FULL PAPER

Mycelial growth of the snow mold fungus, *Sclerotinia borealis*, improved at low water potentials: an adaption to frozen environment

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Abstract The snow mold fungus, *Sclerotinia borealis*, shows optimal growth at 4°C on potato dextrose agar (PDA) and can grow even at subzero temperature. Its mycelial growth was improved on frozen PDA at −1°C and on PDA containing potassium chloride (KCl) (water potential, −4.27 to −0.85 MPa) or D(−) sorbitol (−3.48 to −0.92 MPa). Its optimal growth temperature shifted from 4 to 10°C on PDA amended with KCl or sorbitol, indicating that inherent optimal growth occurs at high temperatures. These results suggest that *S. borealis* uses concentrated nutrients in the frozen environment and that such physiologic characteristics are critical for the fungus to prevail at subzero temperatures.

Keywords Cold adaptation · Osmophile · Psychrophile · Soil freezing

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Introduction

Snow molds are cold-adapted fungi and can grow under snow, attacking perennial grasses, forage crops, and winter cereals in the Northern Hemisphere (Smith et al. 1989; Hsiang et al. 1999; Hoshino et al. 2009). They are taxonomically diverse, including Oomycota, Ascomycota, and Basidiomycota.

Many living organisms have various biochemical and ecological strategies to protect themselves from intracellular freezing. Antifreeze proteins (AFPs) bind to ice-crystal surfaces to inhibit their growth and lowers the freezing point, leading to the protection of cells from freezing injury (Duman et al. 1993). AFPs and antifreezing substances are produced by several cold-adapted organisms, including fungi (Duman and Olsen 1993; Newstead et al. 1994; Snider et al. 2000; Hoshino et al. 2003a, b; Kawahara et al. 2006; Raymond and Janech 2009). We found that basidiomycetous snow molds produce extracellular AFPs (Hoshino et al. 2003a, b) and hypothesized that, in basidiomycetous snow molds, AFPs keep the extracellular environment unfrozen (Snider et al. 2000; Hoshino et al. 2003b, 2009).

Sclerotinia borealis Bubák & Vleugel [syn. *Myriosclerotinia borealis* (Bubák & Vleugel) L.M. Kohn;

Sclerotinia graminearum Elenev: Solkina] is an ascomy-cete and prevails where soil freezing is severe (Tomiyama 1955; Röed 1960; Nissinen 1996). The fungus, however, does not produce extracellular AFPs (Snider et al. 2000; Hoshino et al. 2003a, b). Consequently, we assumed that *S. borealis* has another physiologic strategy to adapt to the frozen environment. Tomiyama (1955) cultured *S. borealis* and *Typhula incarnata* Lasch: Fr. on both frozen and unfrozen potato dextrose agar (PDA) plates that were kept outside in Sapporo, Hokkaido, northern Japan during winter; mycelial growth of *T. incarnata* was inhibited on frozen PDA, but *S. borealis* grew faster on frozen PDA than on unfrozen PDA. However, his experiments were not carried out under controlled conditions, and his results have not been reproduced by others.

Higher plants tolerate freezing stress by avoiding either extra- or intracellular freezing (Hoshino et al. 1999). The latter mechanism includes tolerance of freeze-induced cell dehydration through enhanced osmotic water potential (Kacperska 1993). Many ascomycetes survive and grow under high osmotic stress (Grant 2004). A few reports showed that *S. borealis* adapted in low water potential condition (Tomiyama 1955; Bruehl and Cunfer 1971; Namikawa et al. 2004); however, no discussion is given regarding freezing resistance of this fungus. Osmophile and osmotolerance in fungi provide us with clues to elucidate the adaptation mechanism of *S. borealis* to freezing.

In this study, we re-examined Tomiyama's experiments under controlled conditions and elucidated the physiologic characteristics of *S. borealis* under a frozen condition to present a hypothesis on the adaptation strategy of *S. borealis* to a frozen environment.

Materials and methods

Fungal strains and media

Snow molds, including *Pythium iwayamai* S. Ito, *Microdochium nivale* (Fr.) Samuels & I.C. Hallett var. *nivale*, *Racodium therryanum* Thüm., *S. borealis*, *S. nivalis* I. Saito, *S. trifoliorum* Erikss., *T. incarnata*, *T. ishikariensis* S. Imai and an unidentified basidiomycete known as the supponuke pathogen of winter wheat (inferred to be *Athelia* sp. based on ITS sequence, A. Kawakami unpublished results) (Shimizu and Miyajima 1990) from our laboratory collection were used in this study, and all isolates were deposited in Osaka Prefecture University (OPU), Sakai, Japan. Isolate numbers in OPU are shown in Table 1.

Cultures other than those of *P. iwayamai* were maintained on PDA (Difco, Becton Dickinson Microbiology Systems, Spark, MD, USA) at 4°C. *P. iwayamai* was maintained on corn meal agar (Difco) at 4°C.

Table 1 Mycelial growth of various snow molds on frozen potato dextrose agar media at −1°C

Taxon	Fungus	Locality	Strain no.	Mycelial growth rate (mm/month)	
				Frozen[a]	Control (untreated)
Oomycota	*Pythium iwayamai*	Hokkaido, Japan	OPU1466	0.0 ± 0.0	15.2 ± 3.2
Ascomycota	*Microdochium nivale* var. *nivale*	Hokkaido, Japan	OPU1467	0.0 ± 0.0	7.2 ± 0.5
	M. nivale var. *nivale*	Ås, Norway	OPU1468	0.0 ± 0.0	12.5 ± 2.1
	Racodium therryanum	Hokkaido, Japan	OPU1469	14.5 ± 2.8	17.5 ± 3.5
	Sclerotinia borealis	Hokkaido, Japan	OPU1471	20.4 ± 9.4	13.2 ± 8.5
	S. borealis	Sakhalin, Russia	OPU1481	39.1 ± 8.5	21.0 ± 8.8
	S. borealis	Tomsk, Russia	OPU1482	35.8 ± 7.3	25.3 ± 8.6
	S. borealis	Ekaterinburg, Russia	OPU1483	23.9 ± 6.2	14.1. ± 5.3
	S. borealis	Rovaniemi, Finland	OPU1470	30.7 ± 8.3	12.9. ± 7.8
	S. nivalis	Novosibirsk, Russia	OPU1472	0.2 ± 0.0	10.2 ± 0.4
	S. nivalis	Hokkaido, Japan	OPU1473	0.2 ± 0.0	8.2 ± 0.2
	S. trifoliorum	Hokkaido, Japan	OPU1474	0.1 ± 0.0	12.8 ± 2.5
Basidiomycota	*Typhula incarnata*	Nuuk, Greenland	OPU1475	8.5 ± 1.2	25.4 ± 2.8
	T. incarnata	Hokkaido, Japan	OPU1476	7.5 ± 1.5	28.3 ± 3.1
	T. ishikariensis biological species I	Hokkaido, Japan	OPU1477	18.0 ± 1.2	36.9 ± 2.2
	T. ishikariensis biological species II	Aomori, Hokkaido	OPU1478	11.9 ± 0.5	15.5 ± 0.5
	T. ishikariensis group III	Svalbard, Norway	OPU1479	19.0 ± 1.5	32.7 ± 2.2
	Supponuke disease fungus (*Athelia* sp.)	Hokkaido, Japan	OPU1480	0.0 ± 0.0	36.9 ± 2.6

[a] Kept beforehand at −20°C for 1 day

Mycelial growth of snow molds on frozen media

Mycelial discs 5 mm in diameter were cut from the margins of actively growing colonies on PDA plates of the test fungi, placed onto fresh 9-cm-diameter PDA plates, and incubated at 10°C for 1–7 days. After mycelial growth was confirmed, the plates were frozen at −20°C for 1 day. Frozen plates were transferred to −1°C. Regrowth of mycelia was determined every week for 1 month. Linear mycelial growth rate in triplicates was calculated per day after the initial lag period.

Mycelial growth of snow molds under an osmotic stress condition

Mycelial growth at −1°C was determined on PDA that contained 0.4 M potassium chloride (KCl) (−2.04 MPa) or 0.4 M sorbitol (−1.54 MPa) in triplicate. Other details were the same as above.

Effect of osmotic stress on growth temperature relations of *Sclerotinia borealis*

Sclerotinia borealis was grown on PDA plates (9 cm in diameter) containing 11 different concentrations between 0 and 0.5 M KCl or D(−)-sorbitol in triplicate, and incubated at five different temperatures between −1 and 20°C for 1 month. Colony diameter was determined every week. Linear mycelial growth rate was calculated per day after the initial lag period.

Determination of water potential in culture medium

Water potential of PDA containing various concentrations of solutes was determined by a thermocouple psychrometer (Water Potential Measurement System, Tru Pis, Decagon Devices Inc., Pullman WA, USA).

Results

Mycelial growth rate of the fungi other than *S. borealis* was lower on frozen plates than on unfrozen (supercooled) plates at −1°C. *P. iwayamai*, *M. nivale* var. *nivale* and supponuke disease fungus (*Athelia* sp.) did not grow on frozen PDA (Table 1). When frozen cultures of the three fungi were thawed and reincubated at 10°C, two strains of *M. nivale* var. *nivale* and supponuke disease fungus resumed to grow, but *P. iwayamai* did not (data not shown). Mycelia of *P. iwayamai* were destroyed by the freezing treatment.

Five strains of *S. borealis* showed about twofold faster mycelial growth on frozen plates than on unfrozen plates.

Sclerotia were formed normally under both culture conditions. Colony morphologies of *S. borealis* on unfrozen and frozen plates were different (Fig. 1): on unfrozen plates, hyphae were twisted and tangled with each other forming dense aerial mycelia showing appressed colony appearance, whereas colonies became fluffy on frozen plates.

Mycelial growth of the snow molds, except *S. borealis* OPU1471, on PDA plates at −1°C was suppressed with increasing concentration of solutes from −1 MPa (Fig. 2). Optimal mycelial growth of OPU1471 occurred between −1 and −2.0 MPa in KCl-amended plates and between −1.5 and −3.5 MPa in sorbitol-amended plates. Other strains of *S. borealis* showed active growth under osmotic stress. *S. borealis* OPU1470 from Rovaniemi, Lapland of Finland, showed a mycelial growth rate of 41.7 ± 7.5 mm/week on PDA containing KCl (−1.48 MPa) and 32.3 ± 5.2 mm/week on PDA containing sorbitol (−2.25 MPa) (data not shown). *S. borealis* OPU1471 and 1470 formed normal sclerotia on media, and fungal colonies under osmotic stress were the same as those on frozen plates. Mycelial growth of these two strains was also improved on PDA containing 0.4 M potassium sulfate (K_2SO_4), magnesium chloride ($MgCl_2$), or calcium chloride ($CaCl_2$) (Table 2). Sodium salts (NaCl and Na_2SO_4) and magnesium sulfate ($MgSO_4$) were inhibitory to mycelial growth of *S. borealis* strains.

Osmotic stress influenced growth temperature relation of *S. borealis* OPU1471 (Fig. 3). Optimal growth temperature on PDA was 5°C for OPU1471 and 1470. On PDA containing 0.2–0.3 M sorbitol (−1.1 to −1.31 MPa), optimal mycelial growth occurred at 10°C. Higher osmotic stress of 0.4–0.5 M sorbitol (−1.54 to −1.68 MPa) improved mycelial growth of both strains, but their growth-temperature relations became similar to those on PDA. Similar results were obtained from other strains (data not shown).

Discussion

We confirmed the results of Tomiyama (1955): *S. borealis* grew fast on frozen PDA under the controlled condition (Fig. 1; Table 1). Five strains of *S. borealis* from different localities showed intact mycelial growth under the frozen condition, and mycelial growth rate on frozen plates at −1°C was faster than that on unfrozen plates at 4°C, the optimal growth temperature. Our results agreed with the findings from other studies (Röed 1960; Nissinen 1996), showing that this fungus is adapted to harsh winters with soil freezing.

This fungus can survive low temperatures without producing extracellular AFPs (Snider et al. 2000; Hoshino et al. 2003a, b). The ability to grow at low water potentials enables *S. borealis* to grow at subzero temperatures. It has

Fig. 1 Mycelial growth of *Sclerotinia borealis* and *Microdochium nivale* var. *nivale* on frozen and unfrozen potato dextrose agar (PDA) plates. **a** Colony of *S. borealis* OUP1471 on unfrozen PDA plate. **b** *S. borealis* OPU1471 on frozen PDA plate. **c** Colony of *M. nivale* var. *nivale* OPU 1467 on unfrozen plate. **d** *M. nivale* var. *nivale* on frozen plate. Cultures were pregrown at 10°C for 1–7 days and frozen at −20°C for 1 day. They were then transferred to −1°C. Photos were taken 30 days after incubation at −1°C
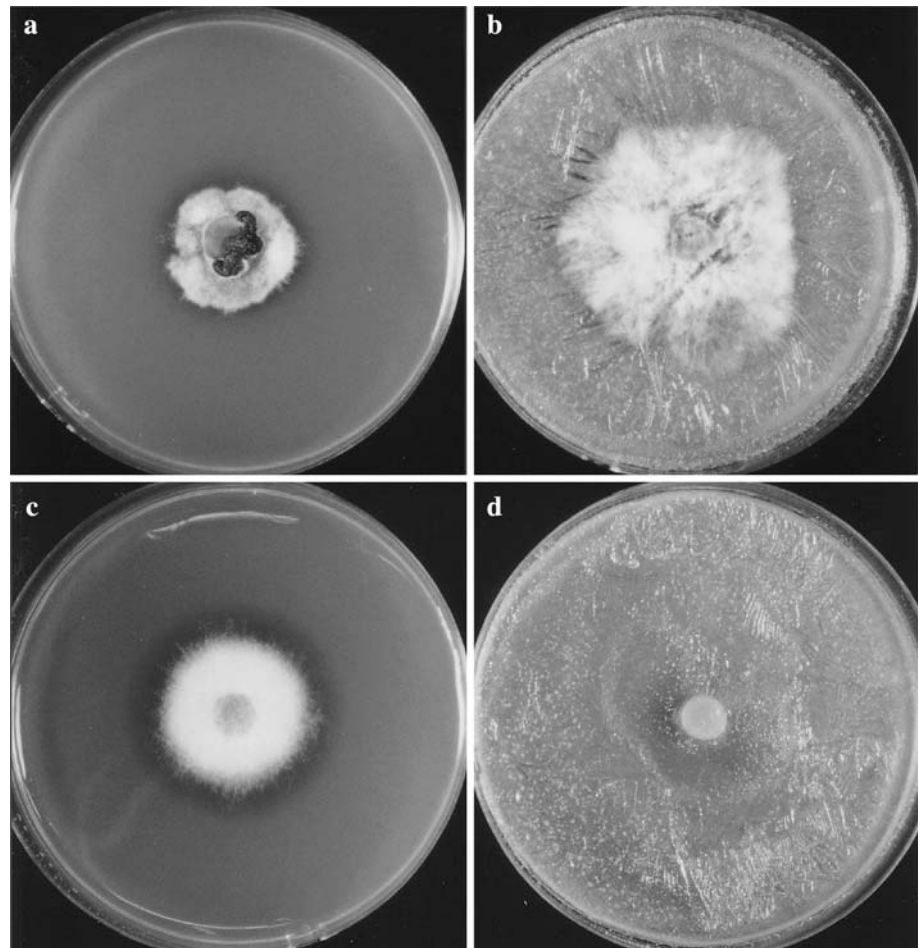

Fig. 2 Mycelial growth of various snow molds on potato dextrose agar (PDA) containing potassium chloride (KCl) or D(−)-sorbitol at −1°C. **a** KCl, **b** Sorbitol. *Sclerotinia borealis* OPU1471 (*open circles*), *Microdochium nivale* var. *nivale* OPU1467 (*closed circles*), *Racodium therryanum* OPU1469 (*open triangles*), *Typhula ishikariensis* OPU1477 (*closed triangles*), and supponuke disease fungus OPU1480 (*open squares*). *Bars* indicate standard deviation
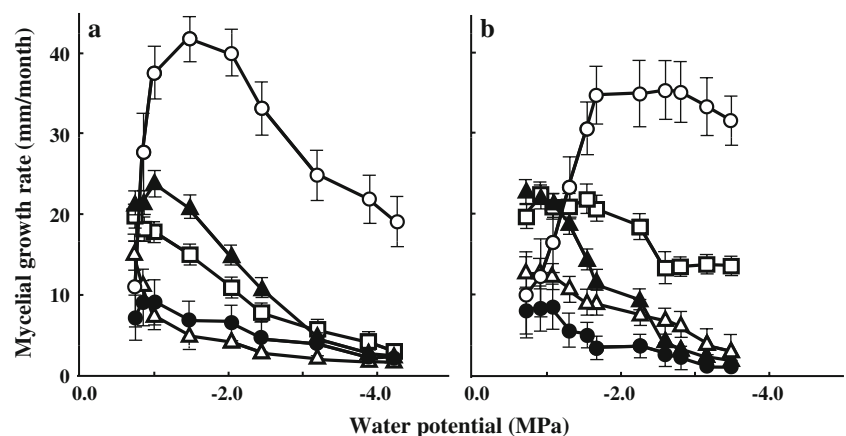
shown that microorganisms in permafrost soil can retain metabolic activities and continue to grow at subzero temperatures (Rivkina et al. 2000). Permafrost soil is known to contain unfrozen water (Ershov 1996), and cold-adapted bacteria grow in the unfrozen water that contains concentrated soluble substrates. Crowns of cold-treated (hardened) grasses also showed a significant decrease in water potential compared with untreated (unhardened) plants (Tronsmo 1986). *S. borealis* adapts to the low water

potential condition of PDA containing twice the amount of medium ingredients (Tomiyama 1955), sucrose, KCl (Bruehl and Cunfer 1971) and D-mannitol (Namikawa et al. 2004). These results suggest that *S. borealis* uses concentrated nutrients in the frozen medium.

Tronsmo (1986) also cultivated *M. nivale* and *T. ishikariensis* at different water potentials in potato dextrose broth supplemented with KCl or polyethylene glycol 6000. Both fungi had a considerable decrease in dry weight

Table 2 Effects of various salts in potato dextrose agar (PDA) plates on mycelial growth of *Sclerotinia borealis* at −1°C

Kinds of salts	Water potential (MPa)	Mycelial growth rate (mm/week)	
		OPU1470	OPU1471
Control (PDA)	−0.73	12.9 ± 7.8	17.5 ± 3.5
+0.4 M KCl	−1.48	32.9 ± 4.3	40.9 ± 4.5
+0.4 M NaCl	−5.65	4.3 ± 3.0	15.5 ± 2.5
+0.4 M MgCl$_2$	−2.24	24.6 ± 2.7	38.6 ± 4.2
+0.4 M CaCl$_2$	−1.73	20.1 ± 2.2	35.2 ± 4.2
+0.4 M K$_2$SO$_4$	−2.10	45.2 ± 3.7	42.8 ± 3.5
+0.4 M Na$_2$SO$_4$	−1.15	2.1 ± 1.0	<1.0
+0.4 M MgSO$_4$	−5.50	8.5 ± 2.5	12.5 ± 3.7

KCl potassium chloride, *NaCl* sodium chloride, *MgCl$_2$* magnesium chloride, *K$_2$SO$_4$* potassium sulfate, *Na$_2$SO$_4$* sodium sulfate, *MgSO$_4$* magnesium sulfate
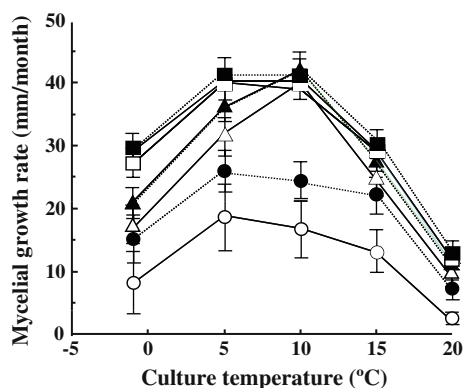

Fig. 3 Effect of osmotic stress due to D(−)-sorbitol added to potato dextrose agar (PDA) plates on growth-temperature relations of *Sclerotinia borealis* OPU1471. Mycelial growth rates on PDA (*open circles* −0.7 MPa), PDA contained 0.1 M (*closed circles* −0.92 MPa), 0.2 M (*open triangles* −1.1 MPa), 0.3 M (*closed triangles* −1.31 MPa), 0.4 M (*open squares* −1.54 MPa), and 0.5 M (*closed triangles* −1.68 MPa), respectively. *Bars* indicate standard deviation

production when water potential of the growth medium decreased from −0.7 to −3 MPa. *M. nivale* and supponuke disease fungus (*Athelia* sp.) did not grow on frozen plates; however, they survive and grow under osmotic stress. Therefore, inhibition of mycelial growth on frozen plates may not be directly related to growth ability under high osmotic stress.

An increase in intracellular osmosis enhanced mycelial growth of *S. borealis* strains (Fig. 2), but sodium ions inhibited their mycelial growth (Table 2). This fungus is regarded as osmophile but not halophile. The high osmotic stress with 0.4–0.5 M sorbitol (−1.54 to −1.68 MPa) shifted optimal mycelial growth temperature of *S. borealis* from 10 to 4°C (Fig. 3). These results suggest that *S. borealis* is well adapted in soil frozen climate.

Ozaki ([1979](#)) observed that *S. borealis* occurred also in non-soil-freezing areas in eastern Hokkaido and suspected that soil freezing was not directly correlated with disease incidence on orchardgrass (*Dactylis glomerata* L.): soil freezing merely indicates the level of predisposition to *S. borealis* in orchardgrass. Less cold-tolerant grasses, such as perennial ryegrass (*Lolium perenne* L.), are vulnerable to the attack of *S. borealis*, even in areas without soil freezing, implying that soil freezing is not always prerequisite for the incidence of *S. borealis*. Decrease in temperature induces biochemical changes in overwintering plants to adapt to cold by decreasing water content. *S. borealis* invades plant tissues and may use concentrated nutrients in hardened tissues only when plants are predisposed to cold. Lower temperature is necessary to induce predisposition in orchardgrass than in perennial ryegrass. Further studies should focus on the behavior of *S. borealis* on plant tissues with high osmotic water potential to elucidate the host–parasite interactions on plants with and without predisposition.

References

Bruehl GW, Cunfer BM (1971) Physiologic and environmental factors that affect the severity of snow mold of wheat. Phytopathology 61:792–799

Duman JA, Olsen TM (1993) Thermal hysteresis protein activity in bacteria, fungi and phylogenetically diverse plants. Cryobiology 30:322–328

Duman J, Wu DW, Olsen TM, Urrutia M, Tursman D (1993) Thermal-hysteresis proteins. In: Steponkus PL (ed) Advances in low-temperature biology, vol 4. JAI Press, London, pp 131–182

Ershov ED (1996) General geocryology. Cambridge University Press, Cambridge

Grant WD (2004) Life at low water activity. Phil Trans R Soc B Biol Sci 359:1249–1267

Hoshino T, Odaira M, Yoshida M, Tsuda S (1999) Physiological and biochemical significance of antifreeze substances in plants. J Plant Res 112:255–261

Hoshino T, Kiriaki M, Nakajima T (2003a) Novel thermal hysteresis proteins from low temperature basidiomycete, *Coprinus psychromorbidus*. Cryo Lett 24:135–142

Hoshino T, Kiriaki M, Ohgiya S, Fujiwara M, Kondo H, Nishimiya Y, Yumoto I, Tsuda S (2003b) Antifreeze proteins from snow mold fungi. Can J Bot 81:1175–1181

Hoshino T, Xiao N, Tkachenko OB (2009) Cold adaptation in phytopathogenic fungi causing snow mold. Mycoscience 50:26–38

Hsiang T, Matsumoto N, Millett SM (1999) Biology and management of *Typhula* snow molds of turfgrass. Plant Dis 83:788–798

Kacperska A (1993) Water potential alterations—a prerequisite or a triggering stimulus for the development of freezing tolerance in overwintering herbaceous plants? In: Li PL, Christersson L (eds) Advances in plant cold hardiness. CRC Press, Boca Raton, pp 73–91

Kawahara H, Takemura T, Obata H (2006) Function analysis and screening of antifreeze material from fungi. Cryobiol Cryotechnol 52:151–155 (in Japanese)

Namikawa Y, Watanabe T, Saito I, Takasawa T (2004) Growth of the psychrophilic snow mold *Sclerotinia borealis* on the agar under xerophilic conditions. Res Bull Obihiro Univ 25:23–26 (in Japanese)

Newstead WJ, Polvis S, Kendall E, Saleem M, Koch M, Hussain A, Culter AJ, Georges F (1994) A low molecular weight peptide from snow mold with epitopic homology to winter flounder antifreeze protein. Biochem Cell Biol 72:152–156

Nissinen O (1996) Analyses of climatic factors affecting snow mould injury in first-year timothy (*Phleum pratense* L.) with special reference to *Sclerotinia borealis*. Acta Univ Oul A 289:1–115

Ozaki M (1979) Ecological study of Sclerotinia snow blight disease of orchardgrass. Bull Hokkaido Pref Agric Exp Stn 42:55–65 (in Japanese)

Raymond JA, Janech MG (2009) Ice-binding proteins from enoki and shiitake mushrooms. Cryobiology 58:151–156

Rivkina EM, Friedman EI, McKay CP, Glinchinsky DA (2000) Metabolic activity of permafrost bacteria below the freezing point. Appl Environ Microbiol 66:3230–3233

Röed H (1960) *Sclerotinia borealis* Bub. & Vleg., a cause of winter injuries to winter cereals and grasses in Norway. Acta Agric Scand 10:74–82

Shimizu S, Miyajima K (1990) Supponuke disease of winter wheat. Ann Phytopathol Soc Jpn 56:141 (in Japanese)

Smith JD, Jackson N, Woolhouse AR (1989) Fungal disease of Amenity Turf Grasses, 3rd edn. E. & F. Spon, London

Snider CS, Hsiang T, Zhao GY, Griffith M (2000) Role of ice nucleation and antifreeze activities in pathogenesis and growth of snow molds. Phytopathology 90:354–361

Tomiyama K (1955) Studies of the snow blight disease of winter cereals. Hokkaido Natl Agric Exp Stn Rep 47:1–234 (in Japanese)

Tronsmo AM (1986) Host water potentials may restrict development of snow mold fungi in low temperature-hardened grasses. Physiol Plant 68:175–179