are an adhesive pad (AP) that held the ascospore on the plant surface and a germ hypha (G) that penetrated directly through the adhesive pad and through the cell wall (CW) of the underlying epidermal cell. The plasmalemma (PL) retracted from the cell wall at the penetration site. Bar = 1 μ m.

Ascospores of *A. anomala* in aqueous suspension adhered rapidly and irreversibly on hazelnut shoots and inert surfaces. The density of spores retained on hazelnut shoots at 1 h after inoculation did not significantly differ ($P > 0.05$) between internode tissue observed immediately or after a vigorous 5-min wash in distilled water (Table 2). The number of spores adhered to the shoot did not change significantly ($P > 0.152$) over the 24 h following inoculation nor did spore density differ between washed and unwashed samples. Nontreated spores adhered (39 spores per mm^2) to hazelnut shoots within 5 min of inoculation, and spore retention did not increase significantly after additional incubation (Fig. 3). Adhesion of ascospores treated with cycloheximide and sodium azide, however, was reduced significantly ($P < 0.05$) (10 and 13 spores per mm^2 , respectively) compared to nontreated ascospores. Heat-killing of spores resulted in high retention on stems after 30 min

of incubation, but the number of adhered spores declined after longer incubation (Fig. 3).

Adhesion of ascospores was greater on shoots ($P > 0.05$) of *C. avellana* and *B. pendula*, with densities of 32.0 ± 17.1 and 20.8 ± 12.6 ascospores per mm^2 , respectively, than on shoots of *C. douglasii* with 4.4 ± 2.2 spores per mm^2 . Ascospores did not adhere to the glabrous shoot surfaces of *A. palmatum* and *P. serrulata*. The spores were distributed on the entire surface of pubescent shoots of *C. avellana*, but they were associated only with glands on *B. pendula* and with stomata on *C. douglasii*. Ascospores adhered to all three inert materials, but adhesion was greater on glass ($34.2 \pm 0.7\%$) than on polystyrene ($17.8 \pm 2.8\%$) or Parafilm ($14.8 \pm 1.1\%$).

Mode of infection. TEM and light microscopy studies documented the infection process at 4, 8, and 10 days after inoculation. As with observations of Calcofluor-stained spores, most ascospores of *A.*

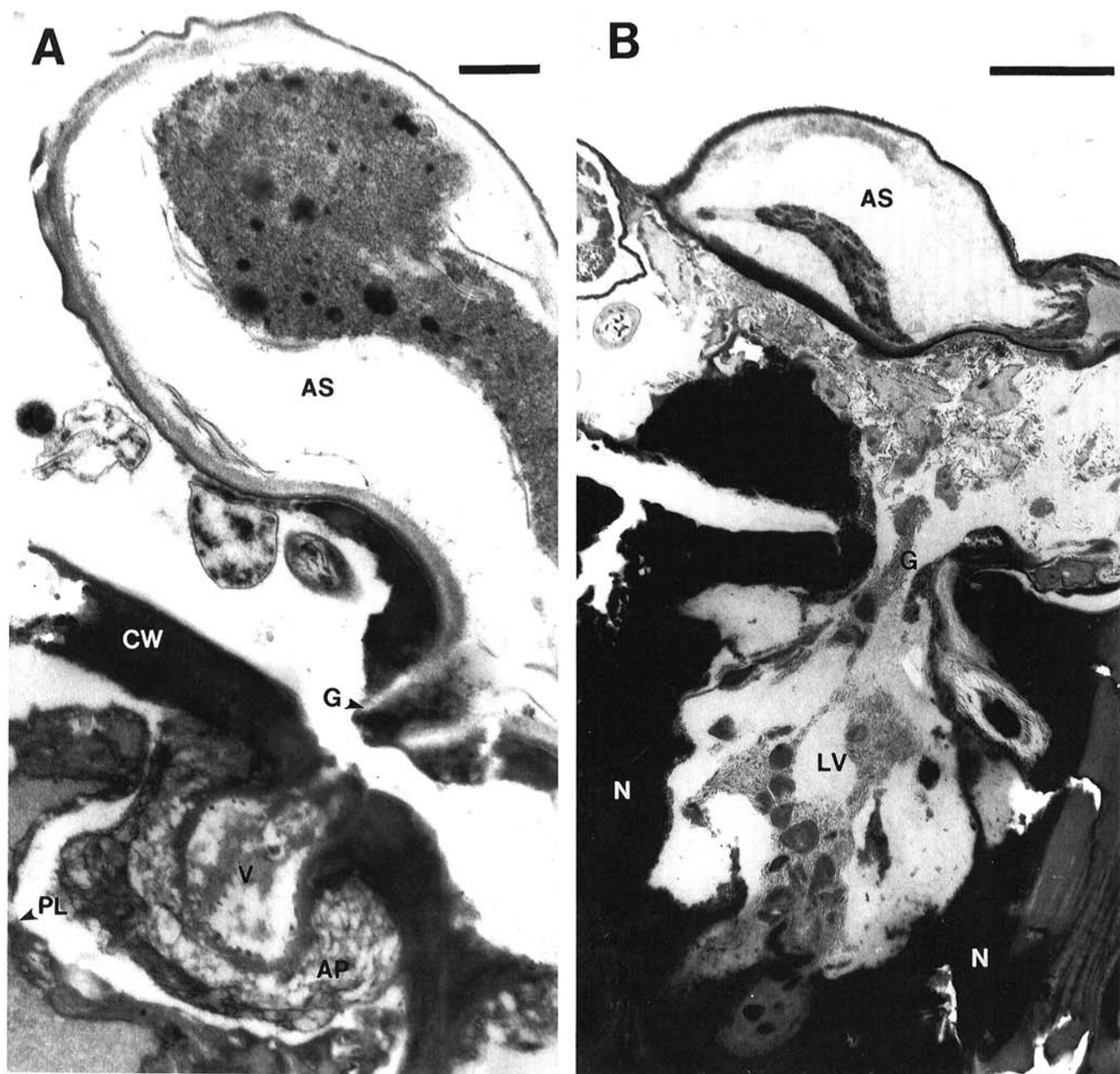


Fig. 5. Transmission electron micrographs of early colonization of susceptible hazelnut epidermal cells by *Anisogramma anomala*. A, Germination hypha (G) penetrated the cell wall (CW) and formed an intercellular vesicle (V) 8 days after inoculation. Plasmalemma (PL) separated from the cell wall and apposition (AP) of callose formed around the vesicle. As the vesicle expanded, the ascospore (AS) became vacuolated and collapsed. Bar = 1 μm . B, Lobate vesicle (LV) in necrotic epidermal cell (N) 10 days after inoculation. Bar = 2 μm .

anomala were found in groups at the bases of trichomes. Both germinated and nongerminated spores were attached to the plant surface by an adhesive pad formed of extracellular material (Fig. 4). A single germ hypha emerged from the lateral wall of the spore and extended through the extracellular material, and by 96 h after inoculation, this germ hypha penetrated directly through the underlying epidermal cell wall. Specialized penetration structures (e.g., appressoria or melanized hyphae) were not observed.

After penetration of the epidermis, the hypha typically expanded into an intracellular vesicle (Fig. 5A). Simultaneously, cytoplasm remaining in the ascospores migrated into the vesicle, and the spore wall collapsed. After 10 days, a lobate vesicle that filled the epidermal cell was observed (Fig. 5B). Hundreds of infection sites were observed with phase contrast microscopy, and approximately 50 of these were examined with TEM. We were not successful, however, in finding secondary hyphae growing from the vesicles as was observed *in vitro*.

Macroscopic necrotic flecks were observed commonly on inoculated shoots at 20 days after application of *A. anomala* ascospores. Five days after inoculation, TEM and phase contrast microscopy also revealed histological changes in otherwise asymptomatic shoots. Papillae and localized necrosis were observed beneath germinated and nongerminated spores (Fig. 6A and B). The plasmalemmae separated from the cell walls, and wall appositions developed around

vesicles in apparent response to hyphal penetration of epidermal cells (Figs. 4 and 5A). As the infection progressed, cells became necrotic and accumulated osmiophilic substances near the cell walls proximal to the developing vesicle (Fig. 5B). Disrupted cytoplasm and separation of plasmalemmae from the walls also were observed in the cells surrounding the infection site. After 10 days, affected tissues were severely disrupted, and a cicatricial layer formed (Fig. 6C), such that the necrotic cells were easily detached from the underlying tissues when sectioning embedded tissue with glass and diamond knives.

Maturation of tissue. Differentiation and maturation of the epidermis of young shoots was marked by a progressive thickening of the cuticle and cell walls, vacuolation, and deposition of osmiophilic material. The undifferentiated epidermis of the internode nearest the apical bud had thin cell walls and cuticle, and cytoplasm occupied most of the cell volume (Fig. 7A). Cells of the second and third internode epidermis were larger and more vacuolated and had thicker laminate cell walls and cuticle layer. Surfaces of the fourth stem internode were light tan, suggestive of early periderm formation. In micrographs, epidermal cells in this tissue were bordered by a thick cuticle, the cell walls were composed of several layers, and the large, central vacuoles contained osmiophilic substances, presumably condensed tannins (Fig. 7B).

Colonization. Hyphae had colonized cambium, phloem, and secondary xylem tissue up to 10 cm beyond the visibly cankered portion of the branches collected during February. No host reaction was observed near hyphae within these tissues, and haustoria-like cells of the fungus were present within phloem and parenchyma cells of the xylem rays (Fig. 8). By April, however, the infected phloem became necrotic, and a canker developed.

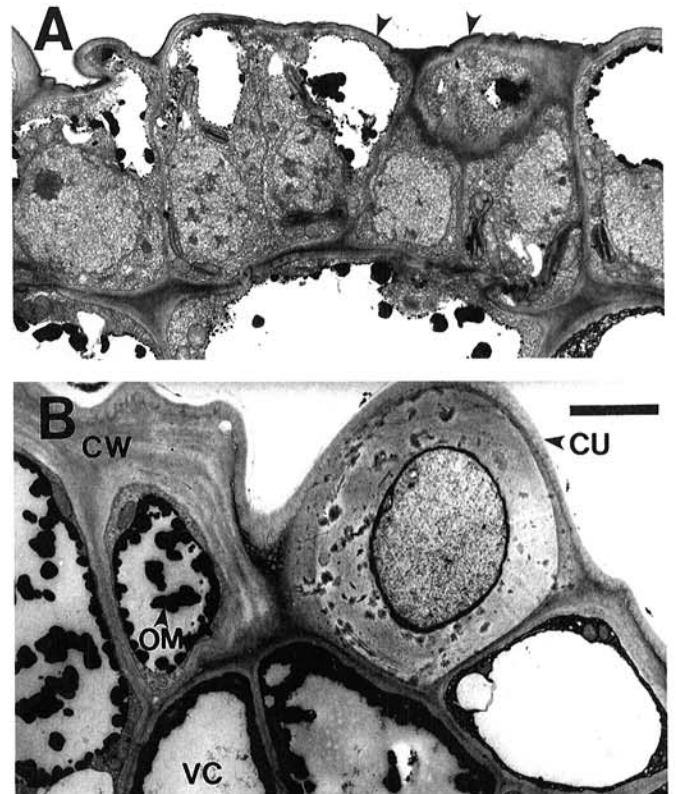
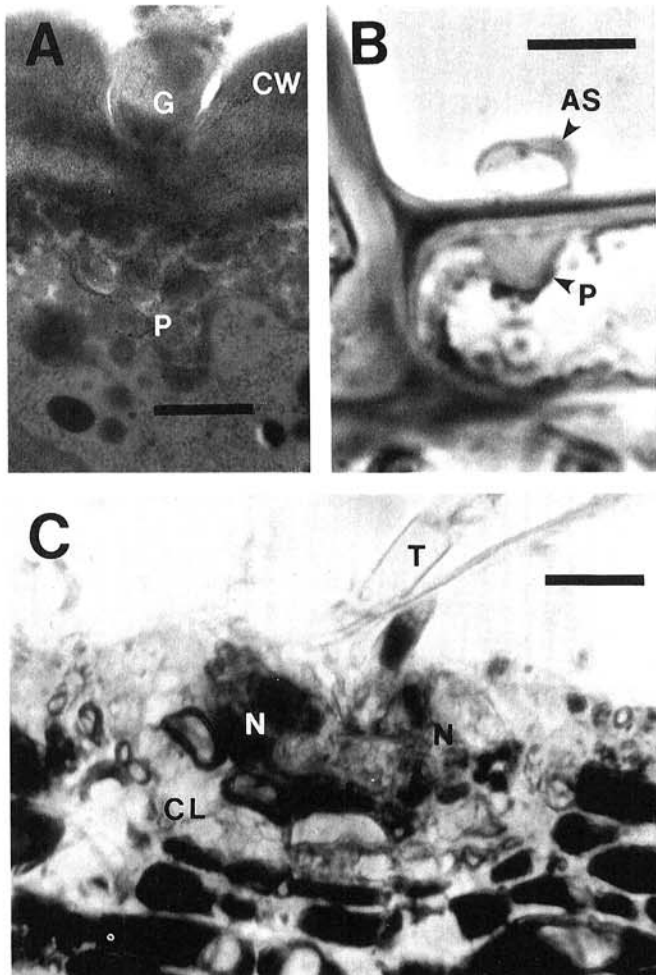


Fig. 6. Host reaction of young hazelnut stem tissue to infection by *Anisogramma anomala*. **A**, Transmission electron micrograph of papilla (P) initiation in response to the germ hypha (G) of *A. anomala* that penetrated the cell wall (CW) of hazelnut epidermis. Bar = 0.3 μ m. **B**, A well-developed papilla formed in an epidermal cell beneath an ascospore (AS). Bar = 10 μ m. **C**, Necrotic tissue (N) and a cicatricial layer (CL) isolated the infection site at the base of a trichome (T) from the healthy tissue at 10 days after inoculation. Bar = 25 μ m.

Fig. 7. Transmission electron micrographs of a longitudinal section of stem tissue of healthy, susceptible hazelnut seedlings. **A**, First internode from apical bud with thin cuticle and nonvacuolated epidermal cells. **B**, Fourth internode with thickened cuticle (CU) and laminated cell wall (CW). Vacuolated cells (VC) contained osmiophilic material (OM) in partially suberized epidermis. Bar = 20 μ m.

DISCUSSION

A. anomala is an obligate biotrophic fungus restricted to several species of *Corylus* (9,28,39). The infection biology of *A. anomala* on European hazelnut, *C. avellana*, appears to share many similarities with obligate biotrophic fungi that cause rust and mildew diseases but has less in common with other related Ascomycetes that cause canker diseases of trees and that are primarily wound colonizers (35). Although infection was first reported to occur through buds wounded by an eriophyid mite (13), it was recently demonstrated that infection occurs only in 1- to 4-week-old shoots and leaf tissue of hazelnut (21,38). In the current study, we documented adhesion of ascospores on and direct penetration of young stem tissue by *A. anomala*.

Ascospores of *A. anomala* are released from perithecia during periods of rain (14,32). Under these conditions, spores must adhere rapidly after deposition or risk being washed off the host by subsequent rain. This study demonstrated that adhesion of ascospores applied to shoots was rapid and irreversible. As the water that contained ascospores wetted the shoots, surface tension drew the spores to the base of the trichomes where germination and penetration occurred. Expansion and maturation of shoot tissue was accompanied by progressively reduced density of trichomes, thickened cuticles, and increased hydrophobicity of the stem surface. These factors all contributed to a lower density of spores being retained on second through fourth stem internodes compared to the more pubescent first internode (Table 1).

Because adhesion occurred in deionized water and spores were not detached from the shoots even after vigorous rinsing, we conclude that substances present on or in the ascospores of *A. anomala*, rather than hydrophobic forces, were responsible for the initial attachment (7). Other studies have shown that glycoprotein adhesives that bind fungal spores tightly can be synthesized and secreted (6,8,29,35,40) or preformed and released (16) as extracellular matrices when the spores contact their host. In our study, irreversible adhesion of ascospores occurred prior to germination. Microscopic examination revealed adhesive pads that attached germinated and nongerminated ascospores to hazelnut shoots within

24 h of inoculation. Similar observations were reported with ascospores of *Venturia inaequalis* on apple leaves (36).

Attachment of ascospores of *A. anomala* on shoots likely involves two phases: spores are discharged from asci covered with a preformed adhesive coating that attaches the ascospore initially, followed by secretion of a more durable adhesive matrix at the point where the germ hypha emerges from the spore. Copious mucilage, which may be the adhesive coating, is produced in hydrated perithecia and asci prior to ascospore release (12). The rapid, irreversible attachment of live ascospores on shoots suggests that a preformed adhesive is present on or in the spore. In addition, ascospores of *A. anomala* killed by boiling initially adhered to shoots of *C. avellana*, suggesting the presence of adhesives on the surface of the spore or the release of preformed compounds during heating. Similarly, uredospores of *Uromyces viciae-fabae* that had been killed by autoclaving produced an extracellular adhesive pad, but unlike live spores, they were readily washed off the host cuticle (6). This study on *U. viciae-fabae* also demonstrated that cutinases and esterase present in the matrix material were destroyed by heating the uredospores, which prevented attachment to the host's cuticle. The importance of such enzymes in adhesion of *A. anomala* is unknown, because adhesion occurred very rapidly on shoot and inert surfaces. During the second phase of adhesion, viable ascospores of *A. anomala* produce an extracellular material that forms the adhesive pad. As with *Nectria haematococca* (22), ascospores of *A. anomala* treated with sodium azide or cyclohexamide, which halt respiration and inhibit protein synthesis, respectively, were less likely to adhere to shoot surfaces than were live spores. Therefore, the first phase of adhesion of *A. anomala* ascospores appears to be passive binding, whereas metabolism is required in the second phase for the synthesis of extracellular materials near the point of germination (22).

Ascospores of *A. anomala* also attached to nonhosts but not to as great a degree as to *C. avellana*. When shoots of several tree species were dipped in an aqueous suspension of ascospores, water and spores were retained in the dense trichomes of *C. avellana* shoots and around the numerous glands on *B. pendula* shoots. However, on the hydrophobic surfaces of the glabrous hosts, *Acer palmatum* and *P. serrulata*, water rapidly ran off, and few spores were retained. Similarly, ascospores had a greater affinity for glass than for hydrophobic polystyrene and Parafilm. The observed differential susceptibility of *C. avellana* internodes to *A. anomala* (21) may be related in part to the number of ascospores that adhered on the surface, with the greatest number retained on the most hydrophilic internode nearest the apical bud.

Observations of Calcofluor-stained ascospores suggested that germination and penetration of host epidermal cells occurred directly beneath the spore. Neither germ hyphae nor appressoria were observed emerging from the 2,000 ascospores on hazelnut stems examined in this study. In addition, direct penetration also is suggested by morphology of germ hyphae in vitro, where emerging germ hyphae expanded into a vesicle within 24 h. Evidence for direct penetration also was obtained by observation of sectioned stem tissue. The germ hyphae emerged from the lateral walls of spores and penetrated through the extracellular matrix directly into the underlying cell. After germination, hyphae of *A. anomala* breached the epidermal cell walls of *C. avellana*, and intracellular vesicles expanded to completely fill the cells in 10 days. These vesicles appear similar in morphology and function to those produced by obligate biotrophic fungi, which require close contact between living cells of the host and parasite (3,10,11,15,27,30,42). For example, many rust fungi that directly penetrate their host produce intracellular vesicles once the penetration hypha breaches the cell wall. In these rusts, infection hyphae grow from the vesicle to colonize adjacent epidermal and parenchyma cells (3,15,27,30). Based on our observations, we can only speculate that something similar occurs in *A. anomala* on hazelnut.

Because of the necrotic cells surrounding the infection sites,

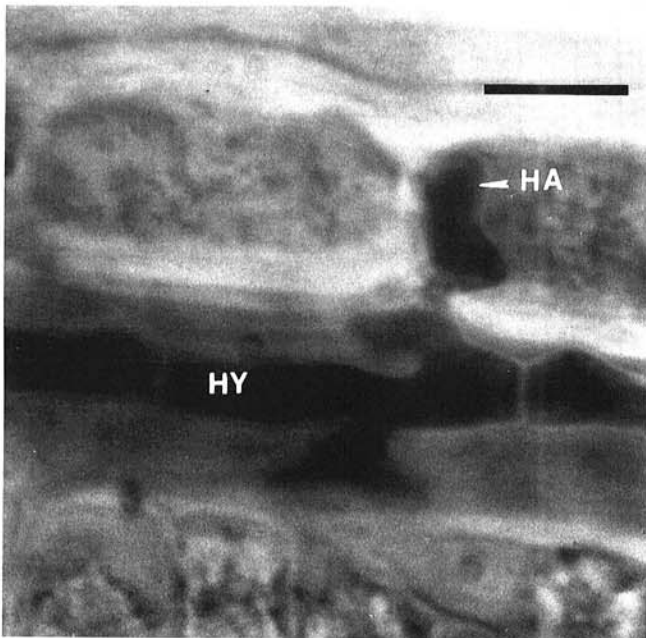


Fig. 8. Colonization of secondary xylem of hazelnut by *Anisogramma anomala* observed with bright field microscopy. Hypha (HY) extended through the secondary xylem, and haustorium-like hypha (HA) formed in the xylem parenchyma cells. Bar = 20 μ m.

proliferation of infection hyphae from vesicles was not observed. The development of papillae (1), apposition of callose, and localized hypersensitive reactions of the host cells in advance of the hyphae appears to restrict colonization (18). Similar host reactions have been observed in incompatible hosts in response to invasion by other biotrophic fungi (15,19). Accumulation of dark-staining materials in over 60% of the infection sites of *Erysiphe graminis* in compatible lines of barley was related to failure of penetration hyphae to establish in epidermal cells (24). Similarly, we observed only failed penetrations and wound reactions in response to hyphae of *A. anomala* in epidermal cells of susceptible hazelnut clones (31), suggesting that successful infections occur rarely. This agrees with inoculation studies in which an inoculum concentration of 5×10^5 ascospores per ml or greater was required to infect 50% of susceptible hazelnut seedlings (21).

As shoot tissue of *C. avellana* matures, it becomes more resistant to infection by *A. anomala*. In a previous study, the unexpanded leaf and stem internode nearest the apical bud were highly susceptible ($\geq 89\%$ infection rate), whereas the fourth internode and leaf were moderately resistant ($\leq 17\%$ infection rate) (21). The decline in susceptibility of stem internodes during maturation corresponded to changes in ascospore adhesion, structural characteristics of the epidermis and cuticle, cell vacuolation, and accumulation of osmiophilic substances. The epidermis of the first internode has a thin cuticle and cell wall that may offer little resistance to the penetration of hyphae. The epidermis of the second through fourth internodes, which were 7 to 30 days older than the first internode, has thickened cuticles and thick, laminate cell walls, which would present a more formidable barrier to penetration by germ hyphae (2,20,25). After penetration of a host cell, biotrophic fungi require an intimate association with living cytoplasm of host cells (42). Epidermal cells of the first internode were filled with cytoplasm and may provide a substrate for establishment of *A. anomala*, while the cells of older tissue were more vacuolate. In addition, osmiophilic compounds that accumulated in epidermal cells of the third and fourth internodes may be fungitoxic (23).

Although wound and hypersensitive responses to *A. anomala* were common early in the infection process and appear to limit infection, hazelnut nevertheless is readily infected in orchards (13,39) and in controlled inoculations (21,38), but high inoculum levels are required. Our observations suggest that colonization beyond initial penetration is rare. Hyphae that colonized beyond the epidermal cells resemble those of biotrophic fungi infecting compatible hosts (21). Our observations of hyphae in asymptomatic wood of infected trees showed that hyphae had colonized the xylem and phloem of infected branches by February. When the trees broke dormancy during March, 10 months after infection, however, infected phloem cells rapidly became necrotic, and the affected area developed into a canker.

The reaction to the presence of *A. anomala* in immature epidermis soon after infection is rapid and may be related to the degree of resistance observed among European hazelnut clones. One *C. avellana* cultivar, Gasaway, has been reported to be completely resistant to eastern filbert blight when inoculated with ascospores (26,31). However, when Gasaway trees were graft-inoculated with infected scions of a susceptible cultivar, Gasaway rootstock was colonized by *A. anomala* (J. N. Pinkerton, unpublished data). After 12 months, the infected wood had formed cankers and stromata. Since the colonization and reproduction of the pathogen on this cultivar was not impaired, resistance in Gasaway appears to be associated with the initial infection process rather than subsequent colonization. The nature of resistance to eastern filbert blight in *C. avellana* clones (31) and in other *Corylus* spp. (4,9,28) is not well understood. Additional knowledge on the mechanisms of host response in *Corylus* spp. to *A. anomala* infection and colonization would be invaluable to hazelnut breeders when selecting clones able to resist this important disease.

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