Sclerotium Germination and Histopathology of Monilinia vaccinii-corymbosi on Highbush Blueberry

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ABSTRACT

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Germ tubes from mature ascospores penetrated succulent blueberry leaves either directly through the epidermis or indirectly through stomates. The fungus hyphae in the leaf, flower, and fruit tissues grew both intra- and intercellularly and caused cellular collapse and necrosis. The stigmatic tissue that surrounds the stylar canal appeared to be the primary tissue through which the fungus entered the ovary. Sclerotium morphology, germination and apothecium development also were studied. Mature sclerotia required chilling below 7 C for a minimum of 900 to 1,200 hr for normal apothecium development.

Additional key words: *Vaccinium corymbosum*, histopathology.

Monilinia vaccinii-corymbosi (Reade) Honey (6) is a pathogenic fungus of considerable economic importance in most highbush blueberry (*Vaccinium corymbosum* L.) -producing areas (4, 12, 13). The principal damage on highbush blueberry is a fruit rot resulting in mummified fruit. However, blighting of twigs and leaves in southeastern North Carolina is more devastating on rabbiteye blueberry (*V. ashei*) than is the fruit rot.

Longyear (9) first reported the mummy berry disease occurring on wild *Vaccinium* species in Michigan and identified the causal organism as *Sclerotinia vaccinii* Wor. Later Reade (16) reported that the mummy berry fungus differed from *S. vaccinii* in host range and sizes of ascospores and conidia, and named the fungus *S. vaccinii-corymbosi.* Honey (6) established the genus *Monilinia* to include those members of the genus *Sclerotinia* commonly possessing a monilioid macroconidial stage and a pseudosclerotium, and reclassified the organism as *Monilinia vaccinii-corymbosi* (Reade) Honey.

In southeastern North Carolina, overwintered sclerotia of *M. vaccinii-corymbosi* break dormancy around the 1st wk in February and develop mature apothecia about 1 mo later (11). Growth of the apothecium initial(s) from the sclerotium is favored by a cool temperature (5 C), the mature apothecium develops at a higher temperature (16 C).

Highbush blueberry plants require a minimum of 800 to 1,200 hr below 7 C during the winter in order to bloom and leaf normally in the spring (3). According to Mainland (10), the number of hours below 7 C in recent years in southeastern North Carolina has ranged from 2,160 at a Bladen County farm during the winter of 1968-1969 to 1,280 in Pender County during the winter of 1973-1974. Since apothecium development and ascospore discharge normally coincide with bud break and new shoot growth, the chilling requirements appear to be similar for the host and pathogen.

These studies were conducted (i) to determine the chilling requirements necessary to initiate sclerotium germination and development of normal apothecia; and (ii) to examine the penetration and infection of blueberry leaves, flowers, and fruits by *Monilinia vaccinii-corymbosi.*

MATERIALS AND METHODS

Overwintered sclerotia of *M. vaccinii-corymbosi* were collected on 21 November, and 22 December 1975, from a highbush blueberry farm located in Bladen County, North Carolina, where a planting of the cultivar Croatan was severely infected with the fungus the previous year. Two hundred dormant sclerotia were selected for germination studies each month. A 300-g sample of a nonsterile highbush blueberry soil with a moisture content of 31% was placed in a 150 × 25 mm glass petri dish. Soil was oven-dried and the percent moisture content (SMC) was determined on a dry weight basis (5) and recorded. Half of the sclerotia collected each month were soaked in distilled water for 72 hr, the other half were not soaked. All the sclerotia (in lots of 25), were placed in eight dishes and sealed in a 0.025-mm (1-mil) polyethelene bag to hold the SMC constant. One dish containing soaked sclerotia and one dish with nonsoaked sclerotia were placed in a Sherer-Gillett CEL 25-7HL constant temperature chamber at 16 C with a 12 hr daylength at a light intensity of 9.6×10^3 lx. The

remaining six dishes were placed at 5 C without light. Two dishes, one with soaked and one with nonsoaked sclerotia, were removed from 5 C after 14, 28, and 56 days and placed at 16 C. Germinated sclerotia and mature apothecia were counted 30, 60, and 90 days after collection. Daily field temperatures were recorded by a hygrothermograph in a standard U.S. Weather Bureau instrument shelter. The number of hours below 7 C up to 21 November 1975, 22 December 1975, and 21 January 1976, was determined. Sclerotia and apothecia used for histological examination were fixed in formalin-acetic-alcohol (FAA), dehydrated in tertiary butyl alcohol and embedded in Paraplast Plus (Sherwood Medical Industries, St. Louis, MO 63103). Sections 5-10 μm thick were mounted on slides with Haupt's adhesive and stained with either safranin and fast green or Harris hematoxylin and Orange G (8).

Mature apothecia were produced in the petri dishes maintained at 16 C. Ascospores were removed with a camel's-hair brush from the top of the dish after discharge and placed on the lower surface of young succulent leaves of highbush blueberry (cultivar Croatan). Rooted cuttings of highbush blueberry (cultivar Croatan) were grown in a peat: sand (1:1, v/v) mixture in 10-cm diameter clay pots in the greenhouse. Inoculated and noninoculated excised leaves were placed on moist filter paper in sterile petri dishes at room temperature (20-25 C). Leaves were removed after 1, 2, 3, and 7 days and placed into FAA.

Percentage germination of conidia was determined after 24 hr by counting 100 spores placed on water agar at 25 C. The HCl-Giemsa method (18) was used to stain nuclei in the ascospores and in the hyphae growing from germinated conidia.

The plant parts (leaves, flowers, and fruits) used in these studies were collected from bushes of the highbush blueberry cultivar Croatan. Conidia were collected from naturally infected leaves with a sterile needle and placed in a drop of sterile distilled water on a clean glass slide. Inoculum was placed on the stigma of a flower with a camel's-hair brush. Several flowers from the same cluster were inoculated, the stem was excised, and the flowers were placed on moist filter paper in a petri dish at 20 to 25 C. Inoculations also were made by placing mycelium from a 14-day-old culture of *M. vaccinii-corymbosi* growing on PDA blocks (2 mm in diameter) onto the stigma of healthy flowers. Inoculated and noninoculated pistils from both inoculation procedures were placed in FAA after 3, 5, and 7 days. Isolations from infected styles were made 5 days after inoculation.

The highbush blueberry plants at the Bladen County location were in full bloom (80%) on 1 April 1976 and infected berries were collected 6 wk later on 15 May 1976. Infected leaves and fruit to be sectioned were placed in FAA, embedded in Paraplast Plus, cut at a thickness of 10 μm, mounted on slides with Haupt's adhesive, and stained with Triarch's quadruple stain (Triarch Incorporated, Ripon, WS 54971).

RESULTS

Germination of sclerotia.—The cumulative number of hours below 7 C up to 21 November, 22 December, and 21 January was 170, 602, and 1,200, respectively. None of the sclerotia collected in November had germinated, whereas 23% of the 392 sclerotia collected in December had germinated. Of the 285 sclerotia collected on 21 January 1976, 84% had germinated. A few mature apothecia were observed in the field at this time.

Sclerotia that had been exposed to 170 hr below 7 C did not germinate (Table 1). Differences in germination were negligible for sclerotia that had been exposed to temperatures below 7 C for over 600 hr, and little or no increase in percent germination was observed after 30 days. As many as 25 protrusions or stipe initials that ranged from 0.5 to 2.0 mm in length were observed on some germinating sclerotia. Apothecium development was observed within 60 days for sclerotia collected in December and stored at 5 C for 0, 14, and 28 days prior to being placed at 16 C. Apothecia did not develop at 5 C. Little or no increase in percentage of apothecial development occurred after 60 days, except for sclerotia stored at 5 C for 56 days. Sclerotia collected in November developed mature apothecia after 60 days. Apothecia produced from sclerotia that had been exposed between 602 and 842 hr below 7 C were small, and deteriorated

TABLE 1. Effect of temperature on germination of sclerotia and apothecium development of *Monilinia vaccinii-corymbosi*[a]

Date sclerotia collected and no. days at 5 C	Total hours below 7 C	Germinated sclerotia (%)		Sclerotia with apothecia (%)	
		Soaked	Nonsoaked	Soaked	Nonsoaked
21 Nov					
0	170	0	0	0	0
14	506	20	0	0	0
28	842	64	32	20	8
56	1,514	56	48	60	45
22 Dec					
0	602	48	45	0	8
14	938	65	45	8	4
28	1,274	50	56	45	40
56	1,946	40	48	50	72

[a]Germination recorded 30 days after sclerotia were collected from a blueberry field. Apothecium development was recorded 90 days after sclerotia were collected.

[b]Sclerotia soaked in distilled water for 72 hr.

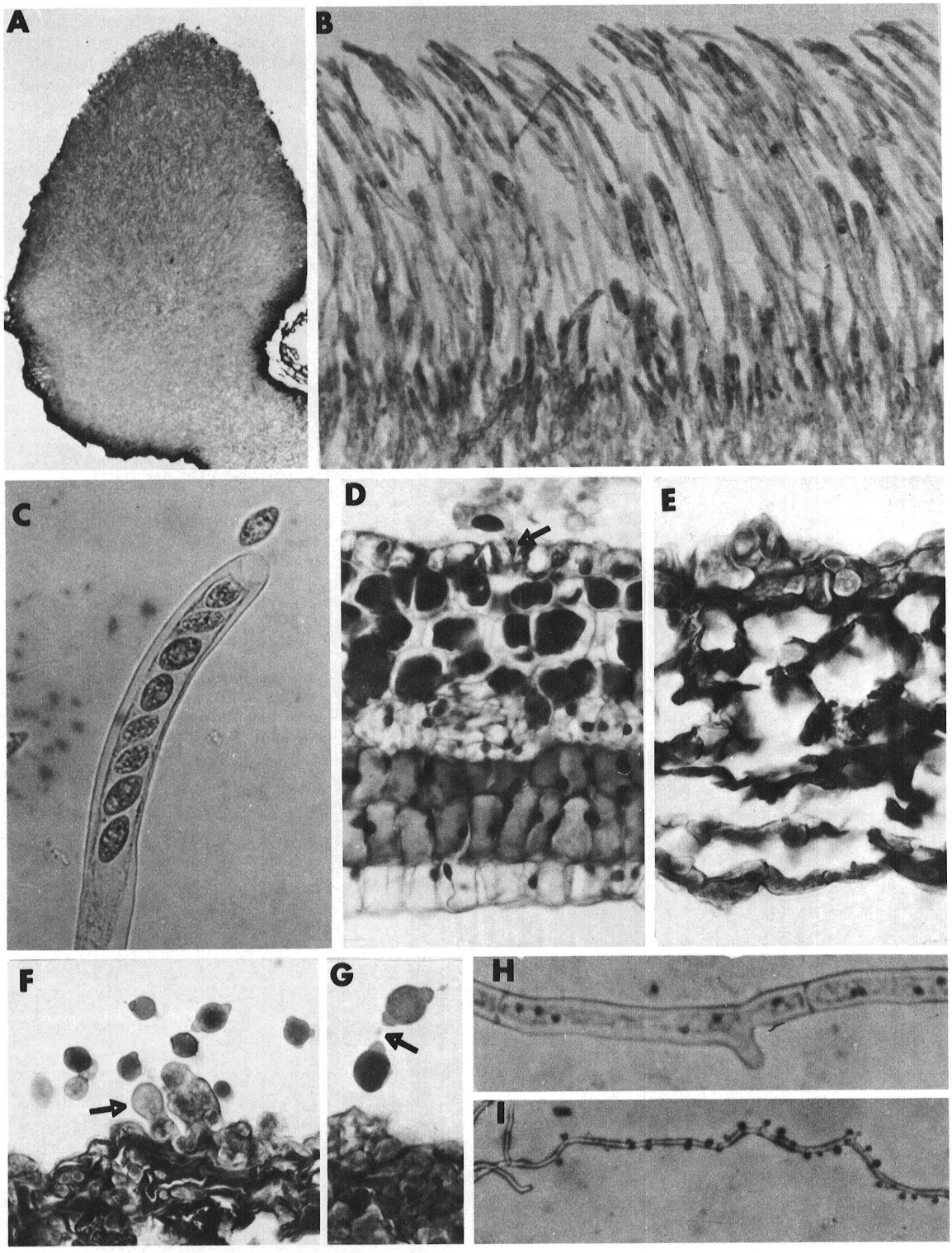

Fig. 1-(A to I). Sclerotium germination, apothecium development, and conidium formation of *Monilinia vaccinii-corymbosi*: **A)** Transverse section of mature sclerotium with stipe initial (×50). **B)** Hymenial layer with paraphyses and developing asci (×400). **C)** Mature ascus and ascospores (×530). **D)** Ascospore penetration through stomate (arrow) of young highbush blueberry (cultivar Croatan) leaf (×450). **E)** Transverse section of infected leaf showing necrosis and hyphal accumulation on leaf surface (×450). **F)** Transverse section of infected leaf with club-shaped conidiophores (arrow) and conidia (×350). **G)** Conidia with disjunctors (arrow) (×500). **H)** Nuclei in hyphae (×1,000). **I)** Hyphae with microconidia (×333).

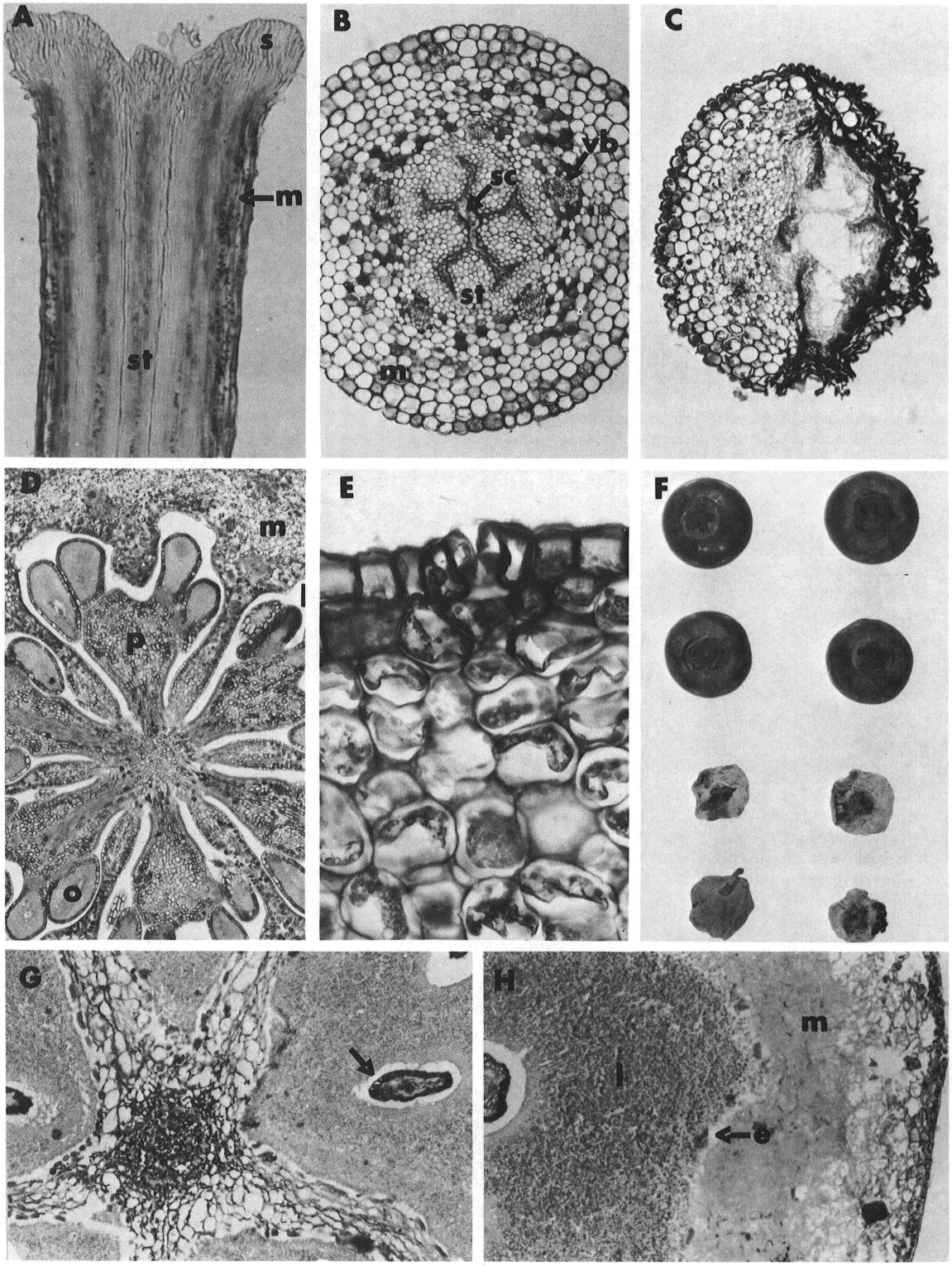

Fig. 2-(A to H). Highbush blueberry fruit infected with *Monilinia vaccinii-corymbosi*: **A)** Tangential section of healthy stigma and style: s = stigma; st = stigmatic tissue; m = mesophyll (×70). **B)** Transverse section of healthy style; m = mesophyll; st = stigmatic tissue; sc = style canal; vb = vascular bundle (×100). **C)** Transverse section of infected style (×100). **D)** Transverse section of healthy ovary; O = ovule; l = locule; p = placentae; m = mesocarp (×50). **E)** Stomate in ovary wall (×600). **F)** Healthy and diseased fruit. **G,H)** Transverse sections of 6-wk-old infected fruit. **G)** Infection of central area of vascular tissue and aborted seed (arrow) in locule (×50). **H)** Fungus tissue in locule (l), breakdown of endocarp (e), hyphae embedded in matrix (m), and outer mesocarp and epidermis (×50).

soon after developing. Sclerotia that had been exposed for a minimum of 938 hr below 7 C produced normal vigorous stipes and as many as 20 apothecia developed from one sclerotium. Sclerotia collected in December and stored at 5 C for 56 days before being placed at 16 C produced the highest percentage of apothecia. Soaking the sclerotia for 72 hr in distilled water made little or no difference in germination or apothecium development.

The mature sclerotium retains a shape similar to the normal fruit, but it is hollow in the center. The structure is composed primarily of fungal tissue. The rind is 20-40 μm thick and consists of large thick-walled cells cemented together by a thick, pigmented deposit. The white inner tissue consists of loosely arranged filamentous hyphae embedded in a dense matrix. The host epidermal cells were sloughed off and did not become a part of the sclerotium. Actively growing hyphae from a germinated sclerotium were 5-7 μm wide and multinucleate. The width of the apothecial initial measured 800 μm at the exit point to 400 μm at its narrowed tip (Fig. 1-A). Hyphal cells in the elongated stipe were loosely arranged, parallel, and thin-walled.

Apothecium.—The hyphal tissue within the excipulum is loosely woven and the elongated hyphae lie more or less parallel to each other and are easily distinguishable. The young binucleate asci that developed from the ascogenous hyphae in the subhymenium averaged 10-15 × 2-3 μm when first recognizable and the two nuclei fused very early (Fig. 1-B). The first indication of ascospore development was the formation of cross-walls and individual cells in the ascus. Ascospores became oval to elliptical in shape, binucleate, and measured 12-15 × 5-7 μm (Fig. 1-C). Mature asci measured 180-200 × 8-10 μm. Paraphyses were rounded at the tips and were 2-3 μm wide. Various stages of ascus and ascospore development were observed in the same apothecium.

Leaf infection by ascospores.—Direct penetration into an epidermal cell and indirect penetration through a stomate were observed 48 hr after inoculation (Fig. 1-D). Necrosis of parenchyma cells surrounding the infection site was observed after 72 hr. Transverse sections of infected leaves showed that growth of the hyphae inside the leaf tissue resulted in cellular collapse, necrosis, and the formation of an ectostroma (Fig. 1-E) with short, club-shaped conidiophores on which large macroconidia measuring 15-25 × 12-20 μm were produced (Fig. 1-F). Conidia were produced in chains connected by disjunctors that measured 2.5-3.0 μm in length (Fig. 1-G). The disjunctor acts as a separating mechanism for the mature conidia and was first described by Woronin (17) in 1888. According to Honey (7), the disjunctors in the upper portion of the chain of conidia are larger (4-5 μm) than those formed at the base (2-3 μm). In many cases, however, conidia were not separated by disjunctors.

Infection by conidia.—Germination of macroconidia after 24 hr was 40%. Numerous multinucleate germ tube branches developed after 72 hr (Fig. 1-H). Small, 3-4 μm in diameter, spherical-shaped, uninucleate microconidia were formed at tips of short lateral sterigmata along the germ tubes and branched hyphae (Fig. 1-I). Three percent of these microconidia germinated on water agar after 24 hr. The entire cell elongated into a hyphal strand.

The style of *V. corymbosum* consists of an outer epidermis, a mesophyll, a central area of stigmatic tissue, and five vascular bundles (Fig. 2-A, B). According to Bell and Burchill (2), the inner tissue of the style and the upper portions of the ovary partitions of *V. angustifolium* are initiated by five broad ray-like elevations that extend from the outer ring of tissue toward the center. The small space in the center where the partitions do not meet, forms the stylar canal. The closely packed cells that form these partitions in *V. corymbosum* measure 5-15 μm compared to the parenchyma cells in the mseophyll that measure 20-30 μm in diameter. Sections of infected styles inoculated with either conidia or mycelium showed some necrosis in the mesophyll tissue just below the stigma after 72 hr. Growth of the hyphae in the mesophyll tissue caused the paraenchyma cells to collapse and die. Within 5 days, the fungus had invaded the stigmatic tissue in the central portion of the style and the cells were almost completely destroyed (Fig. 2-C). Isolations from infected styles yielded cultures of *M. vaccinii-corymbosi.*

The ovary of *V. corymbosum* cultivar Croatan is compound and the ovary wall consists of an outer and inner epidermis and 15 to 25 layers of cells that comprise the mesocarp (Fig. 2-D, E). In contrast to the findings of previous workers (19), stomates are present in the outer epidermis of the young ovary of *V. corymbosum* (Fig. 2-E). About six layers of cells beneath the epidermis stained darker than the innermost cells of the mesocarp. These cells contained a dark purple pigment that gives the fruit its characteristic color (19).

Fungal penetration into the upper portion of the ovary was observed 7 days after inoculation. Some cells in the stigmatic tissue of the central area were necrotic, whereas the surrounding cells appeared healthy. Naturally infected berries observed in the field on 1 May 1976, were light-cream in color. As the fruit approached maturity (15 May 1976), the infected berries became salmon in color rather than the normal blue (Fig. 2-F). Growth of the hyphae in the infected berry tissue was inter- and intracellular. The parenchyma cells and vascular tissue in the central portion of the infected fruit were almost completely destroyed and replaced with fungal hyphae. The immature seeds located within the locules also were infected and distorted as a result of fungal invasion. Growth of the fungus within the locules resulted in the formation of a pseudoparenchymatous fungal tissue (Fig. 2-G). At this stage of sclerotium development, three distinct types of host-fungal tissue could be differentiated (Fig. 2-H): (i) Hyphae in the locules were large, closely packed, and occupied the entire space. The stone cells of the endocarp that surround the locules were completely destroyed. (ii) Hyphae in the fleshy mesocarp adjacent to the locules were smaller in diameter and rather loosely woven in a dense matrix. (iii) The outer layer of host cells adjacent to the epidermis were not completely invaded by the fungus. No evidence of fungal penetration of the epidermal cells was observed 6 wk after infection. Within 3-4 mo the entire host tissue was replaced by the fungus. Isolations from infected berries yielded cultures of *M. vaccinii-corymbosi.*

DISCUSSION

The overwintering structure of *M. Vaccinii-corymbosi* is a sclerotium and not a pseudosclerotium or mummy as

previously stated (7, 11). By definition (1), "a sclerotium is a firm, frequently rounded, mass of hyphae with or without the addition of host tissue or soil, normally having no spores in or on it, and may give rise to a fruiting body." The overwintering sclerotium is composed almost entirely of fungus tissue; it has an outer rind of melanized thick-walled cells cemented together by a thick gelatinous deposit, and a medulla of loosely arranged hyphae embedded in a dense matrix. Several apothecia are usually produced from the sclerotium. Apothecium development may be initiated either very early or after extensive growth (15 mm) by the stipe.

Ramsdell et al. (14), reported that the most favorable temperature for ascospore germination was 15 C, although ascospores germinated well at 5 to 20 C within 6 hr. It appears that ascospores can germinate over a wide range of temperatures, since numerous ascospore germ tubes were observed on inoculated leaf sections at room temperature (20-25 C). Mycelium obtained from single-conidia isolations grew very well on PDA at 25 C.

The fungus usually gained entrance into the ovary of infected pistils through the stigmatic tissue in the central portion of the style that surrounds the stylar canal. Transverse sections of approximately 6-wk-old infected berries indicated that the central portion of the fruit was colonized initially and the outer mesocarp and epidermis were invaded last.

The primary factors influencing sclerotium germination and apothecium development are temperature, moisture, and light. Previous studies (11) have shown that without adequate moisture, sclerotia will not germinate and produce apothecia, even if temperatures are favorable. My results have shown that sclerotia will not germinate unless subjected to temperatures below 7 C for a minimum of 500 hr, even with adequate moisture. Although germination is a prerequisite, it does not always lead to apothecium development. Sclerotia that had received the minimum chilling requirements of 500-600 hr below 7 C germinated but did not produce apothecia. Based upon these and previous studies (11), mature sclerotia require adequate moisture (30-40% SMC), a minimum of 900-1,200 hr at 5-7 C, followed by a temperature of approximately 16 C for normal apothecium development. Light is required for the development of mature apothecia.

Knowledge of the chilling requirements for sclerotium germination and apothecium development would be essential for obtaining the inoculum requisite for screening large numbers of blueberry seedlings for mummy-berry resistance prior to transplanting into the field. This information also would facilitate control of the primary stage of this disease. The primary infections (twig and leaf blight) are caused by the ascospores. Conidia are produced within 2-3 wk on the blighted leaves and are carried by wind or insects to open blossoms. Mummy berry develops as a result of these secondary infections. The fruit rot stage occurs on both the highbush and rabbiteye blueberry; however, damage caused by the primary infections has been much greater on the rabbiteye species. During the past 3 yr, a severe blighting of the leaves, shoots, and flower buds of three rabbiteye cultivars (Delite, Southland, and Tifblue) was observed at the Horticultural Crops Research Station, Castle Hayne, NC 28429. Little or no fruit developed on these blueberry plants as a result of the primary infections. These infections also provided a source of inoculum (conidia) for secondary infections of surrounding highbush blueberry blossoms. Therefore, it is important that the primary stage of this disease be controlled. The fungicides (Benlate 50W, Ferbam 76W, and Captan 50W) that are registered for use on blueberries in North Carolina do not effectively control the primary infection. In the past, mummy berry control in North Carolina has been achieved best by the elimination of apothecia through the practice of clean cultivation or chemical eradication. The most effective control procedure would combine the use of an eradicant ground treatment and the application of protectant fungicides (15). Since accurate weather records are available for different blueberry growing areas in southeastern North Carolina, blueberry growers could be informed about apothecium development as it relates to timing of control procedures.

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