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Non-gramineous hosts of *Myriosclerotinia borealis**

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On the basis of cultural, anatomical, and electrophoretic studies, *Myriosclerotinia borealis* (=*Sclerotinia borealis*) is shown to occur on cultivated non-gramineous plants including *Iris ensata* var. *hortensis* (Japanese iris), *I. pseudoacorus*, *I. hollandica* (Dutch iris), Perko PVH (a hybrid green manure crop between *Brassica campestris* and *B. chinensis*), *Allium fistulosum*, and *Campanula portenshlagiana*. The fungus did not kill these plants, but produced functional sclerotia, capable of carpogenic germination, on decayed leaves or necrotic lesions of overwintered leaves. The fungus seems to act as a saprophyte colonizing senescent leaves and/or as a weak parasite on plants injured by freezing during winter. In culture, the fungus produces discrete tuberoid sclerotia closely attached to the agar surface; rind differentiation is poor on the under surface of sclerotia. Medullary cells are embedded in a gelatinous matrix showing no distinct intercellular spaces. The ectal excipulum of apothecia produced under artificial conditions is composed of globose cells. *Myriosclerotinia borealis* is thus shown to be very close to *Ciborinia* on the basis of these sclerotial and apothecial characters.

Key Words——*Myriosclerotinia borealis*; non-gramineous hosts; *Sclerotinia borealis*; snow mold.

The snow mold *Myriosclerotinia borealis* (Bub. & Vleug.) Kohn (=*Sclerotinia borealis* Bub. & Vleug.), a destructive pathogen of winter cereals and forage grasses, has been reported from several countries in the northern hemisphere (Jamalainen, 1949; Groves and Bowerman, 1955; Tomiyama, 1955; Bruehl, 1982). This psychrophilic fungus has a lower optimum temperature than other snow mold fungi (Ward, 1966) and can develop on slightly frozen ground under snow. In Japan, the fungus is found in the less snowy areas in eastern and north-eastern Hokkaido, where snow comes later in winter and the depth of ground freezing is larger than the other areas of Hokkaido (Tomiyama, 1955).

Dactylis glomerata L. (orchard grass) was first described as typical host of the fungus (cited in Kohn, 1979). Later, Jamalainen (1949) reported 15 species in 11 genera of Gramineae from Finland. Groves and Bowerman (1955) studied isolates from 20 gramineous hosts in 11 genera from Canada, adding 13 species to those listed by Jamalainen. There have been no reports of hosts in families other than the Gramineae. The new combination of the fungus in the genus *Myriosclerotinia* by Kohn (1979) was based on this limited host range and on the close similarity in sclerotial formation on hosts to the cypericolous- and juncicolous species which were originally included in the genus. Recently, however, *M. borealis* was treated as an imperfectly known taxa in a revisionary monograph of the genus *Myriosclerotinia* (Schumacher and Kohn, 1985).

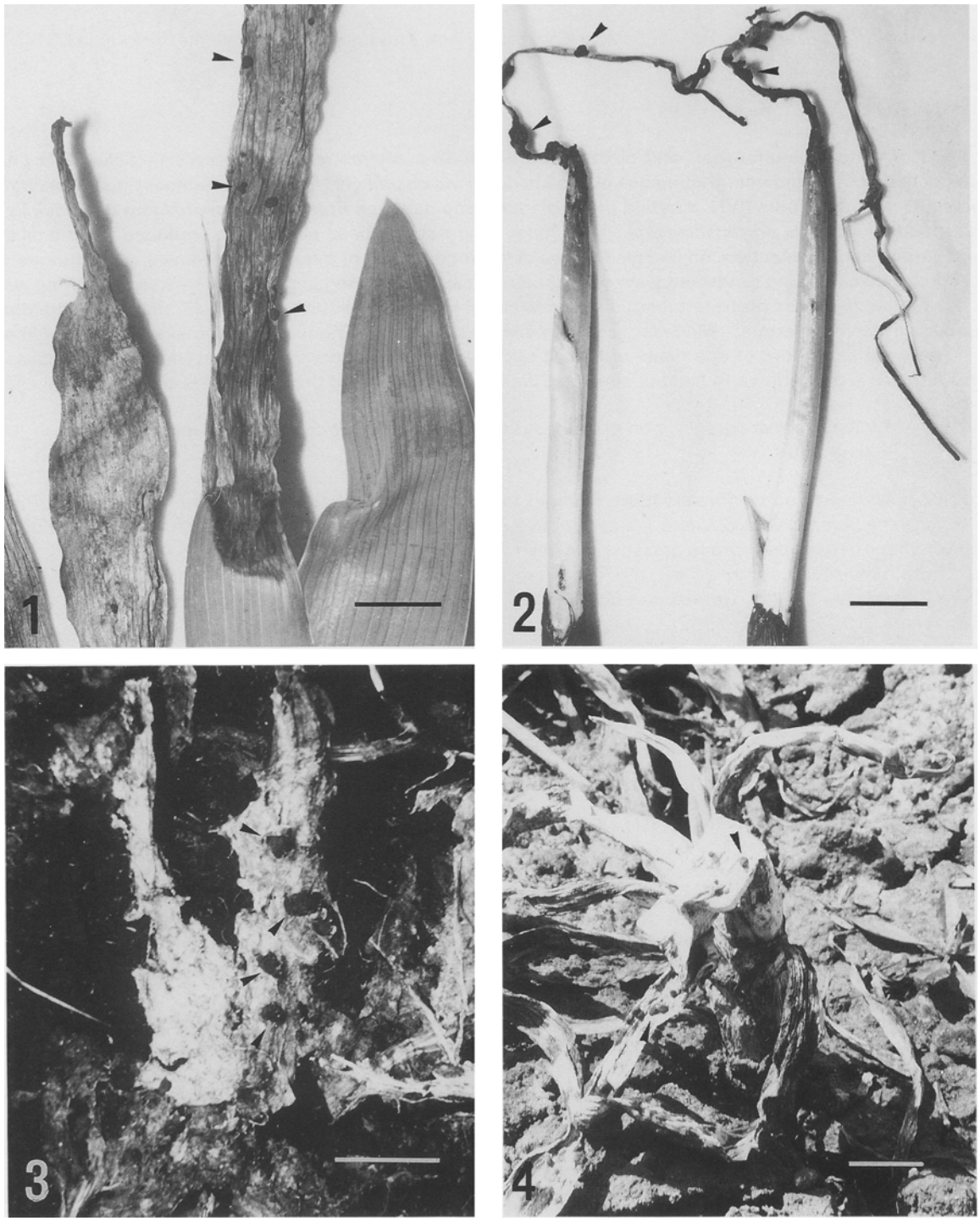
During the study of a *Sclerotinia* species previously reported as "*S. intermedia*", the snow mold pathogen of herbaceous dicots in Hokkaido (Saito, 1997), I noted that some sclerotial anamorphs collected from dead suscepts never outgrew on isolating medium at 20°C. I found that they were psychrophiles which could grow at around 0°C and identified them with *M. borealis*. The purpose of this study is to report the non-gramineous hosts of the fungus and the results of comparative study of non-gramineous isolates with those from gramineous hosts with respect to cultural, anatomical and electrophoretic evidence.

Materials and Methods

Collection and isolation From 1980 to 1985, sclerotia were collected from decayed leaves or stems of plants growing in experimental fields, a herb garden, and perennial flower gardens in the Hokkaido Prefectural Kitami Agricultural Experiment Station, Kunnep, Hokkaido in the spring following snow melt. Sclerotia were surface-sterilized in a 0.5% (as active chlorine) sodium hypochlorite solution and thoroughly washed with sterilized distilled water. They were then cut with sterilized razor blades, placed on potato dextrose agar (PDA) so that the cut surfaces were in contact with the agar, and incubated at 0–3°C. Mycelium from growing margins of colonies was transferred to new PDA plates. All isolates were maintained on PDA slants at 0–3°C. Isolates from *D. glomerata* and *Triticum aestivum* L. (winter wheat) previously identified as *M. borealis* were used for comparison in this study.

*The major part of this study was carried out at the Hokkaido Prefectural Kitami Agricultural Experiment Station, Kunnep, Tokoro-gun, Hokkaido, Japan.

The effect of temperature on mycelial growth Centers of 9-cm Petri dishes containing 15 ml of PDA were inoculated with mycelial disks from colony margins of four non-gramineous isolates, as well as one from *D. glomerata*, grown on PDA at 5°C. Six plates each were incubated at 0, 5, 10, 15 and 20°C. Linear mycelial growth rate was determined by measuring the distance between the edge of the inoculum and the margin of the colony. The measurements were made in four directions per plate following the cross-lines drawn on the



Figs. 1–4. Non-gramineous plants infected by *Myriosclerotinia borealis* showed sclerotia produced on the decayed leaves (arrowheads). 1. *Iris germanica*; 2. *I. hollandica*; 3. Perko PVH (a hybrid green manure crop between *Brassica campestris* and *B. chinensis*); 4. *Allium fistulosum*. Scale lines indicate 20 mm.

reverse of the plates. Data are expressed as the average of the measurements of six plates.

Apothecial production Natural sclerotia collected in spring or obtained from barley grain culture were used. Barley grains were boiled in water for 30 min, then autoclaved in cotton-plugged Erlenmeyer flasks for 20 min at 120°C. The grain medium was then inoculated with agar disks containing mycelium and incubated at 0–3°C. Polyurethane sponges were slit about 2 mm deep at intervals of 10 mm, water-soaked, then autoclaved in deep Petri-dishes (90 × 90 mm). Sclerotia were harvested from about 1 mo-old cultures and inserted into the slits in the sponges. Natural sclerotia were surface-sterilized and seeded in sponges as described above. The dishes were maintained at 20°C, 50 cm below three fluorescent lamps (20 W, daylight type).

Light microscopy of sclerotia and apothecia Sclerotia produced on PDA plates were cut into small blocks (1.5 × 2.0 mm) including rind layers. Apothecia were cut into median longitudinal sections of approximately 2 mm thick with razor blades. All sclerotial and apothecial tissues were fixed and stored in 5% glutaraldehyde buffered with 1/15 M phosphate buffer, pH 7.0, in a refrigerator (2–3°C). The fixed materials were then washed, dehydrated with an acetone series, and embedded in Epon 812 resin in aluminium-foil trays. After polymerization, specimens were trimmed and sectioned vertically with glass knives on a Porter Blum Mt-1 ultramicrotome. Sections 1–2 μm thick were mounted on glass slides coated with glycerine gelatin (Jensen,

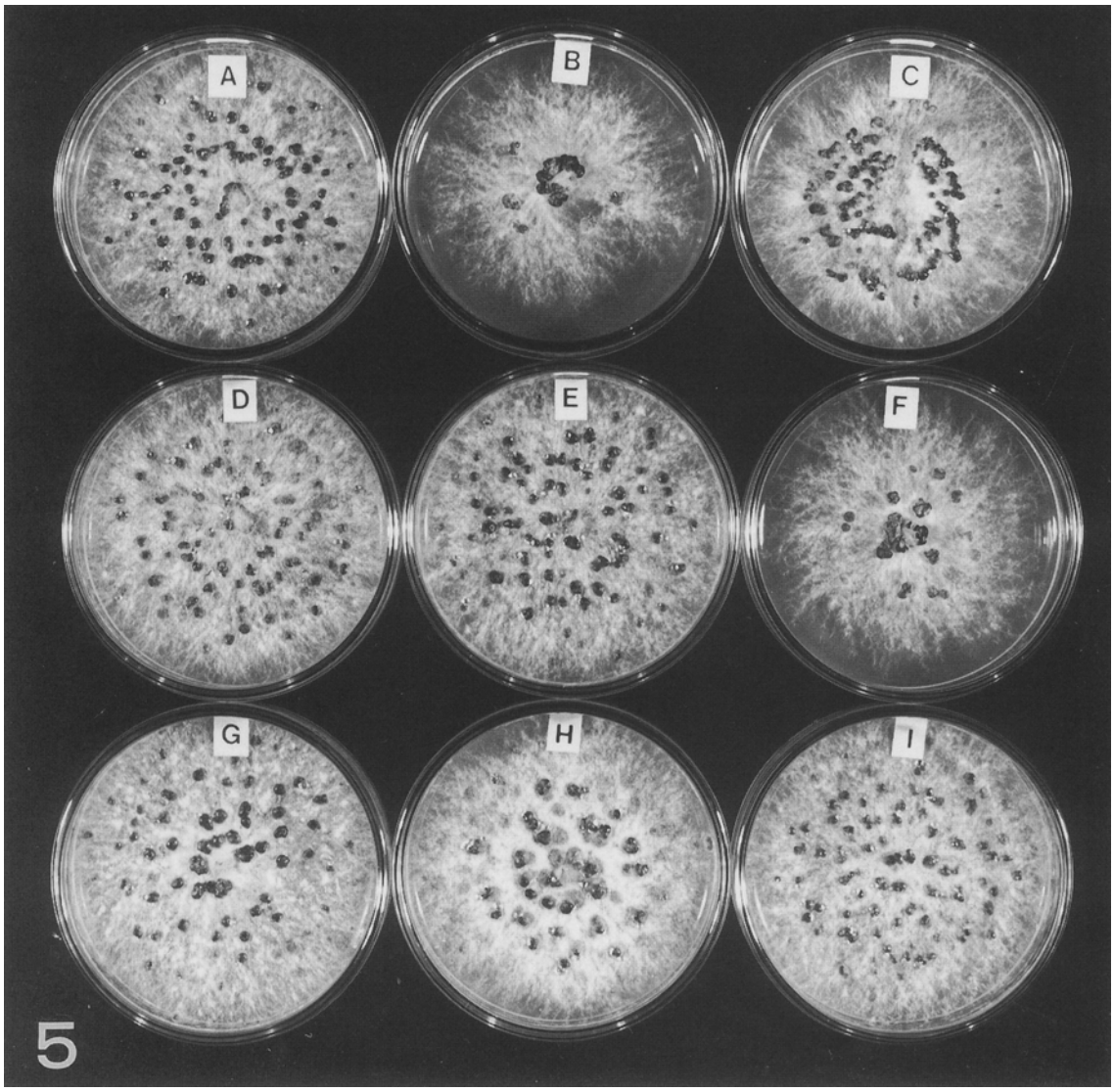


Fig. 5. Cultures of *M. borealis* isolates from various hosts on PDA in 6-cm Petri-dishes, grown at 5°C for 4 wk.
Hosts: A. *Triticum aestivum*; B. *Dactylis glomerata*; C. *Allium fistulosum*; D. *Iris hollandica*; E. *I. germanica*; F. *I. pseudoacorus*; G. *I. ensata* var. *hortensis*; H. Perko PVH; I. *Campanula portenshulagiana*.

1962). Sections were then stained with a solution of methylene blue and azure II, post-stained with basic fuchsin according to the method of Benell et al. (1978), air-dried and mounted with Eukitt (O. Kindler), and observed under a light microscope.

Staining of nuclei in ascospores Hymenial portions of mature apothecia were fixed in a 9 : 6 : 3 mixture of *n*-butanol, glacial acetic acid, and 10% (w/v) chromic acid solution for 3 d at 3–5°C (Lu, 1962). The fixed specimens were then crushed on a glass slide, hydrolyzed in a drop of a 1 : 1 mixture of concentrated HCl and ethanol while gently heating over a burner, rinsed with propionic acid solution, and stained with a mixture of 2% (w/v) hematoxylin and 5% (w/v) iron alum in 50% (v/v) propionic acid solution (Henderson and Lu, 1968).

Polyacrylamide gel electrophoresis (PAGE) Isolates were grown in 300-ml Erlenmeyer flasks containing 75 ml of a defined medium (KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4$, 0.01 g; asparagine, 2.0 g; dextrose, 20 g; Bacto yeast extract, 1 g; distilled water, 1,000 ml) at 2–3°C. For this liquid culture, a floatable inoculum was prepared; small pieces of polyurethane sponges ($7 \times 7 \times 2$ mm) soaked with the liquid medium were put around the growing margin of the colonies on PDA plates, and sponges impregnated with mycelium were transferred into the flasks. After about 3 wk of incubation, the mycelial mats bearing sclerotia were scraped off the sponges, placed on filter paper in glass funnels, then washed 10 times in distilled water. Excess water was removed by blotting with filter paper. Mycelial mats were homogenized in a chilled mortar and pestle after adding a small amount of acid-washed quartz sand and 0.05 M phosphate buffer, pH 7.0. For each gram of fresh weight, 2 ml of the buffer was used. The homogenates were then centrifuged at 3°C for 20 min at $10,000 \times g$. The supernatant was stored in small glass tubes at −30°C; for the experiments, the supernatant was brought to 0°C and used within a few hours. The amount of proteins was determined by Lowry's method modified by Bensadoun and Weinstein (1976) with bovine serum albumin as the standard. PAGE was performed in polyacrylamide slabs ($1.5 \times 140 \times 170$ mm) composed of a 2.5% spacer gel at pH 6.7 and resolving gel at pH 8.6. An equal volume of the phosphate buffer containing 20% (w/v) glycerin was added to the supernatant, then 50-μl aliquots of these 1/2 strength samples were loaded into the gel slots. Electrophoresis was carried out at 0–3°C with a current of 20 mA for 3 h using Tris-glycine buffer, pH 8.6 as the electrode buffer. Protein concentration of aliquots in each slot varied from 13.4 (isolate from *D. glomerata*) to 33.1 μg (isolate from *T. aestivum*). Gels were then stained for protein in saturated Coomassie Brilliant Blue R-250 (Nakarai Chemicals) in 12% (w/v) TCA for 3 h and distained with 12% TCA over night. For the determination of esterase isozymes on PAGE, 30-μl aliquots of the diluted samples were loaded and run at 0–3°C with a current of 20 mA for 4 h. Isozymes were located by dipping slabs in a mixed solution (1/10 M phosphate buffer, pH 5.5, 50 ml; 1-naphthyl acetate, 25 mg; Fast Blue B salt, 40 mg) for 2 h at room temperature (Backhouse et al., 1984).

Results

Symptoms of plants after snow melt In the spring of 1980, following the snow melt, sclerotia were collected from *Iris ensata* var. *hortensis* Makino & Nemoto (Japanese iris) and Perko PVH (a hybrid green manure crop between *Brassica campestris* L. and *B. chinensis* L.). In 1982, sclerotia were collected from *I. germanica* L. (German iris) and *Campanula portenshlagiana* Roem. & Schit. In 1985, sclerotia were collected from *Allium fistulosum* L.

Sclerotia were often collected from dead leaves or stems of overwintered plants; despite the obvious infection, these plants survived the winter and began to grow vigorously from overwintering rhizomes in the spring, when temperature began to rise. This was especially obvious in *I. ensata* var. *hortensis* and *I. pseudoacorus* L. In contrast, necrotic lesions appearing at the same time as sclerotial formation were occasionally found on living leaves of *I. germanica* and *I. hollandica* hort. (Dutch iris) (Figs. 1, 3). The enlargement of the lesions appeared to cease with the increase in temperature. In *A. fistulosum*, though all of the plants survived from their leaf sheaths, most of the leaf blades were living immediately prior to the first snow but they were found to be decayed and infested with sclerotia at snow melt in early spring (Fig. 4). In Perko PVH, sclerotia were often found on old dead leaves (Fig. 2). Although most individuals of Perko PVH survived and grew to bloom, some of them were found to be dead. However, whether cold injury or infection by *M. borealis* caused the death is obscure. Although dicotyledonous plants in this area are often killed by *Typhula* species, the type of sclerotium produced suggested that there was no infection by these species.

Cultural appearance of isolates On PDA, white mycelium develops with little aerial growth. The colonies of 7 non-gramineous isolates were similar in appearance to those of isolates from gramineous hosts which were previously identified as *M. borealis* (Fig. 5). Sclerotial initiation and maturation occurred as the colonies continued to grow vegetatively. Typically there were no patterns of sclerotial distribution on mature colonies; although sclerotia occasionally coalesced around the inoculum in some isolates, this was not a consistent character. A consistent character was the formation of sclerotia close to the agar surface, so that it was difficult to detach them without agar. It is noteworthy that transfer of the fungus with mycelium from old stock cultures often produces abnormal colonies which formed darkly pigmented, stroma-like structures partially submerging in agar medium. In general, this fungus seems to be variable in cultural characteristics even when maintained at low temperature. However, re-isolation from sclerotia in old cultures often avoids abnormalities in the subculture. In addition, when inoculum was taken from the inside of a colony, mycelial growth was often abnormal or retarded, as pointed out by Ward (1966).

Effect of temperature on mycelial growth The growth of all isolates from four non-gramineous hosts and *D. glomerata* was totally inhibited for their growth at 20°C (Fig. 6). The slowest growth of colonies was observed at 0°C for all isolates, but this temperature was not totally inhibitory because the isolates grew throughout the experiment. The isolates differed slightly in their response to temperature from 5 to 15°C, but all appeared to have an optimum temperature of 5°C. Thus, all isolates from four non-gramineous plants as well as isolate of *M. borealis* on *D. glomerata* were apparently psychrophilic, though the optimum temperature for growth was higher than that reported by Ward (1966).

Morphology of sclerotia and apothecia Sclerotia naturally produced on various non-gramineous plants were black, round to elongated with flat under-surfaces, but mostly irregularly shaped, 2–6×2–4 mm. In the sclerotia produced in vitro, the dorsal rind was composed of 2 or 3 layers of semiglobose or brick-shaped cells, 4–16 μm in diam (Fig. 7). It is noteworthy that the rind is thinner than that of *Sclerotinia sclerotiorum* (Lib.) de Bary and *S. minor* Jagger. The pigmentation was often partial on individual cells, usually in the upper wall. Some rind cells retained their contents. The vental rind appeared to be poorly developed in contrast to the dorsal rind (Fig. 8). The medulla was composed of tightly interwoven

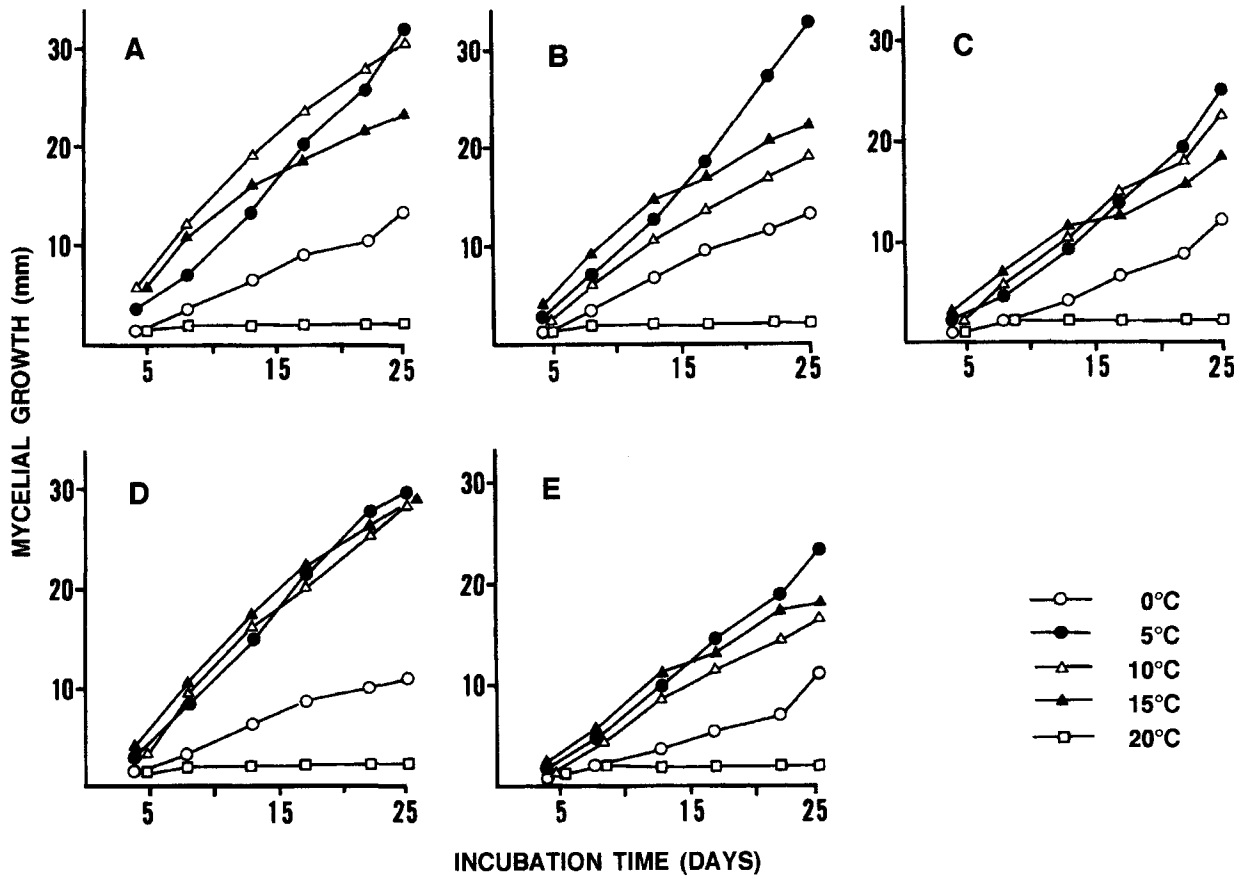
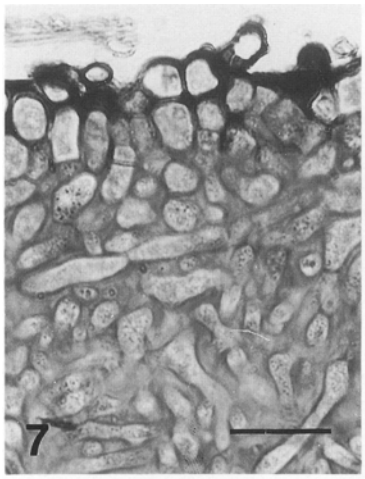


Fig. 6. Effect of temperature on the mycelial growth of *M. borealis* isolates from various hosts.
Hosts: A. *Dactylis glomerata*; B. Perko PVH; C. *Iris pseudoacorus*; D. *I. ensata* var. *hortensis*; E. *I. hollandica*.

Table 1. Dimension of asci and ascospores, and numbers of ascosporic nuclei of isolates of *M. borealis* from various host.

Hosts	Asci[a)]		Ascospores[a)]		Number of nuclei (average)
	Length (μm)	Width (μm)	Length (μm)	Width (μm)	
Dactylis glomerata	207.7±1.66	10.90±0.113	18.92±0.202	7.43±0.057	6–8 (7.2)
Iris pseudoacorus	197.7±1.87	11.40±0.149	18.45±0.166	7.42±0.046	6–8 (7.3)
I. hollandica	204.3±1.64	10.65±0.125	18.21±0.157	7.53±0.066	6–8 (7.2)
I. ensata var. *hortensis*	215.7±8.07	10.76±0.167	18.39±0.164	7.52±0.064	6–8 (7.6)
Perko PVH	203.4±1.43	10.88±0.124	18.52±0.172	7.34±0.07	6–8 (7.1)

a) Average±SD.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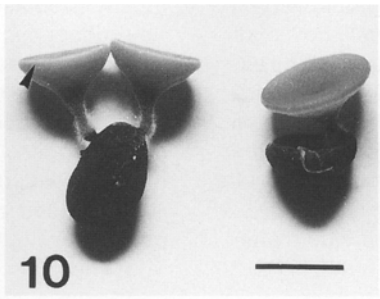
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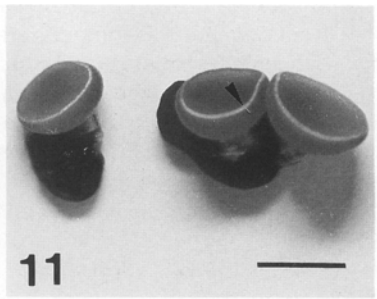
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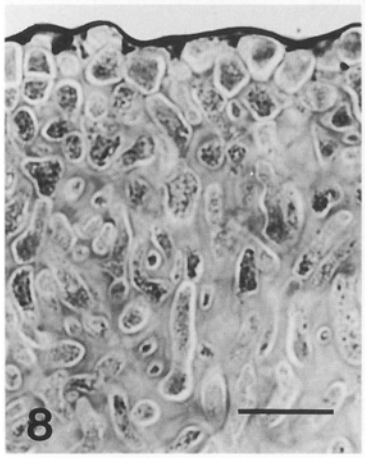
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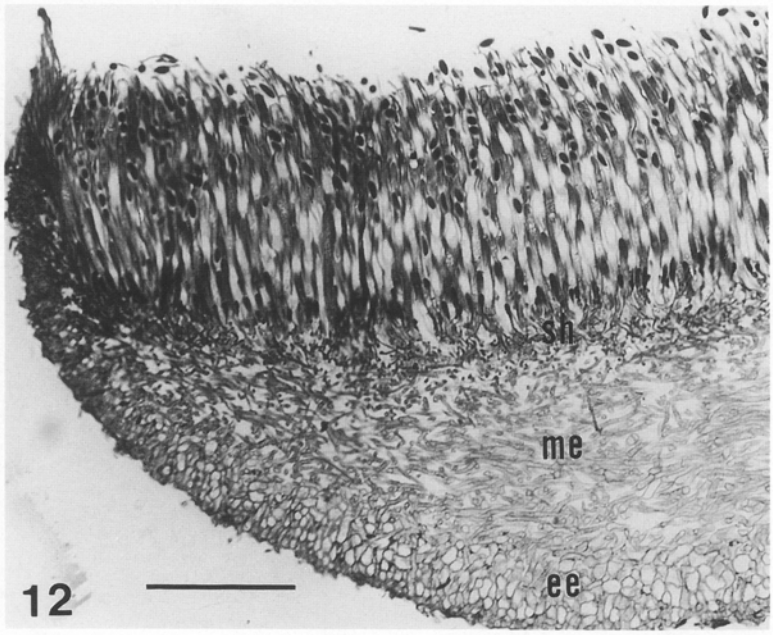
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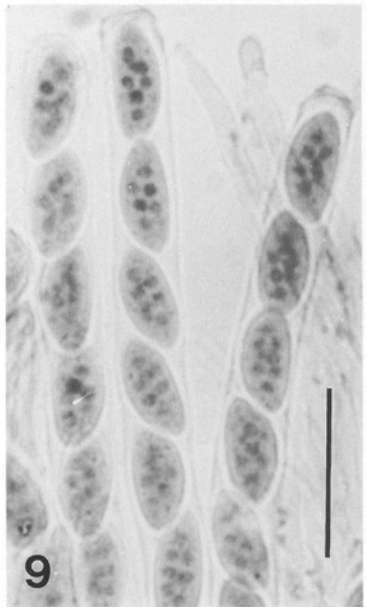
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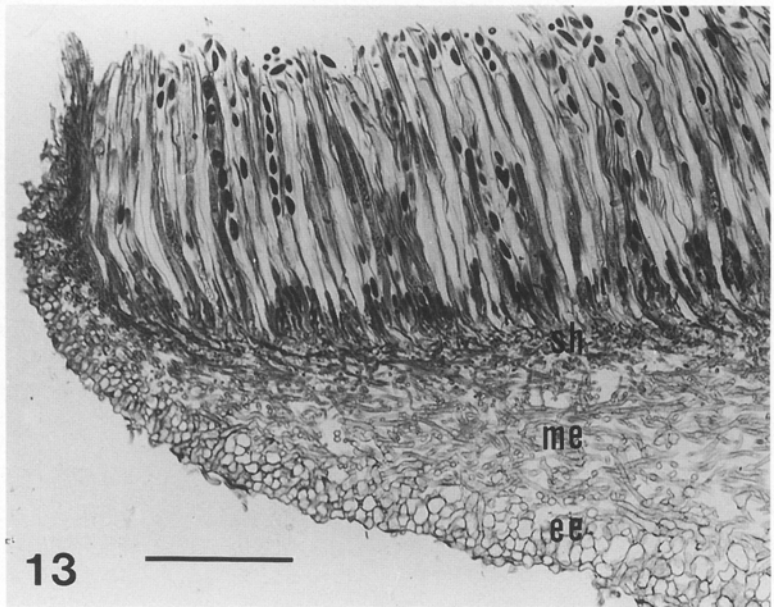
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filamentous hyphae embedded in a gelatinous matrix, so that there were no intercellular spaces between the medullary hyphae. Under the conditions of this study, both sclerotia gathered from the field and cultures in vitro formed apothecia arising singly or in a group. They were cup-shaped, bister coloured (Ridgway, 1912), 2–3 mm in diam with stipes 1–3 mm long. Some apothecia produced a narrow white zone around the apothecial margin (Figs. 10, 11). Apothecial anatomy of isolates from four non-gramineous hosts, Perko-PVH, *I. pseudoacorus*, *I. ensata* var. *hortensis*, and *I. hollandica*, was basically similar to that of *M. borealis* from *D. glomerata*; there was clear tissue-differentiation in the non-hymenial part of apothecia (Figs. 12, 13). The ectal excipulum at apothecial margin was composed of prosenchymatous cells, about 5 μm in width. A bundle of paraphyses often projected beyond the hymenium at apothecial margin (Figs. 12, 13). These projections probably corresponded with the white lines surrounding the apothecial margin that can be seen macroscopically. Whether this is a specific character of *M. borealis* or a variation in the apothecial morphogenesis resulting from the culture conditions is obscure. The medullary excipulum was composed of loosely interwoven, prosenchymatous cells, 4.5–7 μm in width (Figs. 12, 13). The subhymenium was composed of narrow, filamentous cells with dense cytoplasm, about 3 μm in width, forming young asci from croziers. The dimension of asci and ascospores of four non-gramineous plants were almost identical either with those of *M. borealis* from *D. glomerata* or with those previously described by Groves and Bowerman (1955) (Table 1); the asci were 180–250 × 10–12.5 μm and ascospores, 17.5–25 × 7–9 μ. Ascospores were multinucleate as previously reported by Björling (1952); six to eight nuclei were counted in an ascospore for each isolate (Table 1).

Electrophoretic study Seven isolates obtained from non-gramineous hosts, *I. ensata* var. *hortensis*, *I. pseudoacorus*, *I. hollandica*, *I. germanica*, Perko PVH, *C. portenshulagiana*, and *A. fistulosum* were examined, and two isolates from *D. glomerata* and *T. aestivum* were used for comparison. PAGE of soluble proteins of mixed extracts from mycelia and sclerotia showed that all isolates were identical regarding the position of four major bands, though there were some differences in the numbers of minor bands (Fig. 14). Similar minor differences were seen between two isolates from typical gramineous hosts: the isolate from *D. glomerata* appeared to lack two minor bands compared with the isolate from *T. aestivum*. For esterase isozymes, the position of two major bands was identical for all isolates though there were some differences in intensity and number of minor bands; the

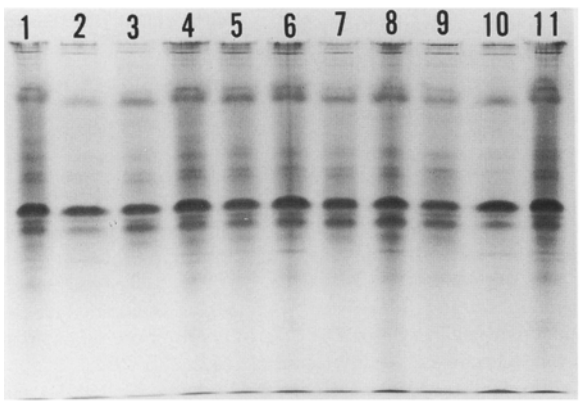

Fig. 14. Polyacrylamide gel patterns obtained by electrophoretic separation of total soluble proteins of 7 non-gramineous isolates of *M. borealis*, compared with isolates from *T. aestivum* and *D. glomerata*.
Lanes: 1 and 11. *T. aestivum*; 2 and 10. *D. glomerata*; 3. *Campanula portenshlagiana*; 4. *Allium fistulosum*; 5. *Iris germanica*; 6. *I. hollandica*; 7. *I. ensata* var. *hortensis*; 8. Perko PVH; 9. *I. pseudoacorus*.

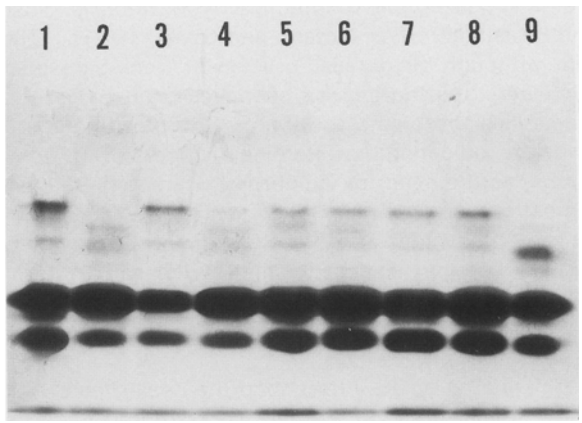

Fig. 15. Patterns of esterase isozymes in 7 non-gramineous isolates of *M. borealis* after polyacrylamide gel electrophoresis, compared with isolates from *T. aestivum* and *D. glomerata*.
Lanes: 1. *D. glomerata*; 2. *C. portenshlagiana*; 3. *T. aestivum*; 4. *I. germanica*; 5. I. *hollandica*; 6. *I. ensata* var. *hortensis*; 7. Perko PVH; 8. *I. pseudoacorus*; 9. *A. fistulosum*.

Figs. 7, 8. Vertical section of mature sclerotium of *M. borealis*. Abbreviations: M, medulla; R, rind.
7. The upper-surface of a sclerotium of the isolate from *D. glomerata*. 8. The under-surface of a mature sclerotium of the isolate from Perko PVH. Scale lines indicate 25 μm.

Fig. 9. Multinucleous ascospores in asci. Scale line indicates 25 μm.

Figs. 10, 11. Mature apothecia of *M. borealis*. Note white lines around the apothecial margin (arrowheads).
10. Isolate from *D. glomerata*. 11. Isolate from *I. pseudoacorus*. Scale lines indicate 3 mm.

Figs. 12, 13. Vertical section of a mature apothecium of *M. borealis*. Abbreviations: sh, subhymenium; me, medullary excipulum; ee, ectal excipulum.
12. Isolate from *D. glomerata*. 13. Isolate from Perko PVH. Scale lines indicate 50 μm.

isolate from *D. glomerata* appeared to be lacking one band (Fig. 15).

Discussion

Although *M. borealis* is typically pathogenic to gramineous plants, the present investigation reveals that the fungus can also act as a saprophyte and/or a weak parasite on some non-gramineous plants producing functional sclerotia to form apothecia. The involvement of dicotyledonous plants is of particular interest. On *I. ensata* var. *hortensis* and *I. pseudoacorus*, the fungus appears to be a saprophyte colonizing non-living leaves as substrates. These two irises are capable of overwintering by their rhizomes in cold districts, but their leaves gradually senesce prior to snow. Although there might be other saprophytes capable of colonizing senescent leaves of these plants, *M. borealis* probably can compete with them under winter conditions. On the other non-gramineous hosts, the fungus seems to act as a weak parasite because most leaves of the plants appeared to remain alive at least until they were covered by snow. Under cold conditions, previously healthy plants are subjected freeze stress. Even in the typical gramineous hosts of *M. borealis*, predisposition by freezing stress is the major factor in the initiation of the disease (Noshiro, 1980). Furthermore, it has been noted that wheat cultivar or grass species of least tolerance to freezing injury are most susceptible to infection by *M. borealis* (Noshiro and Hirashima,1978; Amano and Ozeki, 1981). Therefore, although the detailed process of weak-parasitism is not clear, freezing injuries of leaves or stems probably predispose the plants to infection by the fungus. The evidence indicating non-gramineous hosts of *M. borealis* reinforces the need for supporting characters in addition to host range when delimiting species. In fact, the host range of many species of the Sclerotiniaceae may be only partially known. Except for the few cases of putatively obligate parasitism (Whetzel, 1945), host range should not be used as a single character at species or genus rank.

Insel et al. (1985) described the coalesced development of sclerotia in *M. borealis* and stressed this as distinguishing the fungus from some sclerotium-forming, mesophilic fungi. In the present study, such coalesced sclerotial formation was also seen in some isolates, but this does not seem to be a consistent and inherent character of the fungus. Thus, the fresh isolates of *M. borealis* which have not undergone repeated subcultures usually produce discrete sclerotia scattered over the agar medium. *Myriosclerotinia borealis* seems to be variable regarding sclerotial formation under conventional storage conditions at low temperature around 0°C.

The structure of mature sclerotia of *M. borealis* differed in several ways from those of *Sclerotinia* sensu stricto (Kohn, 1979). The rind was thinner than that of the latter. Cells were frequently seen in which deposition of dark pigments did not occur throughout the wall. Such rind cells, which often retained their contents, presumably were still metabolically active. This was more pronounced on the under-surface of sclerotia, probably due to the close attachment of these sclerotia to the subtending mycelium and agar. A similar rind structure was observed in sclerotia of *Ciborinia allii* (Sawada) Kohn and a *Ciborinia* species on *Gentiana* (unpublished observation by Saito). In the medullary region, component cells were seen to be embedded in a gelatinous matrix, which is suspected to be β-1,3 glucans (Saito, 1974, 1977). Thus, in *M. borealis*, there are no intercellular spaces like those seen in the medulla of *S. sclerotiorum* and *S. trifoliorum* Erikks. (Saito, 1974; Arseniuk and Macewicz, 1992). Similar evidence was found in sclerotia of *Botrytis cinerea* Persoon (Willetts and Bullock, 1982) and of *Ciborinia* spp. (Saito, unpublished observation).

The expanded host range of *M. borealis* on leaves of non-gramineous, spring-flowering plants strongly suggests affinities with *Ciborinia*, a possible generic accommodation suggested by Schumacher and Kohn (1985). The studies reported here support the theory that the affinities of *M. borealis* are with the leaf-infecting species of *Ciborinia* rather than the culm-inhabiting species of *Myriosclerotinia*.

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