Infection of Aboveground Parts of Bean by Pythium aphanidermatum

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ABSTRACT

Zoospores of *Pythium aphanidermatum* were shown to aggregate on leaves and stems of *Phaseolus vulgaris* at the bases of trichomes, against glandular trichomes, around trichome sockets, at stomata, at the junction of epidermal cells, and at wounds. Upon aggregation, the zoospores encysted, and the germ tubes made contact with host tissue, within 30 min after inoculation at 24 C. One-day-old mycelial fragments, however, required 10 h to establish appressoria. Once hyphae became established in plant cells, pathogenesis proceeded at the same rate, regardless of whether the inoculum had been zoospores or mycelial fragments. The first aerial hyphae on colonized suscepts usually arose from cystospores and subcuticular or intracellular hyphal bulges.

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Zoospores of several pythiaceous fungi are attracted to certain sites of plants, then encyst, germinate, and penetrate the host. The behavior of zoospores toward plant roots has been substantiated (4, 5, 13, 14, 17, 32), and accumulation of zoospores at the regions of elongation (3, 7, 16, 28, 29, 31) and maturation (10, 17, 20), and at wounds (9, 21, 22, 28, 29, 31) on roots has been reported. Encystment of zoospores on above ground parts of plants (15, 26, 27) has been reported in *Phytophthora* spp., but not in *Pythium* spp.

The etiology and economic importance of Pythium blight on bean have been reported (1, 8, 11, 19, 30), but the zoospore tactic and tropic responses toward above ground parts of plants have not been studied. In this investigation, using *Pythium aphanidermatum* (Edson) Fitz., the pathogenesis of zoospores was compared with mycelial fragments on above ground parts of *Phaseolus vulgaris* L.

MATERIALS AND METHODS.—The *Pythium aphanidermatum* culture used in this study was isolated from above ground parts of diseased bean plants (*Phaseolus vulgaris* L. 'Tendercrop'). To produce zoospores, the isolate was grown on lima bean or corn meal agar for 2 to 30 days at 28 C. Five disks (4 mm in diam) from the culture were then transferred to a petri dish containing 20-40 small sections of bean leaf (0.7 mm^2) in 13 ml of sterile distilled water. After incubation for 24 h at 31 C, this water was replaced with 7 ml of sterile distilled water and maintained at the same temp. The zoospores were collected 6 h after the water replacement.

To produce mycelium without fruiting bodies, the fungus was grown in potato-dextrose broth (PDB) for 24 h on a shaker at 24 C. After removing the originally inoculated agar plugs, the mycelium was macerated in a Waring Blendor for 30 s at low speed, and for 2 min at high speed, while a total volume of 100 ml of sterile distilled water was added in small aliquots. The size of mycelial fragments varied from microns to millimeters.

Bean plants (*P. vulgaris* 'Pinto III') were grown in a flat of vermiculite for 10 days at 28 C in a growth chamber. After removal of the first two true leaves, the epicotyl was removed and inoculated by dipping it into a heavy suspension either of zoospores or 1-day-old mycelial fragments for 30 min. Inoculated epicotyls were incubated in petri dishes containing wet filter paper at 24 C for periods ranging from 30 min to 24 h.

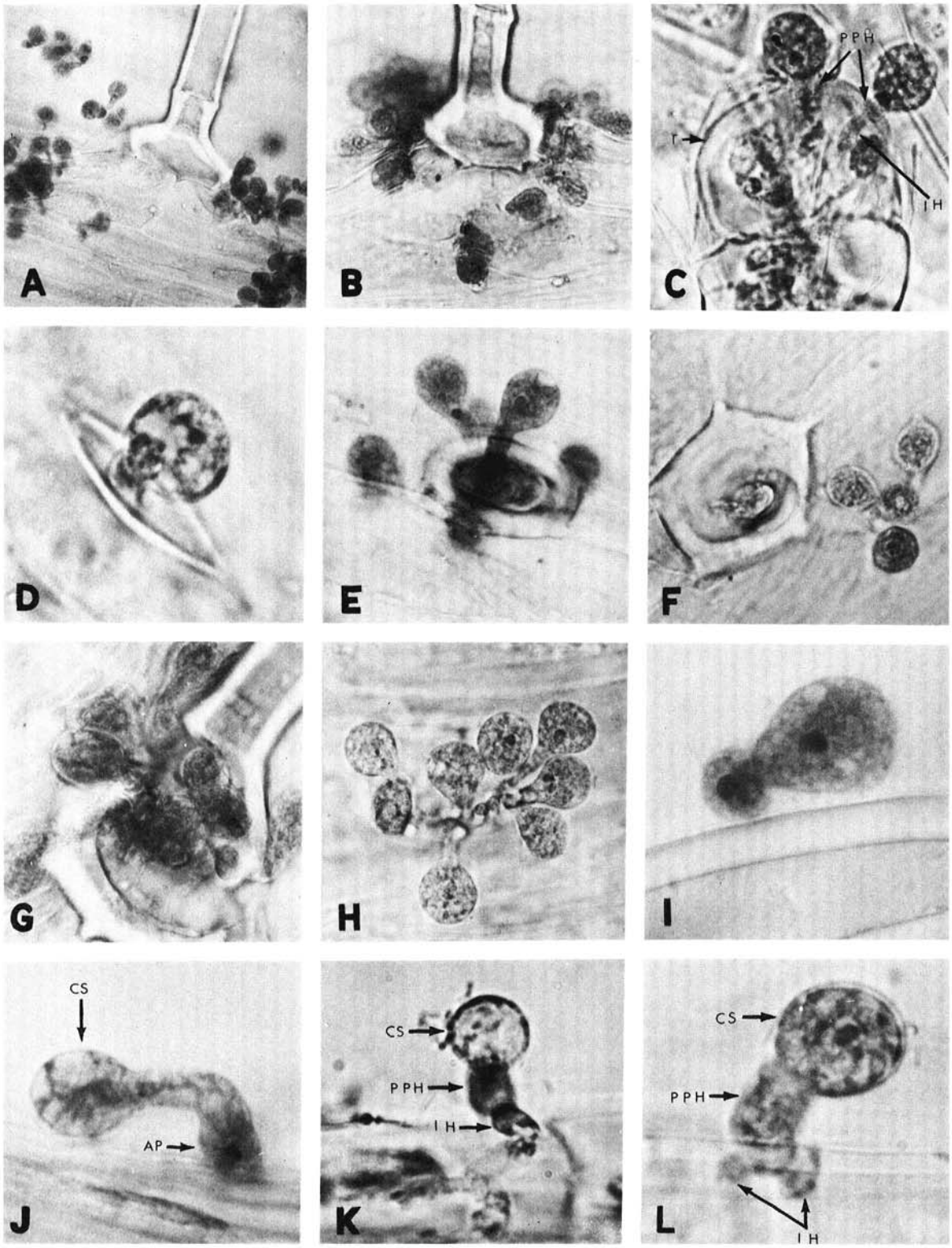
After the various incubation periods, plant segments were fixed in formaldehyde-acetic acid-alcohol (FAA) for 3 days. For rapid observations, plant samples were heated in FAA to remove the chlorophyll. FAA-fixed plant tissues were washed with water and stained with cotton blue-lactophenol. Plant segments were hand-sectioned to locate and follow the development of the fungus.

In another portion of this study, 28 cultivars of *P. vulgaris,* grown in the greenhouse for 1 mo, were spray-inoculated with either zoospores or mycelial fragments. The inoculum concn was approximately 10^3 in 1 ml water/plant. After 4 days incubation in a moisture chamber at 28 C, plant segments were removed and similarly stained and examined.

RESULTS.—*Behavior of zoospores at infection courts.*—When a suspension of zoospores was sprayed on the surface of aboveground parts of bean plants, they often aggregated at suitable infection courts such as the bases of trichomes (Fig. 1-A, B), at the junction of epidermal cells (Fig. 1-F), against glandular trichomes (Fig. 1-C), at stomata (Fig. 1-D), at wounds (Fig. 1-G), and around trichome sockets (Fig. 1-E, F). When a freshly cut cross-section of bean stem was placed in a petri dish containing 13 ml of zoospore suspension (3×10^3 zoospores/ml), zoospores aggregated inside the pith where most of the zoospores remained motile for a few seconds to minutes, then encysted (cystospores) within 1 to 2 s. Some zoospores moved into and out of the pith. Fewer aggregated colonies were observed on the epidermal layer of the stem cross-section than in the pith. The mass movement of zoospores to the pith was almost completed within 5 min after placing the cross-section of stem in the zoospore suspension at 29 C.

Cystospore germination.—Zoospores aggregated, encysted, and produced germ tubes which became prepenetration hyphae (germ tubes of cystospores oriented to infection courts) within 30 min after inoculation. The first karyokinesis was observed as soon

as the prepenetration hyphae became established; one nucleus usually remained in the cystospore and the other nucleus migrated into the developing prepenetration hyphae (Fig. 1-I). Immediately following aggregation and



encystment, all the observed cystospores formed prepenetration hyphae (3-8 μm in diam) oriented toward the respective infection court [Fig. 1-(A to H)]. If a particular cystospore was affixed on the infection court, the force exerted by the prepenetration hyphae upon the cuticle made the round cystospore assume the shape of a very young mushroom (Fig. 3-J). The prepenetration hypha sometimes appeared as an appressorium-like structure from which infection hyphae developed (Fig. 1-C, K, L). Occasionally, the prepenetration hyphal tip appressed on the surface of the plant tissue and became an appressorium (Fig. 1-J).

Penetration of suscept tissues by prepenetration hyphae.—The prepenetration hyphae of cystospores penetrated directly through the plant cuticle (Fig. 1-C, K, L; 3-J), natural openings (Fig. 1-D, E, F), or wounds (Fig. 1-G). The penetrating hyphae were usually constricted at the point of entry into the respective cell (Fig. 1-C, K, L; 3-J). A distinct infection peg originating from the prepenetration hyphae was not observed. A prepenetration hypha produces one (Fig. 1-C, K) or two (Fig. 1-L) infection hypha(e).

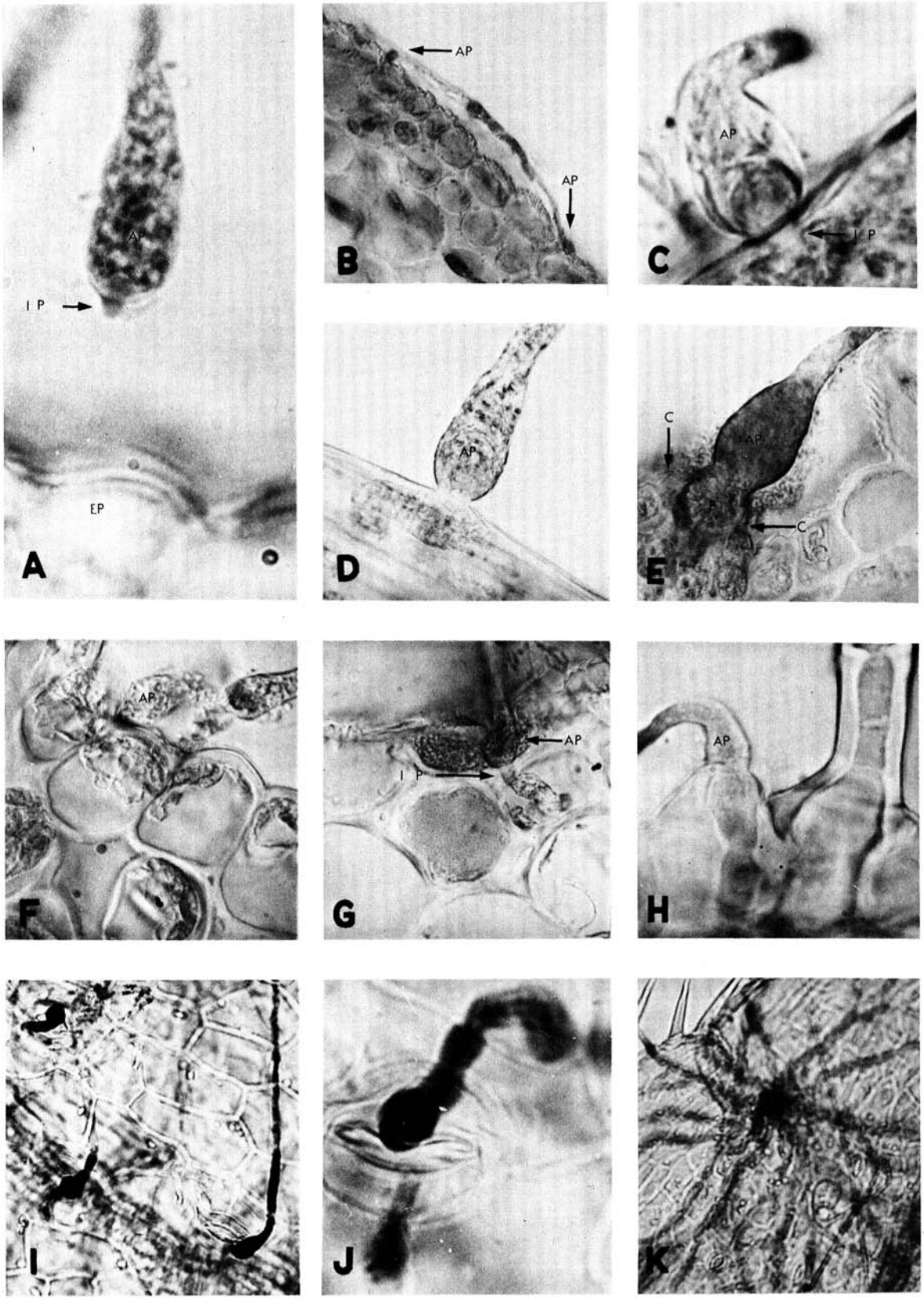
Penetration of suscept tissues by mycelium.—The infection processes of the mycelial fragments were different from the previously described infection processes of zoospores. Hyphae on plant surfaces branched randomly and their tips formed appressoria (Fig. 2-B). The diameter of the appressoria varied from 6 to 43 μm (Fig. 2-A to I). An infection peg (Fig. 2-A) developed from the bulbous appressorium and penetrated directly into the epidermal cell (Fig. 2-C, D, G, H), guard cells of stomata (Fig. 2-I), between epidermal cells (Fig. 2-F), and at the bases of trichomes (Fig. 2-H). In one case, the appressorium (43 μm in diam) formed a subcuticular bulge (35 μm in diam) on the epidermal cell wall prior to penetration of the cell (Fig. 2-E). Occasionally, when an appressorium contacted the plant epidermis the adjacent cuticle would raise and separate from the epidermal cell wall. Formation of appressorial infection pegs was observed 12 h after inoculation of plants with mycelial fragments. A hyphal tip sometimes branched to become an infection cushion instead of becoming an appressorium. At times, the mycelium penetrated directly through the epidermis, trichome sockets, or pores of stomata without appressorium formation.

Colonization of suscept tissues and development of signs.—Cytoplasmic granulation of suscept cells was observed when prepenetration hyphae or appressoria were in contact with suscept tissues. The cytoplasmic granulation in epidermal and cortical cells was oriented toward the appressorium (Fig. 2-F). Penetration of the suscept cell walls by prepenetration hyphae or infection pegs of appressoria required approximately 2 h at 24 C. The infection hyphae usually progressed intracellularly through one-to-three cells immediately around the infection court. An infection hypha inside one cell sometimes twisted or coiled before penetrating into and colonizing adjacent cells. A new hypha frequently arose and branched from the twisted or coiled hyphae and advanced in another direction (Fig. 3-B). After one-to-three plant cells were colonized, a hyphal tip inside the suscept cell grew towards the aerial portion of the plant. This took place approximately 4 h after zoospore inoculation, or 14 h after 1-day-old mycelial-fragment inoculation. At this time, the cystospore on the surface of the plant formed a hyphal tip which coiled and emerged from the cystospore (Fig. 3-J to L). Aerial hyphae emerging from cystospores either infected adjacent plant cells or became filamentous sporangia. The cystospore shell may remain in situ as the hyphal tip grows out of the shell (Fig. 3-K, L), or the cystospore shell might be carried along on the tip of the growing hypha (Fig. 3-L).

Emergence of aerial hyphae from infected tissues was observed about 8 h after inoculation of plants with zoospores, or 18 h after inoculation with mycelial fragments (Fig. 3-C to I). Aerial hyphae usually emerged from the bulges (8-22.3 μm in diam) which were often found in epidermal cells, glandular trichomes, and guard cells, and might also be found in parenchyma cells of hollow pith and cortex and in the innermost layer of xylem near hollow pith. The bulges sometimes originated from the area of coiled or twisted hyphae (Fig. 3-B, F). Subcuticular bulges were frequently found only when hyphae emerged from epidermal cells (Fig. 3-C to E). Some aerial hyphae emerged without formation of the bulges (Fig. 3-I) and were often constricted at the cell wall and cuticle, giving the appearance of a double-bulbous structure (Fig. 3-H). These emerging bulbous, cytoplasmic-dense hyphae, which were morphologically unlike sporangia, released zoospores in the presence of free water.

Fig. 1-(A to L). Aggregation of zoospores of *Pythium aphanidermatum* at various infection courts of aboveground parts of *Phaseolus vulgaris.* **A)** Aggregated zoospores on the surface of a stem (× 275). **B)** Aggregated zoospores at the base of trichome (× 450). **C)** Glandular trichome (T) being penetrated by infection hyphae (IH) developed from prepenetration hyphae (PPH) of cystospores (× 780). **D)** A prepenetration hypha from a cystospore going through an open stoma pore (× 1,330). **E)** Aggregated zoospores around a trichome socket and prepenetration hyphae penetrated a trichome socket (× 720). **F)** Aggregated zoospores at the junction of epidermal cells and a zoospore inside a trichome socket (× 560). **G)** Aggregated zoospores at a wound and prepenetration hyphae grew towards the wound (× 810). **H)** Aggregated zoospores developed prepenetration hyphae toward an infection court (× 760).

(I-L) Karyokinesis and development of infection hyphae from prepenetration hyphae of cystospores. **I)** The first karyokinesis of cystospore on a trichome; one nucleus in cystospore and the other in the developing prepenetration hypha (× 1,590). **J)** An appressorium (AP) developed from a germ tube of cystospore (CS) showing a nucleus at the tip of appressorium (× 1,250). **K)** An infection hyphae (IH) developed from a prepenetration hypha (PPH) of a cystospore (CS) showing the constriction at the point of penetration into a suscept cell (× 1,010). **L)** Two infection hyphae (IH) developing from a prepenetration hypha (PPH) of a cystospore (CS) (× 1,560).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Hyphae inside suscept tissues generally advanced longitudinally to the axis of the stem through cortex, epidermis, and pith and were constricted at the cell walls (Fig. 2-J, K; 3-A). The rate of colonization of pith was faster than that of the cortex or epidermis until the fungus reached a node. At this point, the advance of the mycelium was restricted and the fungus progressed from one internode to an adjacent internode by colonizing the stipules.

Sclerenchyma tissues were rarely colonized even though the fungus had ramified throughout the adjacent cortex and pith. The hyphal bulges and oospores were sometimes seen inside vessel elements near the pith after inoculation with zoospores or mycelial fragments and 4 days of incubation in a moist chamber at 28 to 30 C. Mycelium, sporangia, oogonia, antheridia, and oospores occur in pith and cortical tissues colonized by the fungus. The characteristic fluffy mycelium was usually seen on the plant surface after the cortical tissues were completely colonized. Sporangia were usually heavily concd at the marginal areas of the blight lesions where the dactyloid sporangial tips appeared to be cushion-shaped.

Guard cells of stomata on stipules were preferentially attacked by the fungus (Fig. 2-I, J). From the point of infection, a radial mycelial growth pattern could be observed in infected leaves and stipules (Fig. 2-K). Zoospores were occasionally observed 4 days after inoculation with mycelial fragments, but only on the stipules. All forms of fungal structures were observed on or in the stipules; however, dactyloid sporangia with cushion-shaped tips were the most commonly observed. Mycelium on leaves and stipules preferentially attacked veinal areas. Mycelial bridges between trichomes were commonly observed. Vascular discoloration at nodes might be seen if internode areas were infected.

DISCUSSION.—Previous studies indicated that appressoria were formed prior to penetration by germinating cystospores of *Phytophthora infestans* (27) and *Pythium aphanidermatum* (20) on potato leaves and bentgrass roots, respectively. In this study, most of the germ tubes of cystospores of *P. aphanidermatum* did not form appressoria on bean leaves and stems, whereas mycelia on those structures did produce appressoria. Formation of an infection cushion from mycelia, similar to that reported in *Rhizoctonia solani* (6, 18), was observed.

The formation of appressoria required 10 h at 24 C and 3 h at 30 C when mycelial fragments were used as inoculum, whereas prepenetration hyphae were observed 30 min after zoospore inoculation at 24 C. The formation of prepenetration hyphae from zoospores might require less than 30 min, but earlier observations were not attempted.

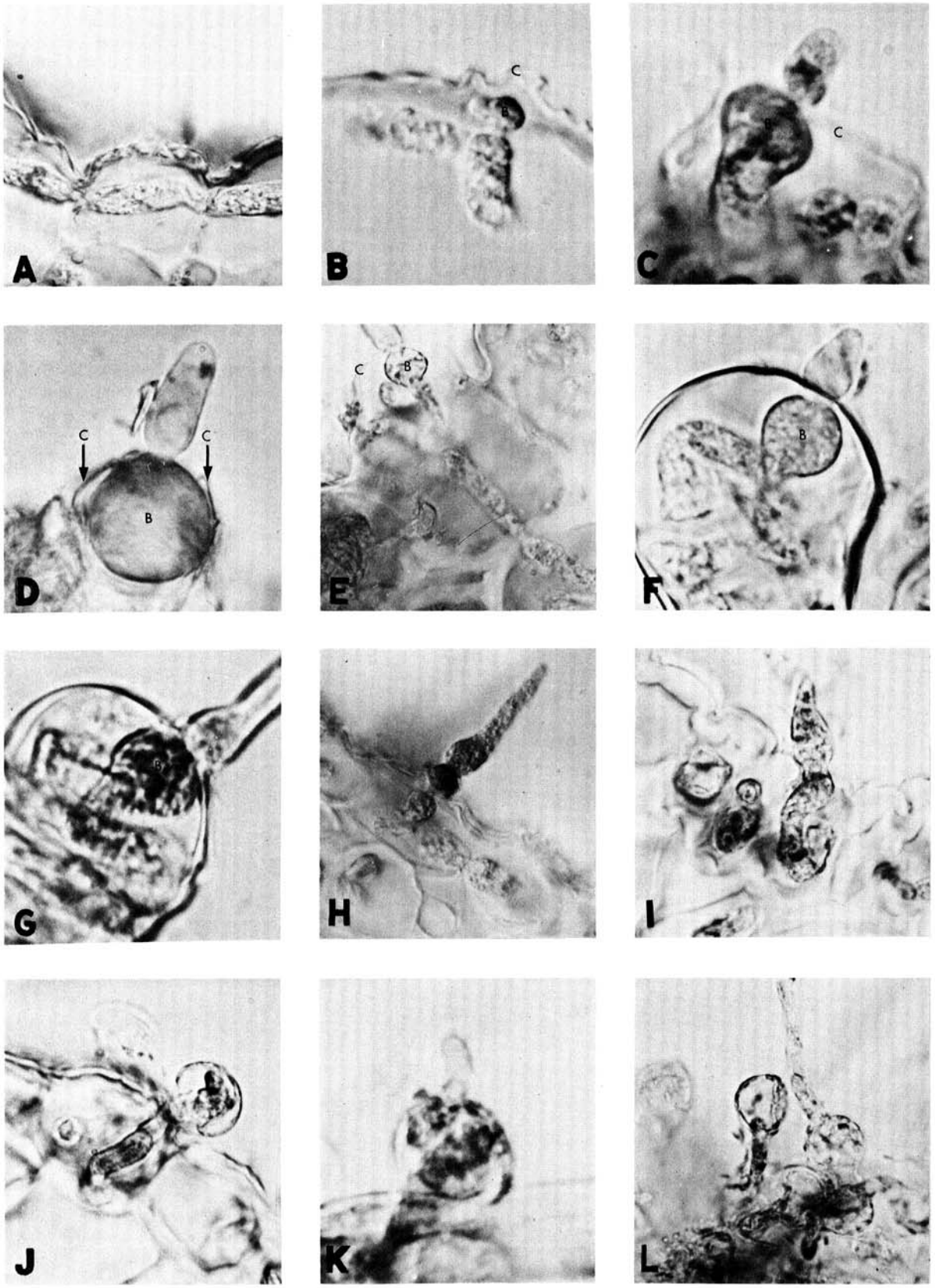
The protoplasm from a cystospore of *P. infestans* has been reported to flow through the germ tube into the appressorium emptying the cystospore (27); however, the flow of protoplasm from cystospore to prepenetration hypha of *P. aphanidermatum* was not observed in this study. The first karyokinesis was observed when a prepenetration hypha was established. Aerial hyphae were also formed from cystospores.

Appressorial pegs or outgrowths from prepenetration hyphae penetrated the cuticle and lower layers. A definite appressorial peg (Fig. 2-A, C) was observed, whereas a prepenetration hyphal peg (Fig. 1-L, 3-J) was not as clearly detected. Formation of an infection peg from an appressorium that originated from a cystospore of *P. aphanidermatum* was observed on a bentgrass root hair by Kraft et al. (20).

The mode of penetration into host plants by mycelium of *Pythium ultimum* (23, 24), *P. debaryanum* (2, 12), and *P. sylvaticum* (25) has been reported previously, but not that of *P. aphanidermatum*. An infection peg was reported to arise from an appressorium of *P. ultimum* on a peach root 5-8 h after inoculation of roots with mycelium at room temp (24), and penetration of bean leaves by an appressorium-like swelling of *P. debaryanum* mycelium was reported to occur 48 h after inoculation at 28 C (2). In this study, an appressorium formed 10 h after inoculation with mycelium of *P. aphanidermatum* at 24 C and 3 h at 30 C, and 2 h later infection pegs were observed penetrating bean stems. Adegbola and Hagedorn (2) reported that infection hyphae of *P. debaryanum* developed from a "subepidermal vesicle" which originated from an appressorium-like swelling at the point of contact with bean stems. Similar hyphal swellings in mycelium of *P. sylvaticum* in strawberry roots were observed by Nemec (25). However, in this study, the bulge of *P. aphanidermatum* occurred subcuticularly or intracellularly when hyphae emerged from epidermal cells or glandular trichomes and became aerial mycelium. Intracellular bulges also occurred in tissues surrounding the hollow pith. These hyphal bulges later appeared to develop into oospores 4 days after inoculation of plants with mycelial fragments or zoospores at 28 C. Intracellular hyphal bulges in pith or cortical cells resembled oogonia formed in liquid culture of PDB.

Since *Pythium* spp. are soil-borne fungi, there is little doubt that plant infections occur at or near the soil line. The symptoms of Pythium blight on bean plants start on the stem at the soil line or on the lower branches of the

Fig. 2-(A to K). Mode of penetration and colonization of *Phaseolus vulgaris* by mycelium of *Pythium aphanidermatum*. **A)** An appressorium (AP) developed from mycelium showing an infection peg (IP); appressorium detached from epidermis (EP) of a stem (× 1,620). **B)** Terminal and intercalary appressorium (AP) formation by mycelium on a stem (× 345). **C)** An appressorium (AP) with an infection peg (IP) in contact with the cytoplasm of a plant epidermal cell (× 1,950). **D)** An infection hypha developed from an appressorium (AP) branching dichotomously (× 650). **E)** Bulging (B) of an infection peg of appressorium (AP) subcuticularly (C) (× 720). **F)** The granulated cytoplasms of epidermal and cortical cells are oriented toward appressorium (AP) located at the junction of epidermal cells (× 690). **G)** Constriction of an infection peg (IP) from an appressorium (AP) at the point of plant cell penetration (× 690). **H)** An appressorium (AP) and an infection hypha adjacent to a trichome (× 810). **I)** Appressoria on guard cells of stomata on a leaf (× 375). **J)** An infection hypha with constrictions in epidermal cells and a guard cell (× 1,100). **K)** Radial growth habit of mycelium from an infection court in a leaf (× 160).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plant. The possibility of infection occurring initially above the lower branches of plants has not been substantiated. The propagules which initiate infections can be mycelial fragments, sporangia, zoospores, and/or oospores. Zoospores are released from oospores (19) as well as from sporangia. Zoospores may swim in a film of water from the lower plant stem to the lowest branches where young parenchyma tissues are then invaded. Zoospores may also be splashed onto upper parts of plants by rain, thus creating the possibility of initial symptoms occurring above the lower branches. Field and greenhouse experiments (19) also indicate that zoospores are much more effective in initiating infections than is mycelium.

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Fig. 3-(A to L). Hyphae of *Pythium aphanidermatum* emerging from infected tissue of *Phaseolus vulgaris.* **A)** A hypha in epidermal cells of stem showing constrictions at cell walls of suscept (× 765). **B)** A hyphal bulge (B) originating from twisted hypha in an epidermal cell toward cuticle (C) (× 1,440). **C)** An aerial hypha originated from the subcuticular bulge; cuticle (C) separated from epidermis (× 1,600). **D)** An aerial hypha emerged from a bulge (B) under plant cuticle (× 940). **E)** Advanced stage of C and D (× 780). **F)** An aerial hypha emerged from a bulge (B) inside a glandular trichome; the bulge (B) originated from a twisted hypha (× 1,350). **G)** Advanced stage of F (× 1,250). **H)** An aerial hypha emerged from double bulges showing constriction at the cell wall and the cuticle of a stem (× 690). **I)** An aerial hypha emerged from an epidermal cell without a hyphal bulge (× 720).

(J-L) Sequential development of aerial hypha from a cystospore. **J)** A cystospore affixed to the epidermal cell of the stem showing a prepenetration hyphal peg which became an infection hypha in epidermis, and the lower wall of the cystospore appears to be buckled inward; a hyphal tip developed in the cystospore (× 925). **K)** Advanced stage of hyphal tip development of J; the hyphal tip inside cystospore coiled and emerged out to become an aerial hypha (× 1,400). **L)** Advanced stage of K, the cystospore (right) remaining in situ; a second aerial hypha (left center) has the cystospore case carried at the tip of the hypha (× 720).

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