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Observations on the Biology of *Ophiodothella angustissima*

Richard T. Hanlin[1*], Jimmy O. Owens[2], Alan H. Icard[3], Anthony E. Glenn[4] and María C. González[5]

Present addresses: [1]Mycology Herbarium, Museum of Natural History Annex, 4435 Atlanta Highway, Athens, GA 30606, USA; [2]Flat Rock Road, Watkinsville, GA 30677, USA; [3]34186 West 90th Circle, De Soto, KS 66018, USA; [4]Toxicology & Mycotoxin Research Unit, Russell Research Center, Athens GA 30604, USA; [5]Instituto de Biología, Universidad Nacional Autónoma de México, Mexico City, CDMX, Mexico

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Corresponding author: María C. González mcgv@ib.unam.mx.

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Abstract: *Ophiodothella augustissima* causes a leafspot disease on shrubs of *Vaccinium arboreum*. In autumn, diseased leaves bearing lesions fall to the ground where they overwinter. These lesions contain perithecial initials that mature with the advent of warm temperatures and spring rains, conditions that also favor the development of new leaves on the host. Mature perithecia contain asci with ascospores that are forcibly ejected upward onto young host leaves, which they infect. These new lesions grow and produce an acervular stage that forms numerous conidia that spread the disease throughout the plant during the summer. The timing of these events is determined by weather conditions, especially rainfall. As

infected leaves dehisce in autumn, they remain beneath the shrubs, where they overwinter, ready to begin the cycle again the following spring.

Key words: Ascomycota, fly speck disease, life cycle, *Ophiodothella vaccinii, Phyllachoraceae*, plant pathogen, *Vaccinium arboretum*

Introduction: *Ophiodothella* is a genus of ascomycetes occuring in both warm temperate (mostly subtropical) and tropical areas. A total of 37 species have been assigned to the genus (Index Fungorum, 2017). Of these, three are considered synonyms of other species and one species has been transferred to another genus, leaving a total of 32 accepted species plus one variety. All species occur as foliar parasites on living plants, with one, *O. orchidearum* E. K. Cash & A. M. J. Watson, also attacking stems, flowers and pseudobulbs. Although *Ophiodothella* species occur on a wide variety of host genera and families, the majority have been reported on hosts in the Orchideaceae and Ericaceae (Hanlin et al. 1992).

Ophiodothella angustissima (Peck) Hanlin & M. C. González (formerly *O. vaccinii* Boyd) (Hanlin and González 2013) is a perithecial ascomycete that causes the foliar disease known as flyspeck on *Vaccinium arboreum* Marshall (sparkleberry), a common understory shrub in forests of the southern United States (Fig. 1). The disease first appears in early summer and continues throughout the growing season, after which leaves dehisce and fall to the ground. For several years studies have been conducted on various aspects of the fungus (Hanlin 1990a,b 2003; Mims et al. 2006) in an attempt to elucidate the life cycle. The results of these studies are summarized below.

Materials and Methods: All of the material for these studies was collected from the same group of plants located in Madison Co., GA (34° 58′ N, 83°18.5′ W). Both living and overwintered leaves of *V. arboreum* containing lesions of various ages were collected at different times of the year, placed in moistened plastic bags, and taken to the laboratory for examination.

Light (LM), confocal (CM), transmission electron (TEM) and scanning electron (SEM) microscopy were all utilized in the study. Light microscopy was conducted on either a Nikon Optiphot equipped with Nomarski differential interference contrast (DIC) or on a Nikon SMZ stereo microscope. Confocal observations were made on a Leitz TCS SP5 microscope. Initial observations were made on material mounted in water, followed by mounts in Melzer's Solution and propiono-orcein.

Material for slides was embedded in both paraffin and plastic. Small squares were cut out of the lesions, killed and fixed in formalin-propionic acid-alcohol solution, then dehydrated through a tertiary butyl alcohol series before infiltration and embedment in Paraplast. Sections were cut on a rotary microtome at a thickness of 6–8 µm and mounted on standard microscope slides with Haupt's adhesive. Following removal of the paraffin in xylene, the sections were stained in 0.5% aqueous hematoxylin. After dehydration, a cover slip was mounted in Permount.

For plastic embedment material was fixed in 2.5% glutaraldehyde buffered in 0.1M Na–cacodylate, post–fixed in 1.5% osmium tetroxide and embedded in Spurr's resin. Sections were cut 1–2 µm thick on a Sorvall MT-2B ultramicrotome, stained in toluidine blue and mounted on glass slides. Material for TEM was fixed as above and, following resin polymerization, ultrathin sections were cut on a Reichert ultramicrotome, collected on slot grids and allowed to dry. They were then poststained in

uranyl acetate, followed by lead citrate, then examined on a Zeiss 902A TEM. SEM material was prepared as for TEM, then critical point dried, mounted on stubs, and coated with gold-palladium for observation. These procedures have been described in greater detail previously (Hanlin and Tortolero 1989).

Photographs were taken with a Nikon F2 camera equipped with a micro lens and photomicrographs were taken with a Nikon HFX camera mounted on the microscope. Electron micrographs were made on Kodak electron image film, SO-163 Estar thick base. Photographs were optimized with Adobe Photoshop® 6.0. Discharge of ascospores from overwintered leaves was confirmed by placing inverted slides coated with petroleum jelly 1 cm above leaves on the ground in the spring.

Results: Infected leaves of *V. arboreum* with lesions that developed during the summer dehisced and dropped to the ground in late autumn. There they remained intact beneath the plants until spring of the following year (Fig. 2). The lesions were blackened due to the presence of melanin pigments secreted by the fungus (Fig. 3), and they contained perithecial initials that are stimulated to mature by the onset of warmer weather and spring rains, conditions that also favor the development of the host leaves. Under favorable conditions, initials quickly develop into perithecia containing mature ascospores. As a perithecium develops, it pushes the surrounding leaf tissue upward, forming a dome-shaped structure when viewed from above (Fig. 4). Perithecia are completely immersed in leaf tissue and contain numerous asci interspersed with filamentous paraphyses that line the inner wall (Fig. 5). The outer surface consists of a black clypeus in the leaf epidermis. A central papilla surrounds the ostiole through which the ascospores are discharged. The ostiolar neck is lined with short, slender periphyses. Occasionally a perithecium will form a secondary ostiole on the lower side of the leaf. Asci are unitunicate, each with an apical ring that stains light blue in Melzer's solution. The eight filiform ascospores usually have a sinuous arrangement within the ascus (Figs. 6, 8). Each unicellular ascospore contains a single, centrally-located nucleus and several small lipid droplets (Fig. 7). Ascospores (Fig. 9) are forcibly ejected upward, where some adhere to the lowermost, newly formed leaves of the host.

Perithecia form only in lesions from the previous season; they have not been observed in other areas of the leaf. Lesion-bearing leaves that were brought into the laboratory in late January and placed in a damp chamber formed mature perithecia and ascospores within 10 days, suggesting that cold temperatures suppress the development of the initials formed earlier. Ascospore discharge could be observed readily with a dissecting microscope. The exact time of ascospore discharge and infection is determined by weather conditions.

Several weeks elapse between the time of ascospore discharge and the first appearance of the disease. Ascospores germinate and the fungus invades leaf tissue. The fungus then permeates the leaf tissues, forming both intra- and intercellular hyphae (Figs. 10–11). Several attempts to germinate ascospores on agar media in the laboratory were unsuccessful. Following infection, small, circular, yellow, chlorotic lesions develop on leaves (Fig. 12). These lesions are discrete and likely are the result of infection by a single ascospore. As the lesions develop, they soon become reddish-orange to yellow-orange in color (Fig. 13), due to the deposition of reddish granules in the tissue. The origin of these granules is unknown. As the lesion increases in size, a single black conidioma forms in the center and is soon followed by the formation of many additional conidiomata in the rest of the lesion (Fig. 14). The conidioma is a modified acervulus that represents the anamorph of the fungus. The acervulus is circular to somewhat irregular in outline and consists of a thin layer of basal tissue

that is formed in the lesion and which bears numerous conidiophores. The hyphae of the acervulus fill the host epidermal cells to form a clypeus, where they secrete a dark pigment. This results in a series of small shiny, raised black acervuli in the lesions (Fig. 15), giving rise to the common name ("flyspeck") of the disease. In the center of the basal layer of hyphae, a small column of compact hyphae arises and extends vertically, pressing against the overlying epidermis, eventually perforating it. As this is occurring, masses of scolecosporous conidia form on conidiophores. The mass of developing conidia push the epidermis up and lift it away from the column, opening a pore through which the conidia escape (Fig. 16). This method of pore formation appears to be unique among the fungi.

The conidia are filiform and one-celled, each with a single nucleus (Fig. 17). Unlike the ascospores, conidia germinate readily on agar media. Germination is polar, with the germ tubes forming at the ends of the spore (Figs. 18–20). Despite germinating, however, germ tube growth on agar was limited and never developed into a mycelium. As the season progresses, leaves successively higher on the shrub become infected, presumably by secondary conidial inoculations. Dispersal of conidia probably occurs mainly by means of air currents and rain splash. Insects may also play a role, however, as the conidia are formed in a mucous matrix inside the acervulus. Daily observations revealed that four to five weeks are required for the disease to reach the uppermost leaves of a 2 m tall shrub. In heavy infections, most of the leaf area will be covered by lesions by late summer (Fig. 21).

By early autumn ascomal initials have begun to form deep in the tissues of the host leaves (Fig. 22). Continued growth of the fungus in the leaves during the summer fills the host cells (Figs. 23–26). At the same time, changes occur in the cells of the fungus. The hyphae that infect the leaves initially are thin-walled and are capable of breaching the host cell by means of a slender penetration peg, which then expands on the other side of the wall to continue growth across the cell (Figs. 27–30). As the season progresses, hyphal cell walls become greatly thickened and the cells are filled with large lipid droplets (Figs. 31–32), a possible adaption to sustain the fungus during the dormant period. Development of the initials ceases with the advent of colder weather, when leaves dehisce and fall to the ground. Leaves remain intact during the winter. When spring arrives, with warmer temperatures and rains, the ascomal initials in the overwintered leaves quickly develop into perithecia with ascospores, thus completing the life cycle.

Conclusions: The area where the study plants are located is surrounded by mixed hardwood-pine forest, mostly *Quercus* spp. and loblolly pine (*Pinus taeda* L.), with numerous shrubs of *V. arboreum* scattered in the understory. Observations over a period of ten years revealed that certain of these shrubs become heavily infected with *O. angustissima* every year, whereas others never show the infection. This suggests that there may be genetic variation among these shrubs and that the fungus is not capable of infecting all of them, as it seems unlikely that spores of the fungus are not dispersed to all of them. A crude experiment on one large shrub that never became infected ca. 15 m from the study area was made in which heavily infected branches were hung in the top of the shrub, with negative results. Although various factors could account for this, it supports the idea of differential resistance in these plants. Another observation was that plants of rabbiteye blueberries located ca. 50 m from the study plants never developed the disease, although they likely were within range of spore dispersal.

Few studies on the life cycle of *Ophiodothella* species appear to have been published. The only comparable studies are those of Parbery (1963a,b) on species of the similar genus *Phyllachora*. His studies of *Phyllachora* species occurring on grasses were mostly conducted

under greenhouse conditions, thus limiting direct comparisons. As in *Ophiodothella*, he (Parbery 1963a) observed that ascospore discharge on plants in the field occurred after periods of rain or high humidity. Inside the host the fungus grew both intra- and intercellularly. He confirmed that clypeus development was concurrent with, but independent of, perithecial development, which is also true in *Ophiodothella*. Parbery and Langdon (1963) demonstrated that pycnidia containing scolecospores that formed in infection sites prior to perithecium formation are genetically connected to the perithecia. Attempts to germinate scolecospores were unsuccessful, but they always formed following host infection by ascospores; they might function as spermatia. In *Ophiodothella* scolecospores are formed prior to perithecium development, but they function as conidia that disseminate the fungus, although the possibility that they also can function as spermatia cannot be ruled out. More detailed studies would be necessary to resolve this question.

These extended observations have revealed many details about the life cycle of *O. angustissima* on *V. arboreum*, yet much remains to be discovered. It is clear that the host and fungus have evolved together, so that conditions that stimulate new leaf formation in the host also stimulate spore formation in the fungus. The development of thick-walled fungus cells filled with oil droplets also appears to be an adaptation to aid in surviving cold winter temperatures, as such wall thickening does not occur in the tropical species *O. casearіae* (Hanlin et al. 2002). More detailed studies would require controlled experiments that were not part of the overall objectives of this project, which were to study the morphology and taxonomy of the fungus.

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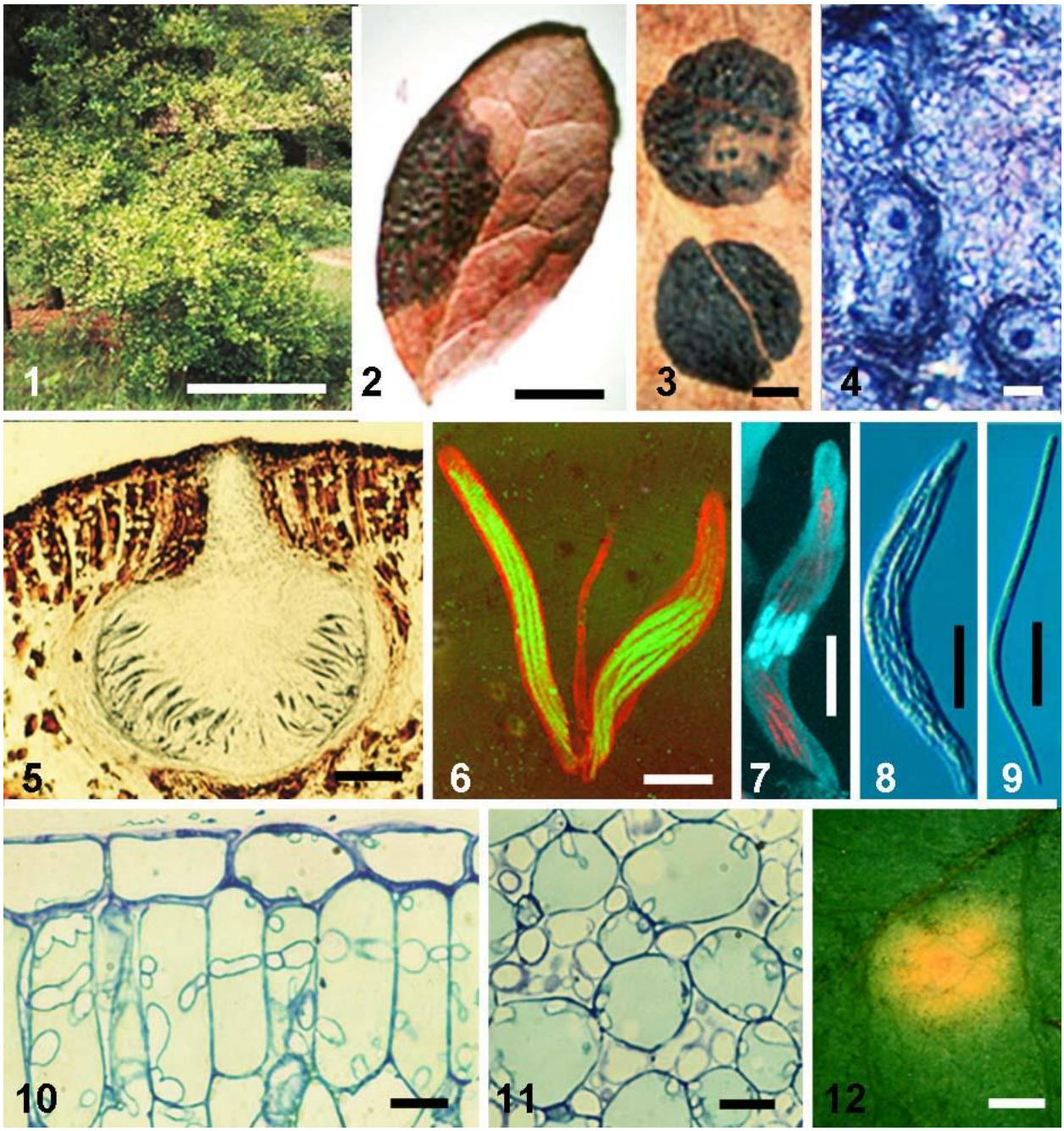

Fig. 1. Shrub of *Vaccinium arboreum* in flower. Fig. 2. Overwintered leaf from ground with lesion. Fig. 3. Two lesions on overwintered leaf. Fig. 4. Close-up of papillate perithecia in lesion. Fig. 5. Vertical section through perithecium with asci and paraphyses. Fig. 6. Two asci with ascospores and a paraphysis. Fig. 7. Ascus showing nuclei in ascospores. Fig. 8. Ascus with ascospores. Fig. 9. Ascospore. Fig. 10. Section through infected leaf showing intracellular hyphae in palisade cells. Fig. 11. Section through mesophyll showing intra- and intercellular hyphae. Fig. 12. Young lesion on newly formed host leaf.
Scale bars: Figs. 1 = 50 cm, 2 = 1 cm, 3 = 5 mm, 4 = 175 µm, 5 = 85 µm, 6–9 = 24 µm, 10 = 20 µm, 11 = 30 µm, 12 = 5 mm. Figs. 6, 7, confocal; Figs. 8, 9 DIC.

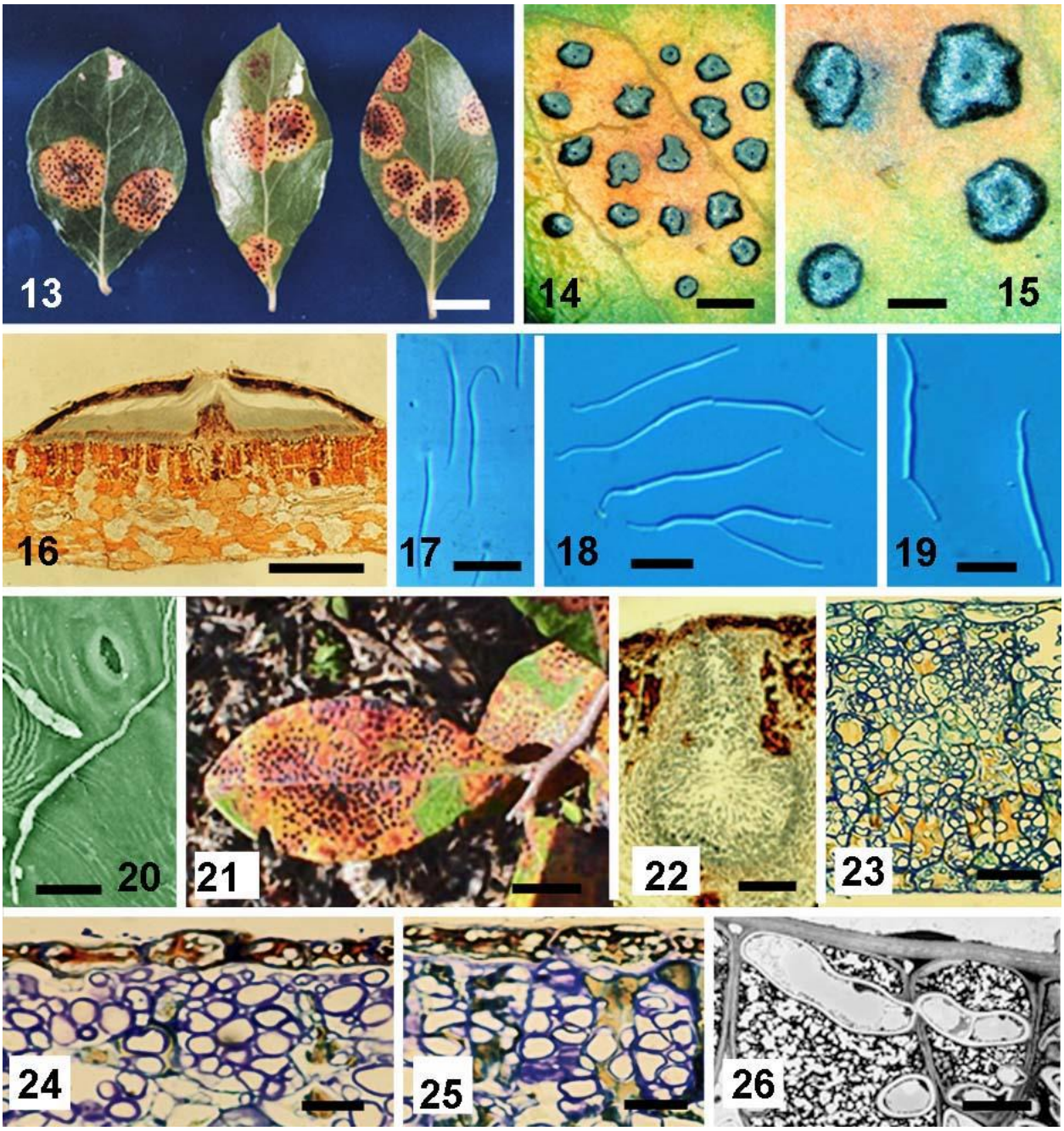

Fig. 13. Host leaves with lesions containing acervuli in early summer. Fig. 14. Close-up of lesion with acervuli. Fig. 15. Close-up of four acervuli. Fig. 16. Vertical section through acervulus showing layer of conidiophores, conidia and central pore-forming column. Fig. 17. Mature conidia. Fig. 18-19. Germinating conidia. Fig. 20. Germinating conidium. Fig. 21. Heavily infected leaf in late summer. Fig. 22. Perithecial initial immersed in leaf tissue in autumn. Fig. 23. Section through late summer leaf completely colonized by fungal cells. Fig. 24. Close-up of fungal cells in mesophyll tissue in late summer. Fig. 25. Same for palisade cells. Fig 26. Section showing thin-walled intracellular hyphae in young lesion. Scale bars: Figs. 13 = 1 cm, 14 = 5 mm, 15 = 1 cm, 16 = 100 µm, 17–20 = 10 µm, 21 = 1 cm, 22 = 2 µm, 23 = 20 µm, 24–26 = 30 µm. Figs. 17 DIC, 20 SEM, 26 TEM.

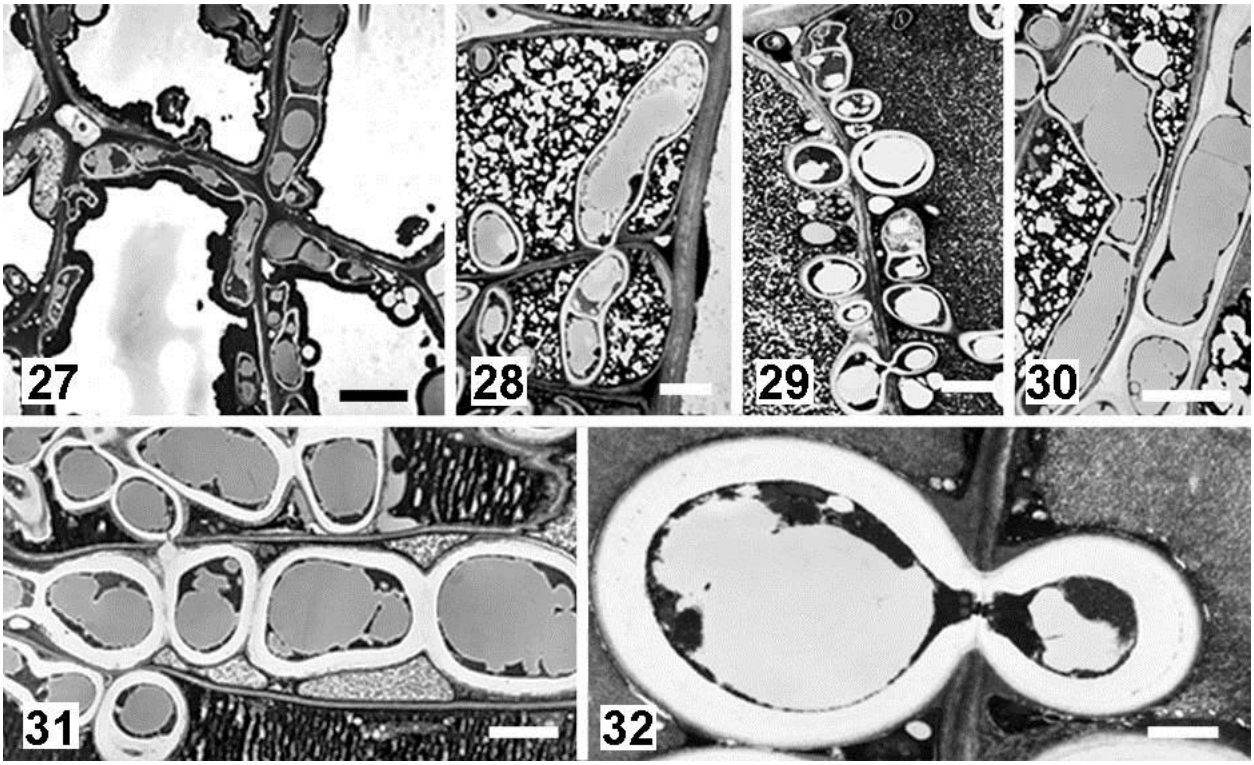

Fig. 27. Thin-walled hyphae lining cell walls of host. Note oil globules. Fig. 28. Intracellular hyphae amid disintegrating cytoplasm. Fig. 29. Cross-section of thin-walled hyphae lining cell wall. Fig. 30. Longitudinal section through intracellular hyphae filled with lipid globules. Fig. 31. Palisade cells of host filled with thick-walled hyphal cells. Fig. 32. Thick-walled hypha with large oil globule breeching cell wall by a narrow penetration peg. Note pore in hyphal septum. Scale bars: Figs. 27 = 5 μm, 28, 29 = 2 μm, 30 = 1 μm, 31, 32 = 2 μm. TEM.