# Developmental Morphology of the Black Knot Pathogen on Plum

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ABSTRACT

Plum trees (*Prunus domestica* 'Stanley') were inoculated with ascospores of *Dibotryon morbosum*, the incitant of black knot disease, and the development of the pathogen within and on the host was studied. The fungus established a well organized tissue within the branch, from the xylem to the epidermis, and on the surface of the hyperplastic host tissues. Within the host the fungus formed a pseudoparenchymatous tissue which was devoid of any type of host cell penetrations. On the host surface the fungus developed a darkly-pigmented, thick-walled ascostroma on which conidiophores formed and within which many erumpent locules arose. Each mature centrum consisted of a basal layer of asci that had grown upward toward the apex among previously formed pseudoparaphyses. Ascospores were two-celled, and were two-ranked within bitunicate asci. Phytopathology 60:1238-1244.

*Additional key words: Dibotryon morbosum*, fungus taxonomy, *Prunus domestica*.

The black knot disease of plums caused by the ascomycetous fungus *Dibotryon morbosum* (Schw.) Theiss. & Syd. is a serious problem on *Prunus domestica* L. 'Stanley' in Pennsylvania. Effective chemical and sanitation control programs have been hampered by a lack of information concerning the biology of the fungus and the conditions essential for the development of disease epidemics in this climate. Smith (8) devised a method of trapping *D. morbosum* ascospores in large numbers and used this method to determine that peak ascospore discharge occurred during mid-May, after the petal-fall stage of growth on plum. Smith & Lewis (9) and Wainwright, in work only partially reported, found a close correlation between black knot infection, ascospore discharge, and rainfall, especially rainfall accompanied by relatively high (13-25 C) temp. The purpose of this paper is to report studies of the developmental morphology of *D. morbosum* from infection to completion of the life cycle and to comment on the nomenclature of the organism.

MATERIALS AND METHODS.—One-year-old plum trees (*P. domestica* 'Stanley') were cut back to 4 or 5 buds on the scion and potted in steam-treated 2:1 soil to sand mixture. The plants were maintained in a greenhouse and were inoculated when 3 inches of growth had occurred.

The inoculum was prepared using knots excised from diseased Stanley trees. These were wetted for 2-3 min in distilled water, then placed over 2% water agar in a petri dish. Ascospores ejected onto the surface of the agar were collected by passing a small amount of sterile distilled water over the surface of the agar and agitating with a rubber-tipped glass rod. The suspension was nonstandardized, but always contained at least 50,000 ascospores/ml.

Prior to inoculation, the plants were washed with a strong stream of water. One ml of the ascospore suspension was pipetted onto each shoot of the plant at two nodes and the internode between. Sterile distilled water was pipetted onto branches of check plants. The inoculated area was identified by a string tag. The plants were placed in a mist chamber for 5 days at 21 C, then removed to a greenhouse bench.

Four branches were collected from inoculated plants and two branches from check plants each month. After the leaves were removed, the branches were surface-sterilized for 5 min in a 10% solution of clorox (sodium hypochlorite, 5.25%) plus a trace of detergent, rinsed 3 times in sterile distilled water, and cut to 100 mm in length. The branches were cut aseptically into twenty 5-mm pieces. Alternate pieces were placed in numbered vials of fixative; the remaining pieces were placed on 1.5% water agar in petri dishes and incubated at 21 C. Microbial growth was recorded and the fungal isolates were identified. Pieces adjacent to those yielding the pathogen were prepared for sectioning. In addition, branches of field-inoculated trees were sampled to obtain several stages in the development of the fungus.

Plant materials were fixed in formalin-aceto-alcohol (FAA) and embedded in paraffin following a tertiary butyl alcohol dehydration schedule (2). Sections were cut at 5 and 10 μ and stained with Johansen's quadruple stain, or with Heidenhain's iron hematoxylin and differentiated with picric acid (2). Photographs were taken on Kodak Plus-X-Pan, Kodak Contrast Process Pan, or Kodak Panatomic-X film.

The fungus was cultured on glucose agar as proposed for morphological studies of *Cladosporium* species (10).

RESULTS.—Symptoms and signs typical of the black knot disease resulted from the inoculation of plants with ascospores of *D. morbosum*. Generally, plants in the greenhouse displayed no rupture of the branch epidermis due to hyperplasia and hypertrophy of underlying tissues until the affected area was 2-4 times the original diam of the branch, whereas, on field-grown plants rupture frequently occurred after only slight

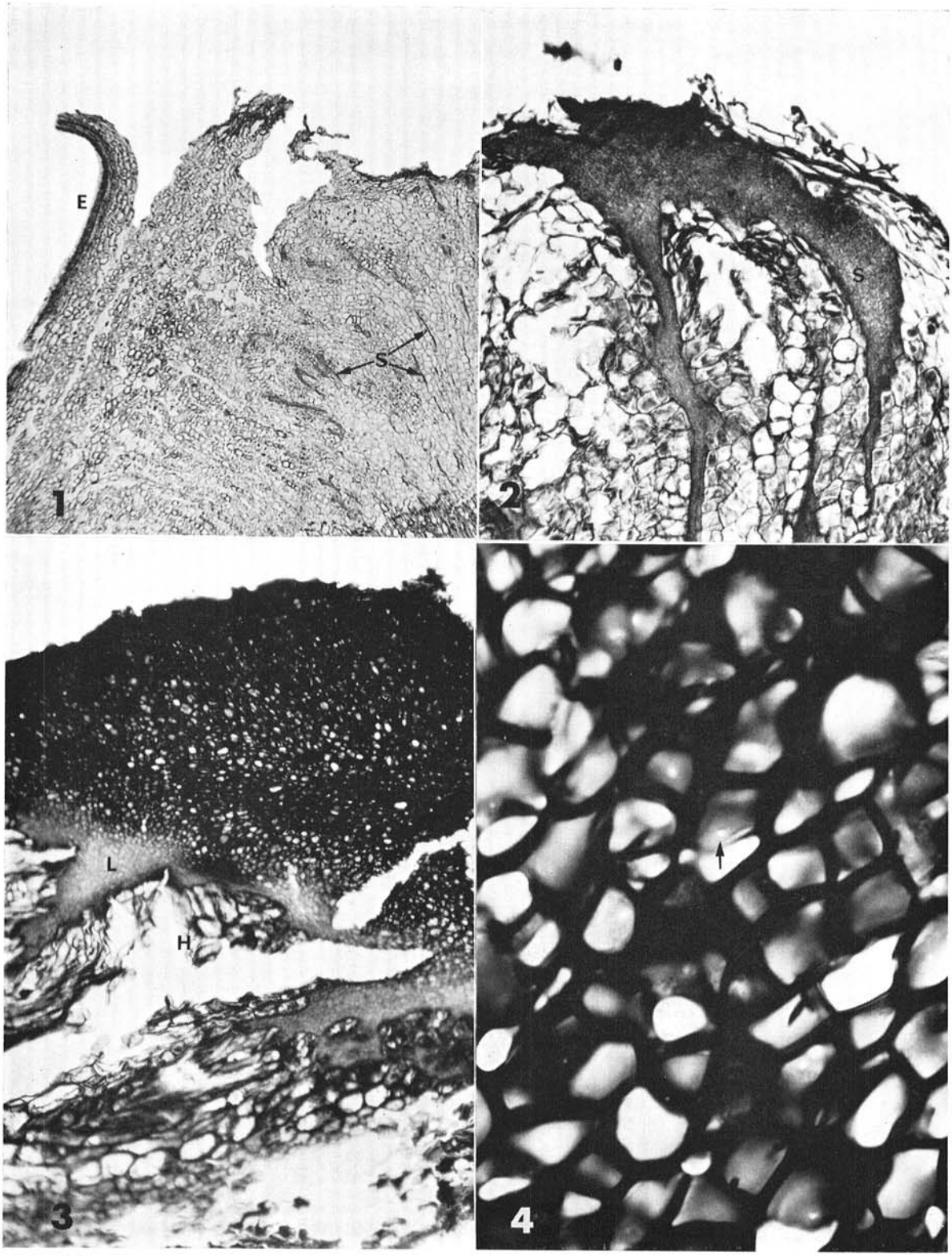


**Fig. 1-4.** Early developmental stages of *Dibotryon morbosum* on *Prunus domestica*. **1)** Cross section of an infected branch showing the split host epidermis (E) and fungus strands (S) in the area of hyperplasia soon after rupture of the epidermis (×40). **2)** Cross section of an infected branch showing the fungus tissues (S) formed at the periphery of the host cortex soon after the host epidermis split (×200). **3)** Cross section of an infected branch showing the stroma of the fungus prior to locule formation. Fungus cells (L) below give rise to thick-walled, apparently aprotoplasmate fungus cells. Host cells (H) are found below the stroma (×190). **4)** Section of stroma showing porelike light areas (arrow) in the walls of the cells. These light areas are characteristic of the stroma of the black knot fungus (×790).

swelling. Also, the perfect stage of the fungus was produced within 5 months on greenhouse-grown plants, whereas the same stage on field-grown plants usually required at least 12 months. The results reported here are as they occurred in the field.

*Early development.*—In early spring, branches infected the previous year exhibited hyperplasia on the side or sides attacked by the pathogen. In the area of hyperplasia closest to the cambium, organized fungal elements were found that extended outward to the periphery of the branch (Fig. 1). The elements consisted of cells forming long, narrow strands of tissue that lay perpendicular to the periphery of the branch. The cells were small but elongate, with thickened walls. The tissue lacked intercellular spaces. This tissue was discrete from the adjacent host cells, with no haustoria or other means of penetration of the host tissues. The fungal cells grew in proximity to the cambial cells or past them to the xylem cells, in the latter case often disorienting the cambial cells. Strands of fungal cells often appeared to terminate at the numerous rays in the area of the cambium.

In the cambial region, the fungal strands were usually one to several cells wide, but the same strands when followed outward into the cortex were many cells wide. The fungal strands were irregular in outline, assuming the shape of the host cells between which they formed.

A cross-sectional view through the hyperplastic region at this stage of development showed many such strands present. In section most strands appeared intermittently between the cambium and the periphery, and were positioned perpendicular to the periphery.

Fungal strands at the periphery of the hyperplastic area initiated lateral growth over the outermost host cells of this area (Fig. 2). Growth of the fungus was both lateral and centrifugal. The fungal cells composing this mass were small, hyaline, isodiametric, and had thickened walls. After this mass became many layers thick, the outer cells were rectangular in shape and oriented with their long axis perpendicular to the periphery of the branch. Except for their different shape and orientation, these outer cells appeared similar to the inner cells. The outer layer of cells was destined to become the basal cells of the conidiophores.

Concurrent with the formation of the conidiophores, the walls of these outer cells became pigmented and thickened. Growth and multiplication of the inner peripheral fungal cells were continuous at this time. Soon the outer fungal cells were black in color (dark brown in transmitted light), had walls thickened to 2 μ, and ranged in size from 4-22 μ. A progression was quite evident from the very dark, thick-walled outer cells to the less pigmented, thick-walled middle cells, to the thin-walled, lightly pigmented basal cells (Fig. 3).

In the transition zone of cell-wall thickening and pigmentation, round, porelike areas were found on the cell walls (Fig. 4), while no such areas were seen in the parent cells. These appeared in the walls between adjacent cells, each cell having them on several such interfaces. The fine structure of these porelike areas is not known. These areas were found in all stromatic cells that were thickened and pigmented. All cells displaying such porelike areas were characterized by the absence of discernible nuclei and cytoplasm.

The conidiophores grew rapidly on the surface of the stroma, developing into a dense green mat.

*The perfect stage.*—The first evidence of centrum formation was detected as light areas near the upper part of the stroma but several cell layers below the basal cells of the conidiophores. These areas were composed of cells less thick-walled and less pigmented than the neighboring cells. Within each area, many thin-walled, hyaline cells developed (Fig. 5). Their continued multiplication caused the stromatic cells immediately above and on either side to be pushed outward (Fig. 6). The hyaline cells composing the centrum were pseudoparenchymatous and lacked intercellular spaces. At this stage, the centrum had pushed out to such an extent that it could have been mistaken for a superficial structure; however, its contents were enclosed completely by the stromatic tissues in which it had developed (Fig. 7). Many centra developed within the stroma.

As the developing centrum pushed outward, its pseudoparenchymatous cells became elongated and flattened, even to the base of the centrum, forming the pseudoparaphyses (Fig. 8). The elongation and flattening was less pronounced in the cells near the apex and these, at maturity, appeared as a convex mass of cells articulating with the cells of the pseudoparaphyses. The lower cells of the pseudoparaphyses became very elongate and thin and were much longer than those cells articulating with the apical centrum cells. In the base of the centra, plurinucleate cells were observed. They were thin-walled with dense contents, and were apparently the ascogonia. Plurinucleate, bifurcating cells, apparently the ascogenous hyphae, radiated from the ascogonium through the basal portion of the centrum. Asci in the early developmental stages had thin walls, dense cytoplasm, and a rather

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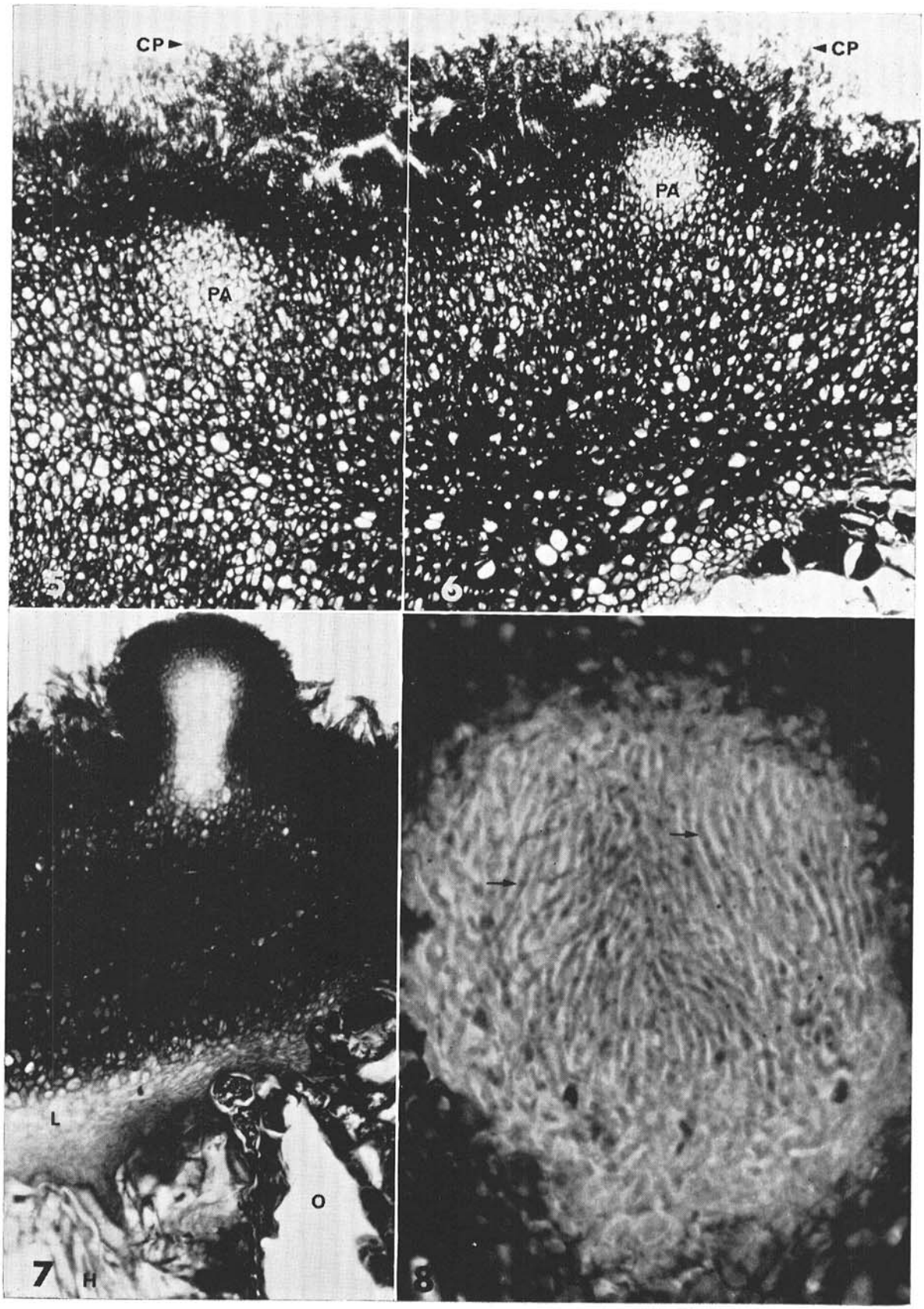
**Fig. 5-8.** Initiation and development of the ascocarp of *Dibotryon morbosum* on *Prunus domestica*. **5)** Cross section of a stroma showing the developing centrum as composed of thin-walled, lightly pigmented pseudoparenchymatous cells (PA) surrounded by darkly pigmented cells. Conidiophores (CP) form a dense covering over the peripheral stromatic cells (×300). **6)** Cross section of a stroma showing the shape assumed by the developing centrum as the pseudoparenchymatous cells (PA) multiply in number. The dark, thick-walled cells above and on the sides are undifferentiated stromatic cells. Conidiophores (CP) arise from the stroma (×300). **7)** Cross section of a stroma showing an erumpent developing centrum composed of thin-walled, hyaline pseudoparenchymatous tissue. Fungus cells (L) at the base of the stroma are growing close to the cells of the host (H). Disintegration of the host cells often leads to hollow areas (O) in the host cortex (×300). **8)** Cross section of a locule showing pseudoparaphyses (arrow) completely developed. At this stage of development asci have not been formed, but occasionally plurinucleate cells are found on the bottom and sides of the centrum. No wall distinct from the stroma develops (×900).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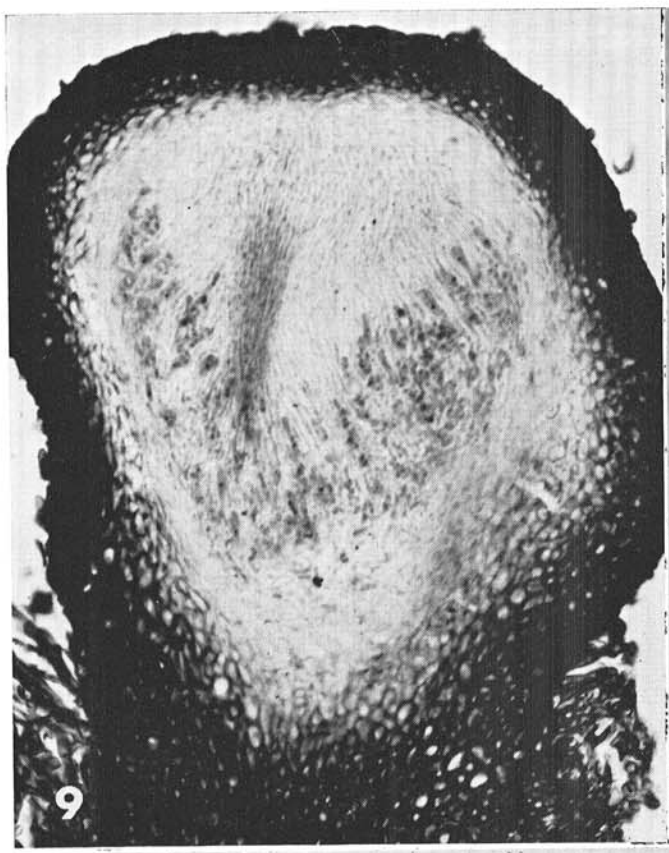
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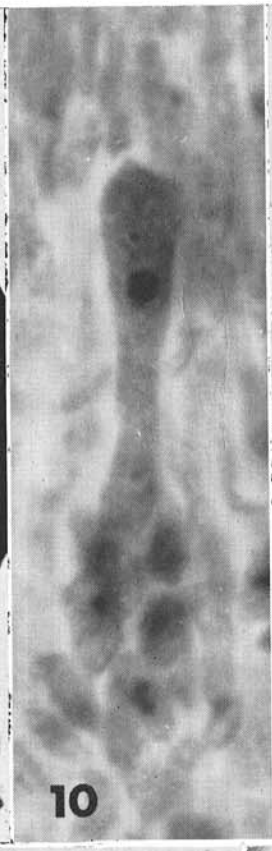
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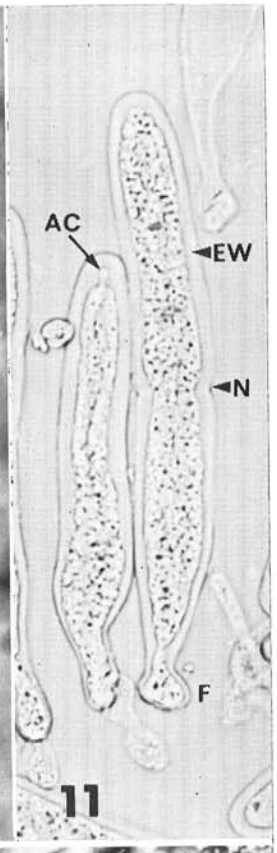
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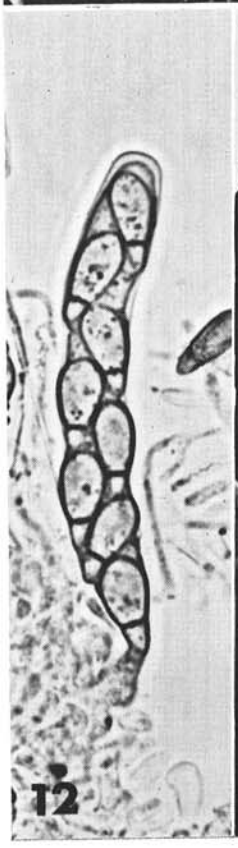
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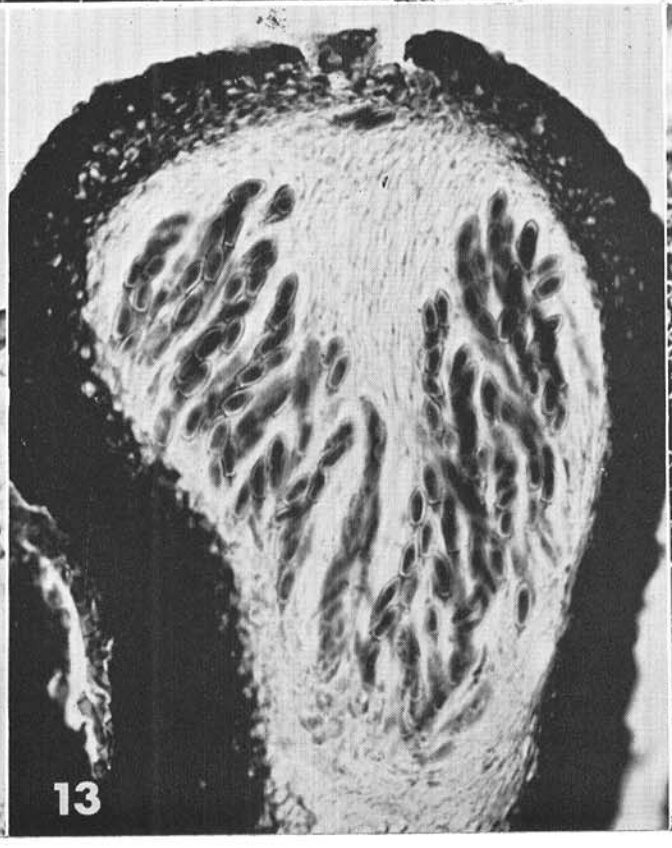
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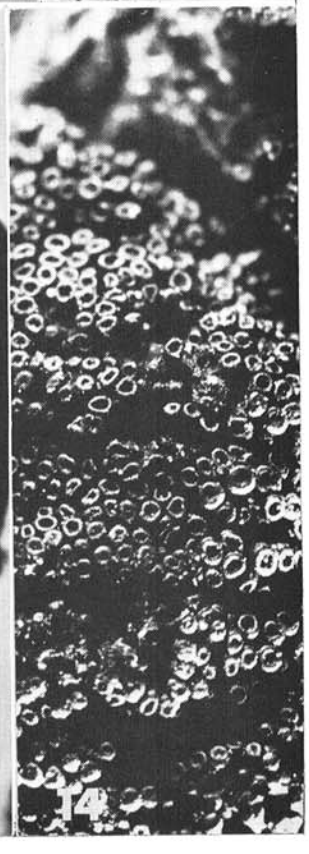
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**Fig. 9-14.** Development of the ascocarp of *Dibotryon morbosum* on *Prunus domestica*. **9)** Cross section of a locule showing newly formed asci growing upward among the pseudoparaphyses in the centrum. The asci arise as a basal layer (×320). **10)** Cross section of a centrum showing an ascus (A) and surrounding pseudoparaphyses (PS). The light halo around ascus is the ascus wall (×2,600). **11)** Immature asci with contents undeveloped. Ascus displaying an apical canal (AC). Extended wall (EW) of the adjacent ascus is marked at the base by a constriction (N). Asci characteristically have a foot-shaped base (F) (×750). **12)** Mature asci with eight 2-celled ascospores. The shape of the ascospores is characteristic of this fungus (×750). **13)** Cross section of a mature locule. Asci have grown upward and matured among the pseudoparaphyses. The pseudoparaphyses all seem to be directed towards the center of the ascocarp apex, where the stromatic wall-like material has broken open. It is from this open area that the asci will protrude and discharge the ascospores. No definite wall lines the centrum. Ascus walls appear as clear areas surrounding the groups of ascospores (×450). **14)** View of knot showing numerous locules. Locules characteristically have depressed centers (×15).

large nucleus (Fig. 9, 10). Before ascospores were delimited, the ascus wall had attained a thickness of 5 μ. It was thickest at the apex where a canal was apparent (Fig. 11). At this stage, the ascus wall was bitunicate.

Development of the ascocarp centrum slowed as winter approached, but asci were present on the bottom and sides of the centrum extending upward among the pseudoparaphyses. Prior to spring, ascospores were delimited in some asci and some ascospores were mature (Fig. 12). The mature centrum was characterized by the presence of asci, both mature and immature, and pseudoparaphyses (Fig. 13). Locules lacked a preformed ostiole, but opened apically with radiating stellate fissures under certain conditions (11). The locules appeared as raised spheres with depressed centers when observed under the dissecting microscope (Fig. 14).

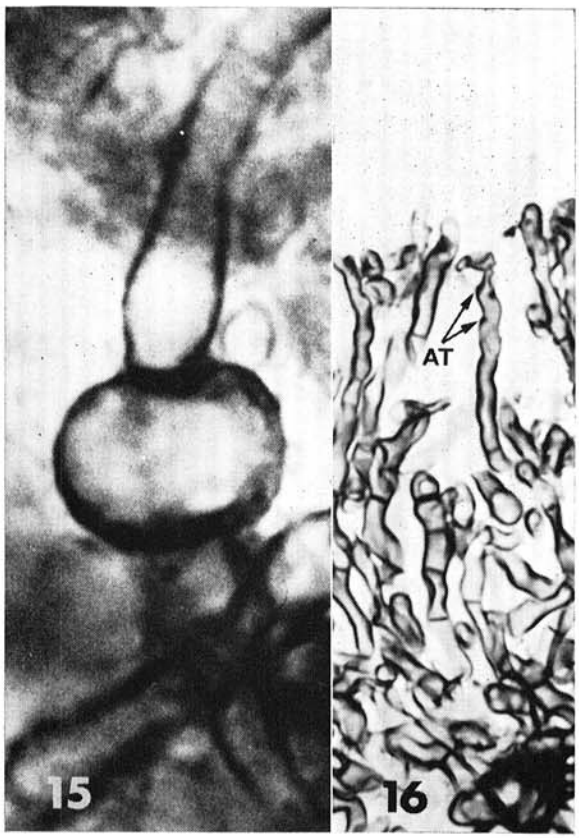


**Fig. 15-16.** Structures of *Dibotryon morbosum*. **15)** Chlamydospore showing its intercalary position on the mycelium (×1,000). **16)** Conidiophores on the host as seen in a cross section of stromata. Points of conidial attachment (AT) are a prominent feature of the conidiophore as is its geniculate structure. Conidia are seldom seen on conidiophores, being dislodged soon after their formation (×775).

The ascospores are two-ranked in the ascus, and are ejected successively from the terminus of the expanded inner ascus wall (11). Asci are bitunicate. The ascospores are two-celled with the upper cell at least two-thirds, and usually three-fourths, the length of the entire ascospore. They measure 16-21 × 5-9 μ. Both cells contain a single nucleus and both germinate by germ tubes.

*The imperfect stage.*—The conidiophores arise from the outer cells of the stroma, are broadly filamentous, nodose-geniculate, and 19-59 × 4-6 μ (Fig. 15). The conidia are borne singly or in chains, and are 3-8 × 2-5 μ. The conidiophores separate from the stroma as a mantle before the ascospores are mature, the shedding occurring during the fall or winter months. After separation, the stroma appears smooth and shiny.

*Chlamydospores.*—Chlamydospores were observed in cultures on media high in carbohydrates. The chlamydospores occurred either singly or in chains (Fig. 16), and were darkly pigmented, rather thick-walled cells. Their shape varied from slightly inflated to spherical; they measured 6-15 μ in diam.

Discussion.—Fungus tissues developing within and on plum branches after inoculation with ascospores of *Dibotryon morbosum* produced fruiting structures that again gave rise to ascospores; this was accompanied by the production of typical black knot symptoms. Our work supports the conclusion reached by Koch (5) that *D. morbosum* is the black knot pathogen and that other organisms present seem to be incidental. While not described here, the phenomenon of ascospore discharge in *D. morbosum* has been described elsewhere (3, 11).

No function was ascertained for the conidia of the fungus. Previous studies have demonstrated that the conidia probably play little or no role in the infection process (6, 8). Although no role was discovered for the chlamydospores, this study indicates that these structures do form in the absence of antagonistic microorganisms and concn of certain inorganic chemicals as was reported earlier (4).

Von Arx (1) transferred the black knot fungus (the type species of *Dibotryon*) to the genus *Apiosporina* typified by *A. collinsii* (Schw.) v. Hoehn. While our description of *D. morbosum* and that found for *A.*

*collinsii* (7) point out certain similarities in mature ascocarp morphology, they also point out certain basic differences in the ontogeny and morphology of such structures. In addition, the black knot fungus lacks certain of the characteristics which describe the genus *Apiosporina*; namely, setae on the locule and an ostiole. Thus, despite the seeming similarities and because of the differences, we feel the genus *Dibotryon* should be retained as a distinct genus. Combination of these two genera should not take place unless the ontogenetic and morphologic differences of ascocarp development are reconciled and the genus description of *Apiosporina* is emended to coincide more closely with the description of its type species.

The imperfect stage belongs in the form-genus *Cladosporium* Link ex Fr. The name *Hormodendron,* sometimes applied to the imperfect stage, is a later synonym for the form-genus *Cladosporium* and would be legitimately combined with *Cladosporium* according to the works of de Vries (10).

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