Mycoscience 38: 227–236, 1997

Sclerotinia nivalis, sp. nov., the pathogen of snow mold of herbaceous dicots in northern Japan

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Accepted for publication 27 May 1997

A new *Sclerotinia*, previously reported as *S. intermedia* from Japan, is described as *Sclerotinia nivalis* on the morphological basis of the sclerotial anamorph and teleomorph produced in culture. The characters assigning this species to the genus *Sclerotinia* are the tuberoid sclerotia superficially produced on suscepts, the small sclerotia produced on aerial mycelium in culture, the interhyphal spaces in medullary tissue of sclerotia, and the globose cells constructing the ectal excipulum of apothecia. It is distinguishable from *S. sclerotiorum*, *S. minor*, and *S. trifoliorum* by the intermediate sized sclerotia in culture, binucleate ascospores, the molecular mass of major proteins of sclerotia, and the patterns of esterase isozymes in sclerotial extracts. Although *S. nivalis* causes snow mold of various dicots, it is a mesophile having an optimum temperature for mycelial growth of around 20°C. It attacks edible burdock (*Arctium lappa*), *Chryhsanthemum morifolium*, *Ambrosia elatior*, carrot (*Daucus carota*), *Angelica acutiloba*, *Ajuga reptans*, and *Plantago lanceolata*.

Key Words——herbaceous dicots; mesophilic fungus; *Sclerotinia*; snow mold.

Tochinai and Sugimoto (1958) studied a snow-mold disease of santonica (*Artemisia maritima* L., the source plant for santonin industry) caused by a sclerotiniaceous fungus, which had been the major problem for santonica cultivation in Hokkaido since the early 1940s. They identified the causal organism with *Sclerotinia intermedia* Ramsey, which was synonymized with *Sclerotinia sclerotiorum* (Lib.) de Bary by Purdy (1955) proposing a broader concept of the species *S. sclerotiorum* based on variability of morphological characters that had been used for identification. The characters of the organism that Tochinai and Sugimoto adopted in their identification are the color of mycelium on the suscept (not always white), temperature response in mycelial growth (faster growth at 0°C than *S. sclerotiorum* and *S. trifoliorum* Erikss.), intermediate size of sclerotia produced at 25 °C (1.9–4.3 mm) and measurements of asci (118.5× 7.3 μm) and ascospores (10.5×4.5 μm). Later, autumn-sown rape (*Brassica campestris* L. subsp. *napus* Hook.) and summer-sown carrot (*Daucus carota* L.) left unharvested in the field were found to be damaged by the same organism under the similar conditions as with santonica (Sugimoto et al., 1962; Narita, 1980). Although there have been no published records, the organism found by Tochinai and Sugimoto was also considered to cause snow-mold disease of pyrethrum (*Chrysanthemum cinerariaefolium* Bocc.), the source plant for pyrethrin industry, which was widely cultivated in Hokkaido in the 1930s (Narita, 1980). Thereafter, the organism has become rare in agricultural sites in Hokkaido because of the decline of cultivation of perennial or biennial dicot crops which could be suscepts. On the other hand, the recent taxonomic revision of Sclerotiniaceae (Kohn, 1979) that synonymized *S. intermedia* with *Sclerotinia minor* Jagger led to the re-examination of the organism designated as *S. intermedia* in Japan, and the organism was suggested to be a new species of *Sclerotinia* (Saito, 1985). Later, this was found to agree with the analysis of restriction fragment length polymorphysm (RFLP) of nuclear and mitochondrial DNA (Kohn et al., 1988). While the organism remained undescribed because of its inertness in fruiting, I have succeeded in producing a teleomorph with isolates from several host plants which equally showed snow-mold symptoms. In this paper, the organism is described as *Sclerotinia nivalis* sp. nov.

Materials and Methods

Isolation Sclerotia collected from various suscepts were surface-sterilized in 1% sodium hypochlorite for 5 min, washed thoroughly with sterilized distilled water, and cut in half with a sterilized razor blade. Sclerotial pieces were placed on PDA (potato dextrose agar) plates in 9-cm Petri dishes, with the cut face in contact with the agar surface. The PDA plates were incubated at 20°C in the dark, and tips of developing mycelium were transferred to PDA slants. The slants were incubated at 20°C for about 3 wk, then transferred to a refrigerator (0–3°C).

Cultural appearance The organisms were grown at 20°C on PDA (Difco), oatmeal agar (75 g of oatmeal was boiled in 1,000 ml of distilled water for 40 min then filtered through a triple layers of gauze. The filtrate was made up to 1,000 ml with distilled water, 15 g of agar was added, and the medium was autoclaved at 120°C for 20 min), oatmeal-yeast extract agar (1.7 g of Difco

yeast extract was added to 1,000 ml of oatmeal agar before sterilization), 5% (w/v) malt extract agar, malt extract-peptone-dextrose agar (malt extract, 20 g; dextrose, 20 g; polypeptone, 1 g; agar, 15 g; distilled water, 1,000 ml) and Czapek Dox solution agar. Thirteen ml of each agar medium was poured into 9-cm Petri dishes.

Temperature response of mycelial growth PDA plates were inoculated with mycelial agar disks (5 mm in diam) and incubated at 5, 10, 15, 20, 25 and 30°C. Diameter of growing colonies was measured daily. The experiment was repeated twice with four replicates.

Production of apothecia Sliced carrot roots (ca. 2 cm thick) in 20-cm Petri dishes were autoclaved at 120°C for 20 min, then inoculated with mycelial agar disks. The inoculated carrot roots were incubated at 19–20°C in the dark for 4 wk. Sclerotia formed were picked off from carrot medium and washed in running tap water for 12 h. They were seeded on saturated sponges in glass containers as described elsewhere (Saito, 1977). The glass containers including sclerotia were kept at 20°C, 100 cm below fluorescent lighting (National FLR 40S-W/MX "36"). For the preparation of dry specimens, mature apothecia were frozen and kept at −82°C, then lyophilized with a freeze dryer (Tokyo Rikaki, Type FD-80).

Histology of sclerotia and apothecia Materials were fixed with 4% glutaraldehyde in 1/15 M phosphate buffer (pH 7), dehydrated in a graded acetone series, embedded in Spurr's low viscosity resin, and cut with glass knives with a Porter Blum MT-1 ultramicrotome. Apothecial sections were stained in methylene blue-azure II and basic fuchsin by the methods of Bennel et al. (1978). Sclerotial sections were stained with Schiff's reagent in PAS-reaction (Saito, 1977). They were also observed on glass slides, being mounted with 0.05% (w/v) toluidine blue in 0.1 M phosphate buffer (pH 4.6) under cover slips. Apothecial sections were also made from freeze-dried apothecia infiltrated with paraffin under vacuum.

Measurements of the teleomorph For measurement, ascospores were made either ejected onto glass slides from a fresh apothecium or prepared from dried specimens. Asci and paraphyses were measured with dried specimens. All materials for measurement were mounted with Melzer's reagent. The iodine reaction of the apical pore apparatus was observed with or without 2% (w/v) aqueous KOH solution. Resin-embedded sections were mainly used for observation of differentiation in apothecial tissue and for measurement of tissue thickness and component cells, and paraffin sections of freeze-dried apothecia were also used to supplement these measurements.

Nuclear staining of ascospores Dissected pieces of apothecia were fixed following Lu's method using a mixture of chromic acid, glacial acetic acid, and *n*-butyl alcohol (Lu, 1962) and stained with propionic-iron alum-haematoxylin (Henderson and Lu, 1968). Ascospores ejected onto glass slides coated with Haupt's adhesive (Jensen, 1962) were also stained by the HCl-Giemsa method (Wilson, 1992) after fixation in Farmer's fluid (Sass, 1951) for 10 min.

Polyacrylamide gel electrophoresis (PAGE) Three isolates of *S. nivalis* from carrot, edible burdock and *Angelica acutiloba* Kitagawa (a medicinal root crop) were cultured on a semi-synthetic agar medium at 20°C. *Sclerotinia sclerotiorum* (Sm5f-1) from kidney bean, *S. trifoliorum* (Str-1) from white clover and *S. minor* from tomato (kindly provided by Mr. S. Saito) were cultured as reference taxa in the same conditions as *S. nivalis*. The medium was constituted as follows: dextrose, 20 g; L-asparagine monohydrate, 2 g; yeast extract (Bacto), 1 g; KH_2PO_4, 1 g; KCl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.0 1 g; distilled water, 1,000 ml. For molecular mass determination of major proteins in sodium dodecyl sulfate (SDS)-PAGE, sclerotia harvested from about 3-wk-old cultures were ground in a chilled mortar with a small amount of quartz sand and 5 ml of 0.05 M Tris-glycine buffer, pH 8.3, for each g of fresh weight of sclerotia. The sclerotial slurry was centrifuged at 2,000 rpm for 15 min and the supernatant was further centrifuged at 20,000 rpm for 20 min. Aliquots of the supernatant (1 ml) were pipetted into 5-ml ampoules, frozen in dry ice-acetone, lyophilized using a freeze dryer (Tokyo Rikaki, Type FD-80) and stored at 0–3°C until use. For six isolates of *S. nivalis*, the patterns of esterase isozymes were compared with those of *S. minor* and *S. trifoliorum*. For this purpose, proteins were extracted from sclerotia obtained in a similar manner as above using 0.05 M Tris-HCl buffer (pH 6.8) as an extraction buffer. Sucrose was then added at the concentration of 50 mM to the sclerotial extracts, which were stored in small test tubes at −30°C until use. All extraction procedures were performed at 0–3°C. Protein concentrations were determined by Bradford's method (Bradford, 1976) using a Bio-Rad Protein Assay kit and bovine serum albumin (Sigma) as standard.

SDS-PAGE of major proteins was carried out in a slab gel (14×14×0.15 cm) containing 12.5% (w/v) acrylamide buffered with 0.375 M Tris-HCl buffer (pH 8.8) and 0.1% SDS (w/v), with a stacking gel buffered with 0.1 M Tris-HCl buffer (pH 6.8) and 0.1% SDS(w/v). Freeze-dried sclerotial extract was dissolved in 10 mM Tris-HCl buffer (pH 6.8) containing 4.5% SDS (w/v) and 5% β-mercaptoethanol (v/v) to give a protein concentration of 1 mg per ml of the solution. The mixed solution was boiled at 100°C for 5 min, then the same volume of 10 mM Tris-HCl buffer containing 40% (w/v) glycerin and 0.002% (w/v) bromophenol blue was added. Protein samples of 20 μl were loaded in gel slots. Electrophoresis was carried out at 2–3°C with a current of 20 mA using 0.025 M Tris-glycine buffer (pH 8.57) containing 0.1%(w/v) SDS as a running buffer. For molecular mass determination, phosphorylase b (94,000 kDa), bovine serum albumin (67,000 kDa), ovalbumin (43,000 kDa), carbonic anhydrase (30,000 kDa), trypsin inhibitor (20,100 kDa), α-lactalbumin (14,000 kDa) (Pharmacia Fine Chemicals) were used as standards. Slab gels were stained with Coomassie brilliant blue. For esterase isozyme electrophoresis, the frozen extracts of sclerotia were melted at 0–3°C, diluted with 0.05 M Tris-HCl buffer (pH 6.8), and the solution containing 20–30 μg

protein was loaded into each slot. Electrophoresis was run at 2–4°C with a current of 25 mA using 0.05 M Tris-glycine (pH 8.3) buffer. For the detection of isozymes, the gel was stained in a reaction mixture (0.1% (w/v) Fast blue RR salt; 0.5 mM 1-naphthylacetate; 0.1 M phosphate buffer, pH 7.0) at 30°C.

Results

Symptomatology From 1981 to 1995, seven dicot plants, including weeds, herbs and root-crops, were found to be affected by the present *Sclerotinia* species in different locations in Hokkaido (Table 1). All these plants showed clear symptoms as soon as snow disappeared in the early spring. Leaves and petioles died and were discolored gray, pale-brown or yellow-brown, and when dried were pressed onto the ground surface and bore black tuberoid sclerotia of 3–4 mm in diam. The snow mold symptoms of carrot are shown in Fig. 1. In addition to such above-ground symptoms, infected root-crops showed conspicuous crown rot symptoms: In carrot, crown of roots were covered partly or wholely with white mycelium, and the internal root tissue was softened or decayed, producing cavities generally filled with white cottony mycelium (Fig. 2). Diseased lesions were also observed on the subterranean parts of root as previously reported by Sugimoto et al. (1962). Sclerotia varied in shape and were generally larger on infected root crowns and in the internal cavities of roots than on leaves and petioles; they often aggregated to form irregularly shaped flattened structures of 3 cm or more in diam. The symptoms of infected edible burdock were basically similar to those of carrot, though they were not typically soft rot (Fig. 3). In *A. acutiloba* infected crown died giving a dry rot, and the spaces between residual fibers were often filled with white mycelium or folded sclerotia (Fig. 4).

Taxonomy

Sclerotinia nivalis I. Saito, sp. nov. Figs. 5–18

Apothecia e sclerotio surgentia, singularia vel nonnulla, stipitata. Discus cupulatus vel umbilicato-discoideus, carnosus, fulvus vel cinnamomeus, 5–9 mm diam; excipulum medullare 150–300 μm crassum, "textura intricata," ex hyphis hyalinis 6–19 μm latis compositum; excipulum ectale 60–100 μm crassum, "textura prismatica" vel "textura globulosa," ex cellulis globosis hyalinis vel brunneolis leptodermis 7–26 × 8–32 μm compositum; subhymenium pallide brunneum, "textura intricata," 30–40 μm crassum. Stipites cylindracei, deorsum attenuati, disco paene concolores sed ad basim infuscati, 5–17 × 1–2 mm, ad maturiatem saepe squamulosi per decorticationem excipuli. Asci cylindracei, versus basim paulo attenuati, apice rotundati, inoperculati, poro apicali jodo non vel obscure cyanescenti praediti, octospori, 114–144 × 6.7–9.2 μm. Ascosporae monostichae, continuae, ellipticae, hyalinae, non guttulatae, binucleatae, 9.2–11.7 × 3.8–5.0 μm. Paraphyses filiformes, hyalinae, romosae, septatae, 90–140 × 1.6–2.5 μm. Sclerotia superficialia, plano-convexa, rotundata vel irregularia, nigra, 0.3 × 3.6 mm; corium nigrum, "textura prismatica," ex cellulis pachypleuris 6–14 μm diam compositum; medulla hyalina, "textura oblita," ex hyphis 3.0–10.5 μm crassis membrana gelatinosa dense obtectis composita. Sclerotia minuta in hyphis aeriis saepe adnascentia. Microconidia globosa, 2–3 μm diam, e phialide hyalina formantia.

Habitat: parasitic on *Arctium lappa* L., *Chrysanthemum morifolium* Ramat., *Ambrosia elatior* L., *Daucus carota* L., *Angelica acutiloba* Kitagawa, *Ajuga reptans* L., *Plantago lanceolata* L.

Holotype: I. Saito ISNAD 23-1, deposited in the Herbarium of the Faculty of Agriculture, Hirosaki University (No. 24055). Apothecia and sclerotia produced on 10 October 1995 in the culture conducted by I. Saito. The isolates used in culture were derived from sclerotial anamorph on *Arctium lappa* (edible burdock), collected on 15 May 1982 by I. Saito in Makubetsu-cho, Hokkaido, Japan.

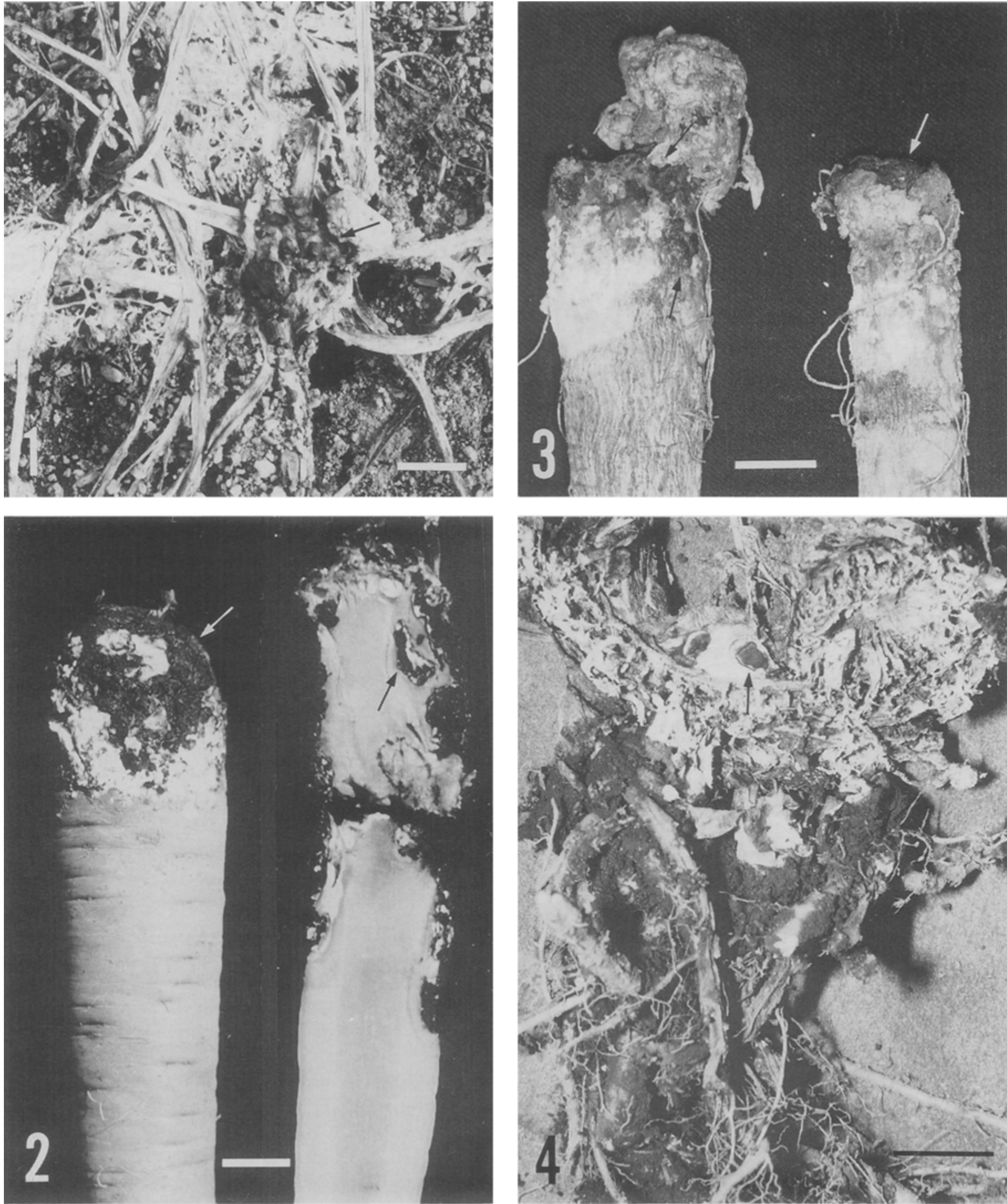
Specimens examined: Apothecia and sclerotia produced in culture with the isolates derived from sclerotial anamorph on *Daucus carota* (carrot) (ISNAD23-2), on *Angelica acutiloba* (ISNAD23-3), on *Plantago lanceolata* (ISNAD23-4), on *Ambrosia elatior* (ISNAD23-5). These specimens were also deposited in the Herbarium of the Faculty of Agriculture, Hirosaki University, with the specimens nos. 24056–24059.

Etymology: Latin, *nivalis*=of snow or snowy, referring to the environmental condition of disease occurrence.

Apothecia stipitate, arising singly or multiply from a sclerotium depending upon the sclerotial size. Disc deep cupulate (Fig. 12) or discoid with a central depression (Fig. 13), or sometimes convex, 5–9 mm in diam, ochraceous or cinnamon in color. Stipes cylindrical

Table 1. List of host plants from which sclerotial anamorph was collected.

	Plants	Location
Compositae:	Edible burdock	Makubetsu, Hokkaido
	Chrysanthemum morifolium Ramat.	Kunnep, Hokkaido
	Ambrosia elatior L.	Sapporo, Hokkaido
Umbelliferae:	Carrot	Kamiiso, Hokkaido
	Angelica acutiloba Kitagawa	Kunnep, Hokkaido
Labiatae:	*Ajuga reptans* L.	Ootaki, Hokkaido
Plantaginaceae:	*Plantago lanceolata* L.	Sapporo, Hokkaido



Figs. 1–4. Symptoms of plants infected by *Sclerotinia nivalis*. Scale bars = 2 cm.

1. Snow mold symptoms of carrot showing dead and discolored foliage, and sclerotia produced on the crown (arrow). 2. Infected roots of carrot: Left, a crown mantled with white mycelium and irregularly shaped sclerotia (white arrow); Right, section of an infected root showing tissue soft rot and sclerotia in a cavity (black arrow). 3. Infected root crown of edible burdock mantled with white mycelium and sclerotia (arrows). 4. Section of a collapsed rhizome of *Angelica acutiloba*. Mature sclerotia are seen in cavities (arrow).

5–17 × 1–2 mm, tapering towards the base, almost concolorous with the outer surface of the disc but usually darkening downward; ectal layer of the stipe often splits horizontally in layers to give shaggy appearance of the stipe at maturity (Figs. 12, 13, 15).

Axial section of apothecium (Figs. 14, 16) shows hymenium ca. 140 μm thick; subhymenium *textura intricata*, 30–40 μm thick, light brown; medullary excipulum 150–300 μm thick, *textura intricata* of loosely interwoven hyaline hyphae, 6–19 μm wide; ectal excipulum

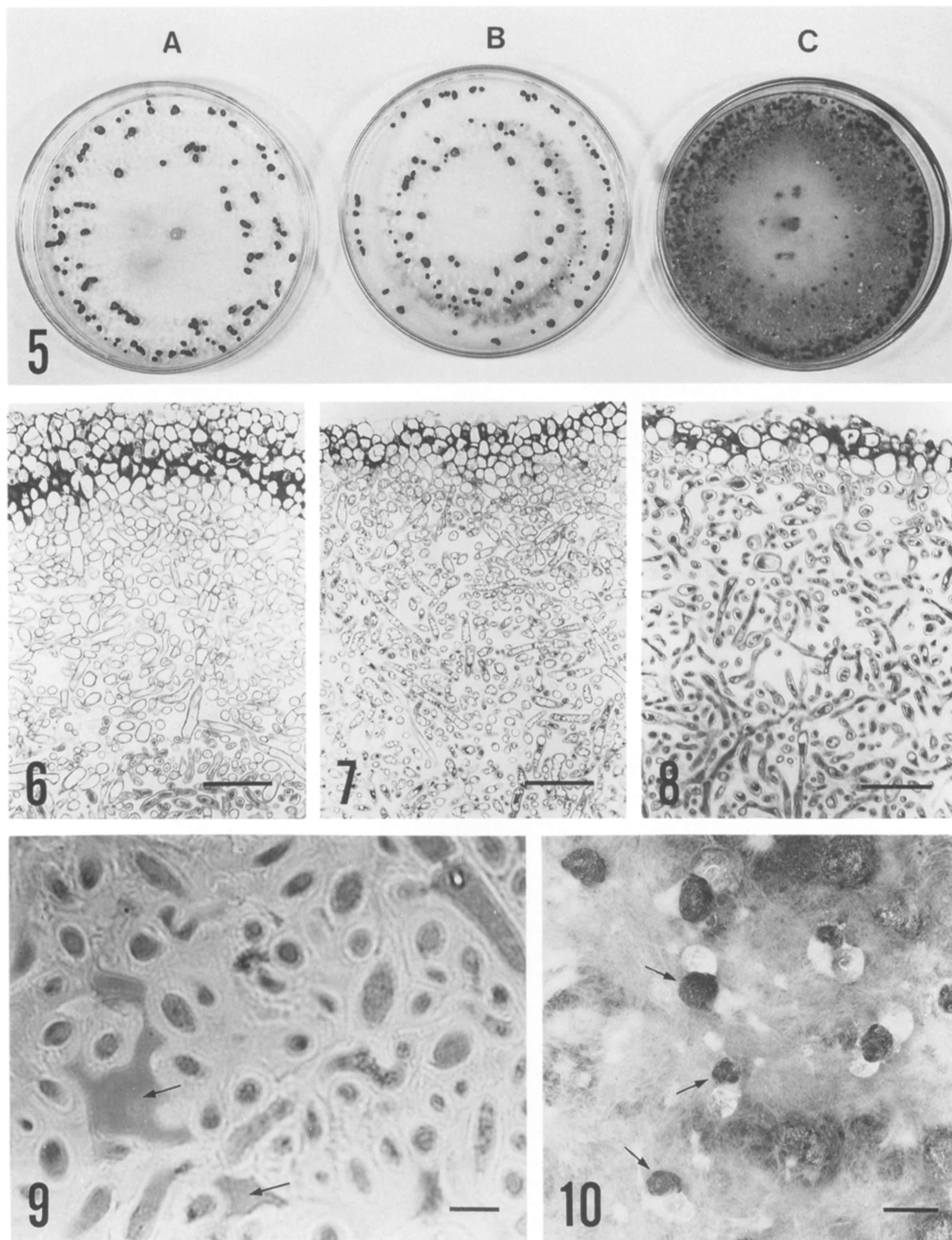


Fig. 5. Cultural appearance of *Sclerotinia nivalis* on various agar media: A, PDA; B, oatmeal agar; C, malt extract agar.

Figs. 6–8. Vertical sections of mature sclerotia. Scale bars = 50 μm. 6. Section of a natural sclerotium on root crown of carrot. 7. Section of a mature sclerotium produced on PDA. 8. Section of a small sclerotium produced on aerial mycelium on PDA.

Fig. 9. Medullary region of a mature sclerotium produced on PDA. Note faintly stained, markedly thickened layers enveloping medullary hyphae and interhyphal spaces (arrows). Scale bar = 10 μm.

Fig. 10. A part of colony on PDA where small sclerotia are produced on aerial mycelium (arrows). Scale bar = 1 mm.

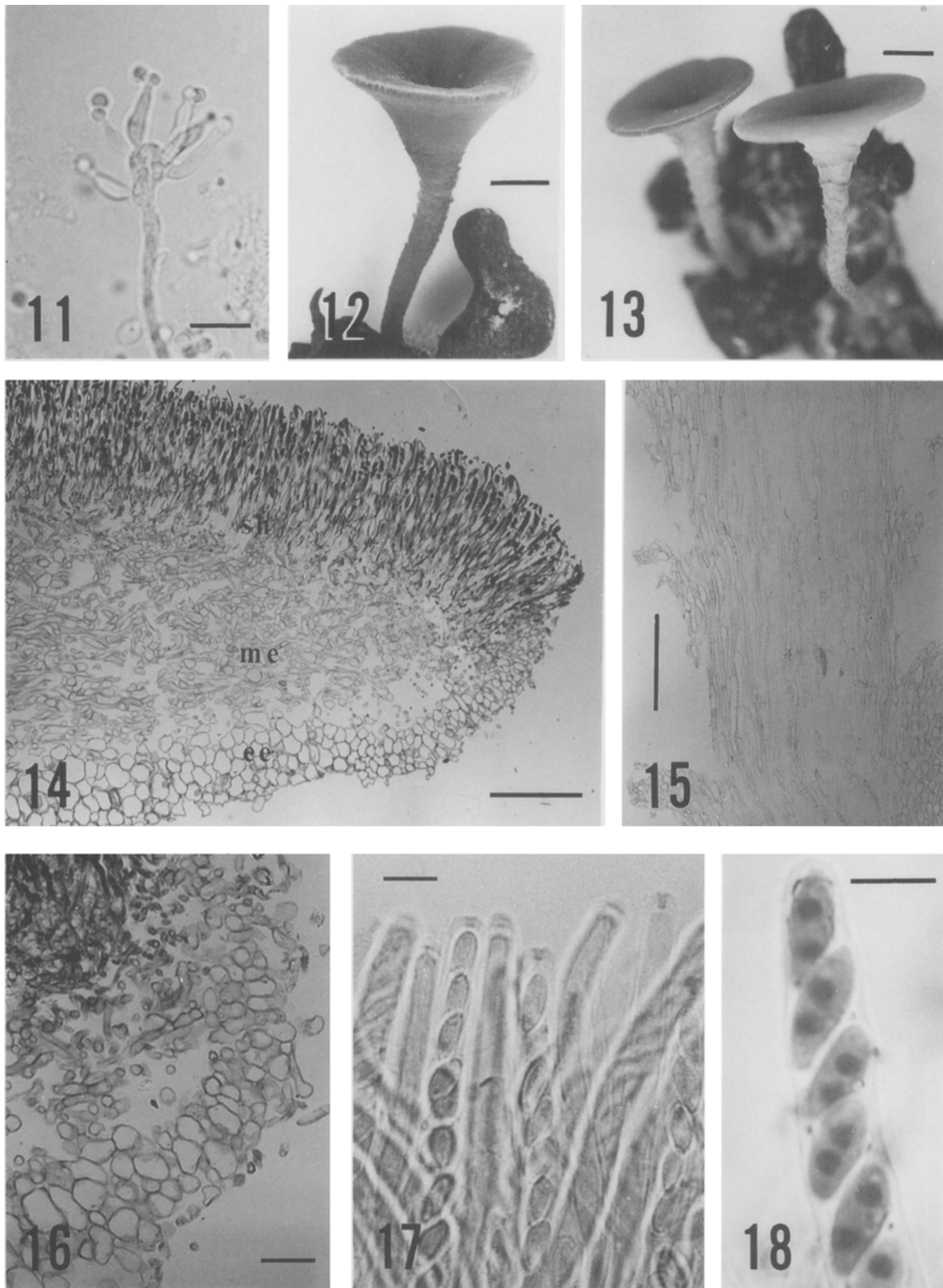


Fig. 11. Microconidia on phialides laterally borne on hyphae. Scale bar = 10 μm.

Figs. 12, 13. Apothecia arising from sclerotia. Scale bars = 1 mm. Note shaggy appearance of stipe surface. 12. Mature apothecium of edible burdock isolate. 13. Mature apothecium of carrot isolate.

Fig. 14. Vertical section of apothecium. Scale bar = 100 μm. Abbreviations; sh = subhymenium, me = medullary excipulum, ee = ectal excipulum.

Fig. 15. Vertical section of apothecial stipe. Note the ectal layer peeled off the medullary portion of stipe, giving shaggy appearance to stipe surface. Scale bar = 200 μm.

Fig. 16. Vertical section of apothecium at margin. Scale bar = 10 μm.

Fig. 17. Asci. Scale bar = 10 μm. Note apical pore apparatus stained with Melzer's reagent in pretreatment with 2% KOH.

Fig. 18. Nuclei in ascospores. Scale bar = 10 μm.

60–100 μm thick, *textura prismatica* or *textura globulosa*, composed of hyaline to light brown, thin-walled globose cells, 7–26×8–32 μm; asci cylindrical, slightly attenuate toward the base, eight-spored, 114–144×6.7–9.2 μm, apex rounded, without pretreatment in 2% KOH apical pore apparatus weakly J+ or J−, with KOH apical pore apparatus apparently J+; ascospores one-celled, hyaline, ellipsoid, non-guttulate, binucleate (Fig. 18), 9.2–1.7×3.8–5.0 μm; paraphyses hyaline, filiform, septate, 90–140×1.6–2.5 μm. Ectal excipulum of stipe *textura prismatica* or *textura globulosa*, composed of light brown thin-walled cells, 8–24 μm in diam, outermost cells often grew to form tomentose hypha. Medullary excipulum of stipe *textura porrecta*, composed of thin-walled, elongated hyaline cells, 8–16 μm wide, arranged parallel to the stipe axis.

Sclerotia entirely covered with black rind of *textura prismatica*, composed of melanized thick walled cells, 6–14 μm in diam. The sclerotia produced naturally on host plants have a thick rind layer of 6–10 cells, of which the outer 4–6 cells are less melanized than the inner 2–4 cells. Cortex of *textura prismatica*, composed of 5–7 non-melanized cells, was seen below the melanized rind layer (Fig. 6). Such zonation in the cortical region of sclerotia was not seen in those produced on PDA, where the rind layer was composed of 2–6 cells (Fig. 7). Sclerotial medulla is *textura oblita* of densely interwoven hyphal cells, 3.0–10.5 μm wide. The medullary hyphae are enveloped with a very thickened gelatinous layer, 2.5–5 μm thick. When the resin-embedded sclerotial sections were mounted in a buffered toluidine blue solution on a glass slide, covered with a cover-slip without washing, the unstained or weakly stained gelatinous layers were clearly seen emerging in the dye-background (Fig. 9). The layers of individual cells mostly do not fuse together, and remarkable intercellular spaces (arrows in Fig. 9) and the boundaries between the cells are therefore visible in the medullary tissue.

Microconidia, in culture, are globose, 2–3 μm in diam, produced on phialides (Fig. 11).

Habitat: Parasitic on leaves, petioles, roots and root crowns of edible burdock (*Arctium lappa* L.), carrot (*Daucus carota* L.), *Angelica acutiloba* Kitagawa, *Plantago lanceolata* L., *Ajuga reptans* L., *Ambrosia elatior* L., *Chrysanthemum morifolium* Ramat., causing snow-mold disease.

Cultural characteristics Colony appearance was observed with isolates from carrot (Fig. 5), edible burdock and *A. acutiloba*, grown on different agar media at 20°C. All three isolates grew well on PDA, oatmeal agar, oatmeal-yeast extract agar, and malt extract-peptone-dextrose agar, while they gave poor or sparse growth on Czapek Dox solution agar and 5% malt extract agar. Colonies on PDA produced sparse aerial mycelium giving a powdery appearance, with small white flocci distributed among sclerotia. After 3 wk, many submerged hyphae became light brown and stained colonies pale olive-buff, mainly in the large center where sclerotia were not seen. Sclerotia 0.6 to 4 mm in diam were produced near the margin in a uniform distribution. They were spherical, subspherical, elongated or fused to form irregular shapes and tightly attached to the agar surface by their under surface, which could be seen through the bottom of the Petri dishes. No haptera or delicate black membrane were seen, both of which *S. trifoliorum* often forms on the edge of colonies (Keay, 1939). On malt extract-peptone-dextrose agar, colonies showed felty appearance and were olive-buff or light grayish olive in color. Sclerotial initials were developed on the colony in similar density to those on PDA, but they mostly remained immature with felty surface and were more or less flat in shape and deep grayish olive or dark olive gray in color. Haptera and the delicate black membrane often developed from the edge of the colony toward the glass wall of the Petri dish. The colony appearance on oatmeal agar was basically similar to that on PDA except that the aerial mycelium was produced more profusely than on the latter substrate, especially in the center of the colony at the earlier stage prior to sclerotial formation. Sclerotia produced on such areas rich in aerial mycelium were easily separated from the colony without any adhesion to the agar media. The amendment of yeast extract to oatmeal agar appeared to stimulate production of aerial mycelium. Sclerotia were not clearly seen from the bottom of Petri dish. It was noteworthy that the organism produced small spherical sclerotia, 0.3–0.5 mm in diam, on aerial mycelium quite freely from the substrate (Fig. 10). The small sclerotia have the same internal structures as larger non-aerial sclerotia (Fig. 8).

Temperature response of mycelial growth Figure 19 shows the radial growth of mycelium on PDA. All three isolates showed no growth at 30°C but gave minimal growth at 5°C. They grew well at 15 and 25°C and most rapidly at 20°C. At 10°C, mycelial growth of all isolates was slower than that at 15 or 25°C. From these results, *S. nivalis* appears to have an optimal growth temperature of around 20°C.

Electrophoretic studies The result of SDS-PAGE of sclerotial proteins is shown in Fig. 20. For *S. sclerotiorum*, *S. minor*, and *S. trifoliorum* used as reference taxa, the present data confirm the previous studies (Tariq et al., 1985). Thus, one major band was seen for each of these three species of *Sclerotinia*. *Sclerotinia minor* was clearly distinguished from the other two species by the major band with estimated molecular mass of 35 kDa and one detectable band near the running-front. For *S. sclerotiorum* and *S. trifoliorum*, the single major protein bands were close each other, with estimated molecular masses of 36 kDa and 36.5 kDa, respectively. These results were fairly reproducible. For *S. nivalis*, three major bands of proteins were observed for each of the isolates from carrot, edible burdock, and *A. acutiloba*; the molecular mass of these bands were estimated at 35.5, 39 and 41 kDa. Thus, *S. nivalis* could be distinguished from *S. sclerotiorum*, *S. trifoliorum* and *S. minor* by the number and the molecular mass of major protein bands on SDS-PAGE.

The electrophoretic patterns of esterase isozymes in sclerotial extracts of *S. nivalis*, *S. minor* and *S. trifolio-*

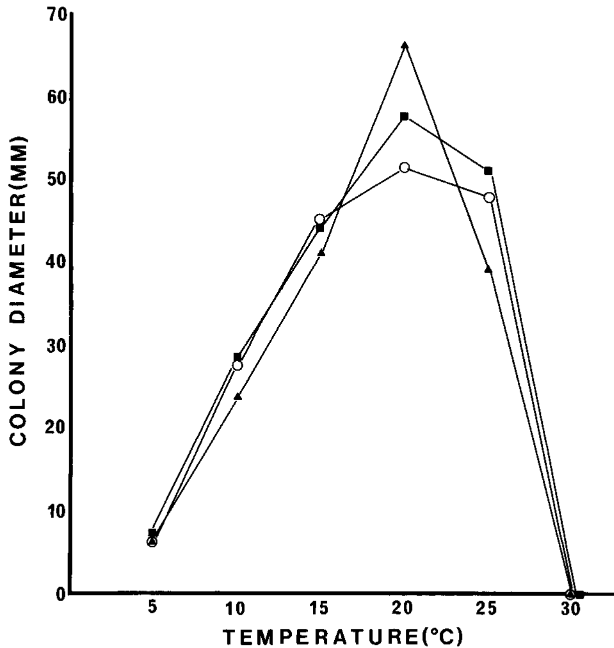


Fig. 19. Average colony diameter of *S. nivalis* after incubation for 3 d at different temperatures.
▲, isolate from carrot; ○, isolate from edible burdock; ■, isolate from *Angelica acutiloba*.

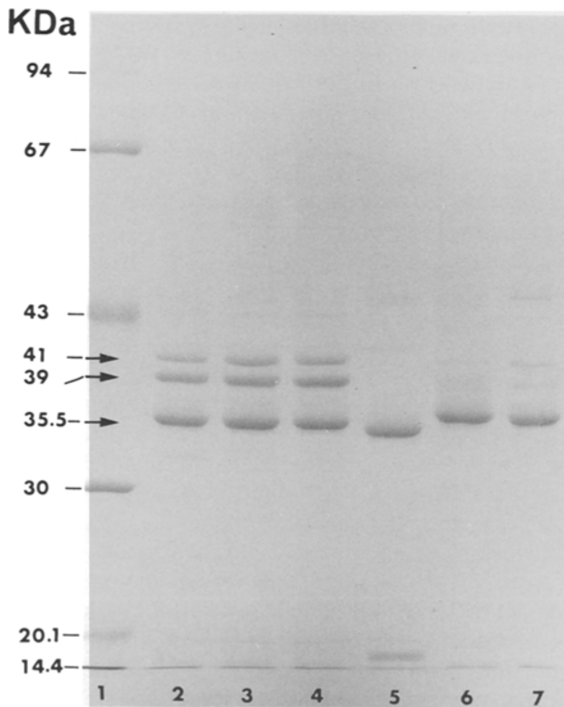


Fig. 20. SDS-polyacrylamide gel electrophoresis of total proteins of sclerotia of *Sclerotinia* spp.
Lane 1, molecular mass standard; lane 2, *S. nivalis* isolate from carrot; lane 3, *S. nivalis* isolate from edible burdock; lane 4, *S. nivalis* isolate from *Angelica acutiloba*; lane 5, *S. minor*; lane 6, *S. trifoliorum*; lane 7, *S. sclerotiorum*.

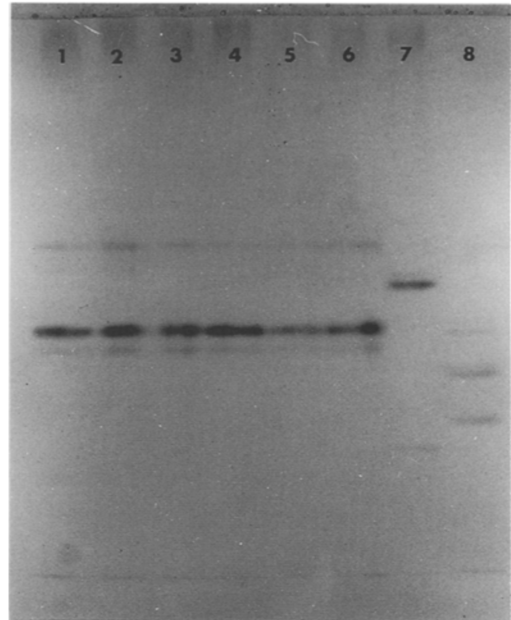


Fig. 21. Patterns of esterase isozymes in the extract of sclerotia of *Sclerotinia* spp. after polyacrylamide gel electrophoresis.
Lane 1, *S. nivalis* isolate from edible burdock; lane 2, *S. nivalis* isolate from *Ajuga reptans*; lane 3, *S. nivalis* isolate from *Chrysanthemum morifolium*; lane 4, *S. nivalis* isolate from *Plantago lanceolata*; lane 5, *S. nivalis* isolate from *Ambrosia elatior*; lane 6, *S. nivalis* isolate from *Angelica acutiloba*; lane 7, *S. minor*; lane 8, *S. trifoliorum*.

rum are shown in Fig. 21. The banding patterns obtained were quite different between three species. *Sclerotinia trifoliorum* showed three faint bands; *S. minor*, one prominent band and one faint band which moved faster; and *S. nivalis*, one prominent band with intense enzyme activity and two faint bands. All six isolates of *S. nivalis* had identical patterns. This enabled me to identify the two anamorphic isolates from *A. reptans* and *C. morifolium* with *S. nivalis*.

Discussion

I concluded from this study that the most appropriate taxonomic position of the present new species is in the genus *Sclerotinia*. This genus was recently delimited on the basis of morphology to include only three closely related species, *S. sclerotiorum*, *S. minor*, and *S. trifoliorum* (Kohn, 1979). The morphological characters which support this ascription of the present species are its tuberoid sclerotia produced superficially on suscepts or on agar media, the internal structures of the sclerotia, and the globose cells constructing the ectal excipulum of apothecia. While *S. nivalis* does not incorporate the remnants of suscept tissue within sclerotia, it usually forms sclerotia close to agar in PDA culture, which sometimes makes it difficult to detach sclerotia from the colony without adhesion of agar. This is not usual in *S. sclerotiorum*, *S. minor*, and *S. trifoliorum*. However, in

S. nivalis, the adhesion of sclerotia and substrate varies with nutritional conditions; in medium suitable for production of aerial mycelium, such as oatmeal agar enriched with yeast extract, mature sclerotia are easily detached without any adhesion of agar. On the other hand, *S. nivalis* produces small sclerotia on aerial mycelium in the colony where larger sclerotia have already been produced. This situation agrees well with the generic features of *Sclerotinia* (Kohn, 1979), as there are no structural differences between these two types of sclerotia. Regarding the internal structure of sclerotia, medullary hyphae are not embedded in a continuous gelatinous matrix but individually enveloped with thickened gelatinous layers, probably composed of β-1,3 glucans (Saito, 1977), which do not fuse with each other and thereby reveal remarkable inter-hyphal spaces in the medullary tissue. This is an important general feature of *Sclerotinia* (Saito, 1977; Arseniuk and Macewicz, 1992), distinguishing it from another group which produces superficial tuberoid sclerotia, *Botrytis*.

Sclerotinia nivalis can be distinguished by its intermediate sized sclerotia (0.6–4 mm in diam) in culture from the large sclerotium forming species, *S. sclerotiorum* and *S. trifoliorum*, and by its binucleate ascospores from *S. minor*, which forms tetranucleate ascospores (Kohn, 1979). *Sclerotinia nivalis* is further distinguished by SDS-PAGE of sclerotial proteins from other species of *Sclerotinia* in terms of three major proteins of the former, estimated at 35.5, 39 and 41 kDa in molecular mass respectively. Consequently, the anamorph state of *S. nivalis* can be easily identified based on the existence of these three major proteins in sclerotia.

The diseases caused by *S. nivalis* are always of the snow-mold type in all host plants previously reported (Tochinai and Sugimoto, 1958; Sugimoto et al., 1962; Narita, 1980), as well as those described here. Of these plants, root-crops such as carrot and edible burdock showed crown rot symptoms which extend to root rot and foliage death. While the disease occurs at low temperature, *S. nivalis* is a mesophilic fungus which has an optimum temperature for mycelial growth in culture of around 20°C. The similar pathogenic and cultural properties to those of *S. nivalis* are known in *Microdochium nivale* (Fr.) Samuels et Hallett, the pathogen of pink snow mold of winter cereals and grasses (Booth, 1971 – as *Fusarium nivale* (Fr.) Ces.). The latter fungus, however, can attack all plant parts causing leaf blight and scab, etc. during the cool, wet period from spring to autumn in Hokkaido (Miyajima and Saito, 1984) and elsewhere (Cook and Bruehl, 1968). While the season of apothecial production by *S. nivalis* in nature is unknown, its fruiting period might be in autumn, because the disease progresses in winter. Considering the inertness in fruiting of *S. nivalis* in the laboratory, the snow mold caused by this fungus is likely to be disseminated by myceliogenic germination of sclerotia in soil rather than by air-borne ascospores. In inoculation experiments with carrot roots in unsterilized soil, sclerotia of *S. nivalis* could germinate myceliogenically and infected carrot during incubation at 0°C (unpublished data). This supports the possibility of soil-borne infection of the organism under snow. Because of the decrease in suppressive activities of most soil microorganisms at temperatures around 0°C, these conditions appear to be suitable for myceliogenic germination of sclerotia of *S. nivalis* as a low-temperature-tolerant mesophile.

Sclerotinia nivalis is pathogenic to many herbaceous dicots belonging to four genera of Compositae, two of Umbelliferae, and one genus each of Labiatae, Plantaginaceae and Cruciferae. In view of its wide host range, *S. nivalis* has great potential to become an economically important plant pathogen. Consequently, it is necessary to study the mechanism of its dissemination.

Acknowledgements——I wish to thank Dr. K. Takaoka (former president of Hokkai Sankyo Co. Ltd.) for his continuous support and encouragement of my research.

I also thank Prof. Y. Harada (Hirosaki University) for his critical review of the manuscript, and Dr. K. Yagi (Hokkai Sankyo Co. Ltd., Agroscience Research Laboratory) for his useful suggestions on the electrophoretic study, and Mr. S. Saito(Tochigi Prefecture, Department of Agriculture) for providing isolates of *S. minor* and Dr. N. Matsumoto (National Institute of Agro-Environmental Sciences), Dr. O. Tamura (Hokkaido Prefectural Tokachi Agricultural Experiment Station) and the late Dr. A. Tanii for their assistance in collection of the sclerotial anamorph.

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