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Sclerotinia trillii, sp. nov., causing a new sclerotinia disease on *Trillium* in Japan

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***Sclerotinia trillii* n. sp., which attacks *Trillium tschonoskii* and *T. smallii* in Hokkaido and northern Honshu, Japan, is described. The characters identifying this species with the genus *Sclerotinia* are large tuberoid sclerotia, produced both on infected plants and in culture, which consisted of only mycelium (true sclerotia) and flesh apothecia produced on them. This species is distinguished from *S. sclerotiorum*, *S. minor*, *S. trifoliorum*, and *S. nivalis* by relatively large sclerotia, cultural colony appearance, and red-brown to yellow-brown, relatively large apothecium, in addition to its parasitic nature on *Trillium*. *Sclerotinia trillii* is a psychrophilic having an optimum temperature for mycelial growth at 15–20°C.**

Key Words——sclerotinia disease; taxonomy; *Trillium*.

A sclerotinia disease was first observed at Tomakomai, Hokkaido, in June, 1996, on *Trillium tschonoskii* Maxim., with the symptoms of grayish brown rot on leaves, peduncle and stem (Figs. 1, 2). Occasionally, periphery of the rotted part changed red purple. The same disease was observed in June, 1997, on *Trillium smallii* Maxim. in Hirosaki, Aomori Pref.

Sclerotinia disease on *Trillium* has not hitherto been reported in Japan. In the USA, somewhat similar diseases caused by *Ciborinia trillii* Batra et Korf and *Sclerotinia sclerotiorum* (Lib.) de Bary have been reported (Batra and Korf, 1959; Holcomb, 1990). The present study revealed that the pathogenic fungus is an undescribed species of *Sclerotinia*. It is described here as *S. trillii* sp. nov.

Material and Methods

Isolation Sclerotia collected from *Trillium tschonoskii* and *T. smallii* were directly placed on clear agar containing 100 ppm streptomycin-sulfate in 9-cm Petri dishes. The plate was incubated at 15°C under lighting for 12 h per day (12 LD, daylight type fluorescent lamp: 15 W). After 4 d, 3×3 mm agar disks were cut from developing mycelia and transferred to fresh PSA plates (potato 200 g, sucrose 20 g, agar powder 20 g, distilled water 1000 ml) for immediate use or to PSA slants for storage. The slants were first incubated for 1 mo at 15°C under 12 LD, then transferred to a refrigerator (7°C).

Isolates used Two isolates were used in this study: No. 2843, isolated from sclerotia on *T. tschonoskii*, collected at Tomakomai; and No. 2917, isolated from sclerotia on *T. smallii*, collected at Hirosaki. For comparison, *Sclerotinia sclerotiorum* from apple leaves (No. 1191) was used in culture and some inoculation experiments.

To study cultural behavior, *Sclerotinia minor* Jagger (Sm-5, from tomato, *Lycopersicon esculentum* Mill.), *Sclerotinia nivalis* I. Saito (ATCC201665, from burdock, *Arctium lappa* L.) and *Sclerotinia trifoliorum* Erikss. (No. 2181, from alfalfa, *Medicago sativa* L.) were also used for comparison. For SDS-PAGE (of major protein in sclerotia), isolate No. 2843, *S. sclerotiorum* (No. 1191), *S. nivalis* (ATCC201665) and *S. trifoliorum* (No. 2181) were used.

Inoculation The pathological character of the isolates were confirmed with two experiments.

Trillium plants: small agar pieces with developing mycelia (5×5 mm) were applied to the stem and peduncle of potted *T. tschonoskii* plants and wrapped with Parafilm. The pots were then placed in a moist chamber in the shade. On control plants, PSA pieces without mycelia were likewise applied.

Fruits of non-host plants: fruits of cowpea (*Phaseolus vulgaris* L.) and eggplant (*Solanum melongena* L.) were inoculated with the trillium fungus (No. 2843), or with *S. sclerotiorum* for comparison. Small agar pieces with mycelia were applied on surface-sterilized fruit surface (wiped with 70% ethanol), wrapped with plastic tape, and the pieces were placed in deep dishes. The dishes were incubated at 15°C under 12 LD for 20 d.

Cultural characteristics To see the colony appearance, the fungi was grown at 15°C under 12 LD on fresh PSA. In addition, wheat grain medium (wheat grain 12 g, distilled water 25 ml) (Drayton, 1934), and carrot piece medium (3.5 cm in diam) were used for observation of growing mycelia. The media were all autoclaved for 20

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Figs. 1, 2. Sclerotinia disease on *Trillium tschonoskii*, showing sclerotia (arrows) in early stage of development.
Fig. 3. *Sclerotinia trillii* (left) and *S. sclerotiorum* (right) on autoclaved carrot pieces, grown at 15°C under 12 LD for 10 d.
Fig. 4. *Sclerotinia* spp. grown on PSA plates for 1 mo at 15°C under 12 LD. (Upper: from left, *S. trifoliorum*, *S. trillii*, *S. sclerotiorum*. Lower: from left, *S. minor*, *S. nivalis*.)
Fig. 5. Sclerotial section of the *S. trillii*, showing black rind (upper) and white medulla (lower) portions. Scale: 10 μm.

minutes at 121°C. Experiments were repeated two times with five replications.

Temperature response of mycelial growth: PSA plates with mycelial disks were incubated at temperatures ranging from 5°C to 30°C under 12 LD, and the diameter of growing colonies was measured at every fifth day.

Production of apothecia Sclerotia of isolate No. 2843

were obtained from PSA plate cultures which had been grown for 6 mo at 15°C under 12 LD, placed on moist quartz sand in a deep dish (9 cm in diam and 9 cm deep), and kept at 4°C in the dark for 4 mo. Then the dish was transferred to 5°C under 12 LD and incubated for a further 45 d. At that time, a few sclerotia germinated to produce apothecial initials 1 mm high. Sclerotia were then soaked in distilled water at 5°C overnight (Drayton, 1934). After seven days, the dish was transferred to 10°C under 12 LD. Ten days later, it was transferred to 15°C under 12 LD. This temperature treatment was adapted from temperature changes during apothecial development in nature.

Observation of apothecial structures Apothecial morphology was observed under a light microscope. Apothecia with sclerotia were air-dried at room temperature (20°C) for 3 h, then cut into sections (18 μm thick) on a freezing microtome (MICROM HM400), the sections being mounted in distilled water, lactophenol, Melzer reagent (iodine-iodine potassium solution) without KOH, cotton blue and olsein acetate reagent. As a color standard, "Methuen Handbook of Colour" (Kornrup and Wanscher, 1984) was used.

Sodiumdodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) Isolate No. 2843, *S. sclerotiorum*, *S. nivalis*, and *S. trifoliorum* were grown on PSA plates at 15°C under 12 LD for about 1 mo to obtain samples of sclerotia. Proteins were extracted based on the procedures of Scott (1981) and Saito (1997). 2.5–2.6 g of sclerotia were ground in mortars with quartz sand and 5 ml of 0.05 M Tris-HCl-buffer (pH 6.8) on crushed ice. Sclerotial slurries were centrifuged at 2,000 rpm for 15 min. Supernatants were centrifuged at 15,000 rpm for 20 min and dispensed in 1-ml aliquots into 1.5-ml tubes with 50 mM sucrose. Those were stored in −30°C until use. Samples of 50 μl were electrophoresed with prestained SDS-PAGE molecular size marker (phosphorylase B, 112 kDa; bovine serum albumin, 84 kDa; ovalbumin, 53.2 kDa; carbonic anhydrase, 34.9 kDa; soybean tripsin inhibitor, 28.7 kDa; lysozyme 20.5 kDa; Biorad Low Range) at 20 mA in 0.025 M Tris-glycine buffer containing 0.1% (w/v) SDS for 1.5–2 h at 0°C on slab gels of 12% polyacrylamide, 100×100×1 mm. After electrophoresis, the gel was stained with Coomassie brilliant blue solution.

Results

Pathogenicity *Trillium* plants: Seven days after inoculation with the *Trillium* fungus (No. 2843), wilt and rot symptoms developed gradually from the inoculated parts, reproducing the same symptoms as seen in nature. In 15–20 d sclerotia were produced on affected parts of the plant.

Non-host plants: Isolate No.2843 produced no symptoms of disease on either cowpea or eggplant. On the contrary, *S. sclerotiorum* produced apparent rot on both plants.

Cultural characteristics The fungus isolated from *T. tschonoskii* and *T. smallii* showed the same colony appearance on PSA, producing white mycelium with some aerial hyphae. In 30 d or more, it produced black, hemispherical sclerotia 3.5–11 mm in diam, which could easily be detached from the colony surface (Fig. 4). The mycelia were 2.5–5 μm wide. Microconidia were produced on old mycelia and on sclerotia, which were hyaline and globose, 2.5–3 μm in diam.

On wheat grain medium: The fungus from *Trillium* produced grayish white mycelia, which were somewhat tufted. In 15–20 d many black and hemispherical sclerotia were produced. Colonies of *S. sclerotiorum* on wheat grain media produced white mycelium and black, amorphous sclerotia.

On sterilized carrot pieces: The fungus from *Trillium*

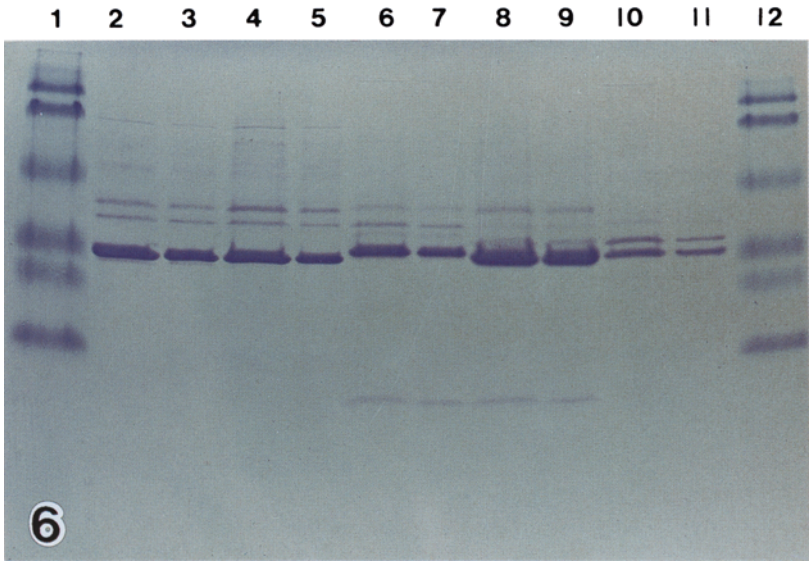


Fig. 6. SDS-PAGE of protein extracted from sclerotia of *Sclerotinia trillii* (lanes 2–5), *S. trifoliorum* (lanes 6, 7), *S. sclerotiorum* (lanes 8, 9), *S. nivalis* (lanes 10, 11). Molecular size marker (lanes 1, 12).

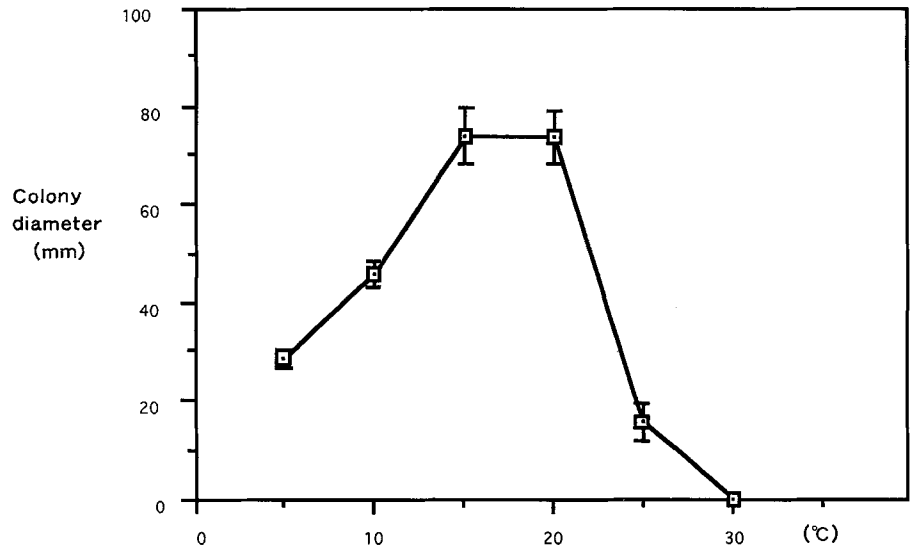


Fig. 7. Colony diameter of the *Sclerotinia trillii* (No.2843) on PSA plates (9 cm diam) at different temperatures, 30 d after the inoculation.

produced felty white mycelium, black hemispherical sclerotia in 2–3 wk. *S. sclerotiorum* produced cottony white mycelium, black sclerotia in 7 d, and the carrot tissue became soft-rotted (Fig. 3).

Temperature response of mycelial growth The fungus from trillium grew well at 15–20°C but did not at 30°C. Mycelia grew even at 5°C (Fig. 7).

Morphology of sclerotia and apothecia Sclerotia were composed of a black rind consisting of 4–6 layers of globosal or subglobosal cells, 7.5–20×6–14 μm wide, and a white medulla consisting of interwoven mycelia, 5–9 μm in diam, with interhyphal spaces. Sclerotial cortex was not observed (Fig. 5). Apothecia were cupulate when young to disciform at maturity, 2–7.5 mm in diam, red-brown (henna color) or yellow-brown (camel), with stipes 4–5 mm tall, darker and hairy at the base (Fig. 8). The exipulum of the disk measured 175–200 μm thick, the subhymenium was 17.5–22.5 μm thick, and the medullary excipulum 90–125 μm thick. The ectal excipulum was 75–100 μm thick, consisting of globose cells. Asci were hyaline, clavate, 180–200×10–15 μm, uniseriately 8-spored, J+. Paraphyses were filiform, 170–200×2–3 μm. Ascospores were ovoid or subglobose, hyaline, continuous, 10–19×7–10 μm (Figs. 9–11).

Electrophoretic patterns of the sclerotial proteins The electrophoretic patterns of major proteins in sclerotial extracts of the *Trillium* fungus, *S. trifoliorum*, *S. nivalis* and *S. sclerotiorum* are shown in Fig. 6. All were different. The *Trillium* fungus and *S. sclerotiorum* had major bands at the same position. The *Trillium* fungus had two far bands of large size. *S. trifoliorum* had three far bands of large size and one far band of small size. *Sclerotinia sclerotiorum* had three far bands of large size and one far band of small size.

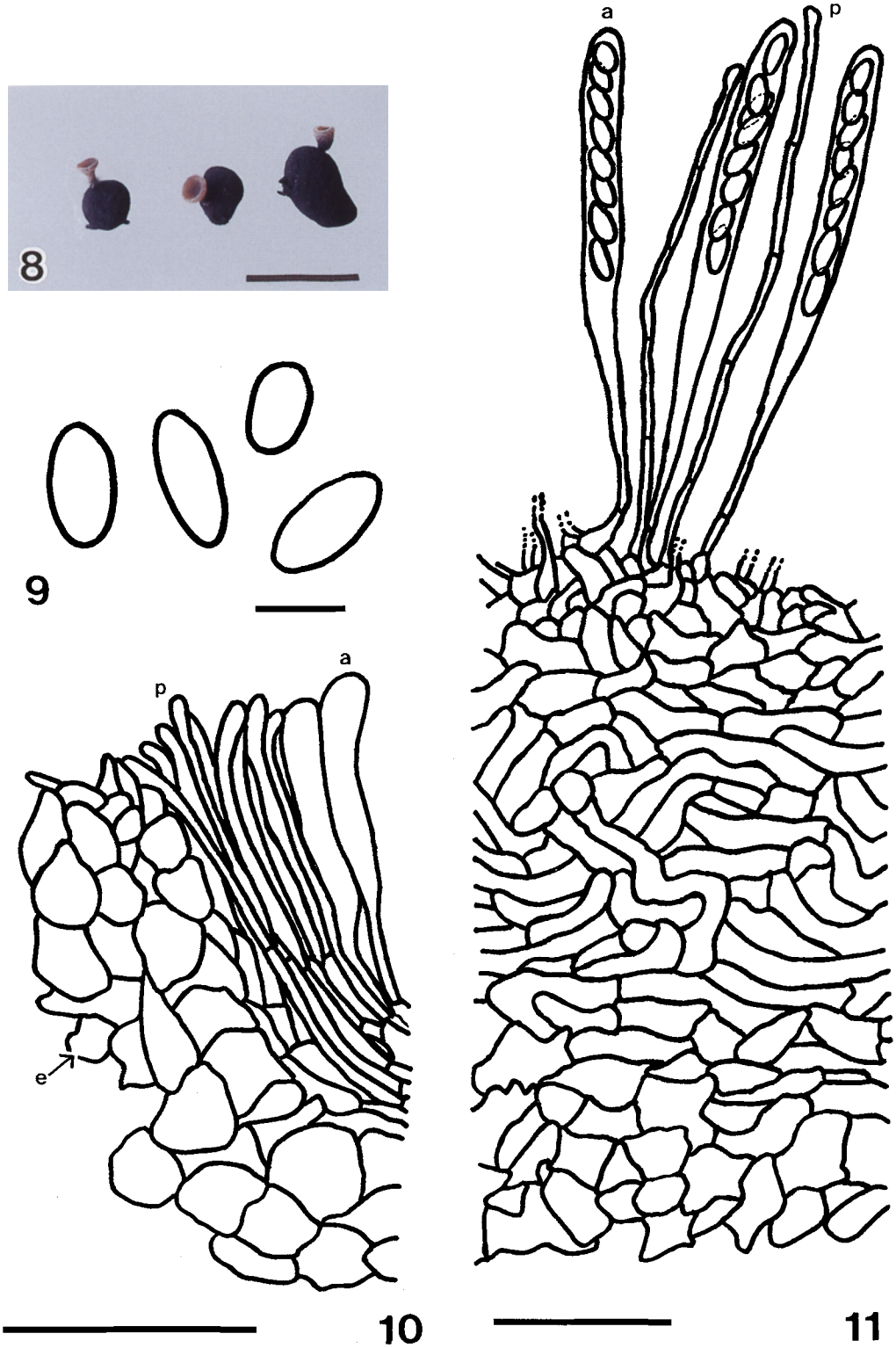
Taxonomy

Sclerotinia trillii Y. Harada et Narumi, sp. nov.
Figs. 1–5, 8–11

Coloniae in PSA niveae, pannosae, ex mycelio 2.5–5 μm latis et hyphis aeriis non vel vix evalventibus constantes, post 15–30 die, in superficie sclerotia 3.5–11 mm diam formantes. Sclerotia nigra, globosa vel subglobosa, 3.5–11 mm diam; cortex pullulans, ex cellulis 4-6-stratosis globosi vel subglobosi 7.5–20×6–14 μm compositus; medulla nivalis, "textura intricata", ex cellulis 5–9 μm composita. Microconidia in hyphis senescentibus et sclerotiis formantia, hyalina, globosa, 2.5–3 μm diam. Apothecia 2–7.5 mm diam, 1.5–5 mm alta, juventate cupulata maturitate disciformia, rufo-brunnea vel flavo-brunnea; excipulum 175–200 μm crassum; subhymenium 17.5–22.5 μm crassum; excipulum medullare 90–125 μm crassum; excipulum ectale 75–100 μm crassum, ex cellulis globosis compositum. Asci hyalini, clavati, 180–200×10–15 μm, octospori, J+ in poro apicali. Paraphyses filiforme, apice rotundatae, 170–200×2–3 μm. Ascosporae continuae hyalinae, obovatae vel ellipticae, uniseriales in ascis, uni- vel biguttulatae, 10–19×7–10 μm.

Holotypus: Apothecia enascentia a sclerotiis in cultura (2843) ex sclerotio in foliis vivis *Trillii tschonoskii* Maxim., Tomakomai, Hokkaido, Japonia, 21 Jun. 1996, Y.Harada leg., in Harbario Universitatis Hirosakiensis conservata (HIROSAKI UNI-23933).

Hab: On *Trillium* tschonoskii Maxim., Tomakomai, Hokkaido, Japan, 21 Juneus, 1996 (No. 2843). On *Trillium smallii* Maxim., Hirosaki, Aomori Pref., Japan, 6 Juneus, 1997 (No. 2917).



Figs. 8–11. *Sclerotinia trillii*. 8. Apothecia arising from the sclerotia. 9. Ascospores. 10. Component cells of ectal excipulum in apothecial margin (a: ascus p: paraphyses e: ectal excipulum). 11. Apothecial section, showing asci (a), paraphyses (p) and subhymenial tissues. Scales: 10 mm for 8; 10 μm for 9; 100 μm for 10; 50 μm for 11.

Discussion

The fungus on *Trillium* was categorized as a *Sclerotinia* species according to Kohn's key (Kohn, 1979), because of its true sclerotia, no anamorphic state and apothecial characters like globose cells in the ectal excipulum and

Table 1. Comparison of *Sclerotinia trillii* with hitherto known *Sclerotinia* species.*

Species	Host range	Season of apothecium formation	Apothecia		Size		
			diameter (mm)	color	sclerotia (mm)	asci (μm)	ascospore (μm)
S. sclerotiorum	Polyxeny	Spring-early summer	2–8(–10)	Ochraceous –cinnamon	3–10	(110–)130–150(–160) × 6–10	(9–)10–14 × 4–5(–6)
S. trifoliorum	Forage Legumens (Broadbean)	Autumn	3–7(–10)	Grayish sepia –umber	4–8.5	140–200 × 10–12	10–20 × (4–)6–9(–11)
S. minor	Polyxeny	Autumn	2–9	Cinnamon –umber	1–3	(110–)125–180 × 7–11	8–17(–20) × (4–)5–7(–9)
S. nivalis	Herbaceous Dicots	Only in culture	5–9	Ochraceous –cinnamon	0.6–4	114–144 × 6.7–9.2	9.2–11.7 × 3.8–5.0
S. trillii	*Trillium*	Only in culture	2–7.5	Henna-camel	3.5–11	180–200 × 10–15	10–19 × 7–10

*Morphological data for *S. sclerotiorum*, *S. trifoliorum*, and *S. minor* are from Kohn (1979), and for *S. nivalis* from Saito (1997).

J+ reactions in apices of the asci. Kohn (1979) studied the morphology of *Sclerotinia* and proposed three distinct species: *S. sclerotiorum*, *S. trifoliorum*, and *S. minor*. Saito (1997) described *S. nivalis* as an additional species.

The characteristics of *S. trillii* and other *Sclerotinia* species are compared in Table 1.

Pathologically, *S. trillii* is so far known to occur only on *Trillium*.

In cultural characteristics, *S. trillii* is distinguished from other *Sclerotinia* species by its poor aerial hyphae on wheat grain medium and sterilized carrot piece, its hemispherical sclerotia on wheat grain medium, and its inability to melt carrot piece medium.

In SDS-PAGE; *S. trillii* was distinguished from other *Sclerotinia* species in the band patterns of major sclerotial protein. Saito (1997) stated that *Sclerotinia* spp. have different patterns. The electrophoresis data of this fungus, therefore, also indicated that *S. trillii* was a distinct species of *Sclerotinia*.

Based on field observations and laboratory experiments, the life history of *S. trillii* was thought to be as follows. Sclerotia germinate to produce apothecia in early spring. Then ascospores would go to flowers, peduncles, or stem of *Trillium*. This happens during blooming and petal-fall stages, because *Trillium* bloom in early spring and disease symptoms appear soon after blooming in spring to early summer. Tissue rot of *Trillium* plants develops in early summer, followed by the appearance of black sclerotia, which would oversummer and overwinter.

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