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REVIEW

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Cold adaptation in the phytopathogenic fungi causing snow molds

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Abstract Snow molds are psychrophilic or psychrotrophic fungal pathogens of forage crops, winter cereals, and conifer seedlings. These fungi can grow and attack dormant plants at low temperatures under snow cover. In this review, we describe the biodiversity and physiological and biochemical characteristics of snow molds that belong to various taxa. Cold tolerance is one of the important factors related to their geographic distribution, because snow molds develop mycelia under snow cover and because they should produce intra- and extracellular enzymes active at low temperatures for growth and infection. Basidiomycetous snow molds produce extracellular antifreeze proteins. Their physiological significance is to keep the extracellular environment unfrozen. The psychrophilic ascomycete *Sclerotia borealis* shows normal mycelial growth under frozen conditions, which is faster than that on unfrozen media at optimal growth temperature. This fungus does not produce extracellular antifreeze proteins, but osmotic stress tolerance enables the fungus to grow at subzero temperatures. In conclusion, different taxa of snow molds have different strategies to adapt under snow cover.

Key words Antifreeze protein · Cold-active enzyme · Frost resistance · Psychrophile · Snow mold

What are snow molds?

Snow molds are psychrophilic (cold-loving) or psychrotrophic (cold-tolerant) fungal pathogens of forage crops, winter cereals, and conifer seedlings. These fungi can attack dormant plants at low temperatures under snow cover (Boyce 1961; Smith 1986; Smith et al. 1989; Hsiang et al. 1999; Iriki et al. 2001). The environment under snow protects overwintering plants from freezing, as it maintains darkness, humidity, and low temperature, which is described in detail by Matsumoto and Hoshino (2008). "Snow molds" or "snow mold fungi" is a generic name including diverse fungi belonging to various taxa (oomycetes, ascomycetes, and basidiomycetes). Table 1 summarizes major snow molds reported in the Northern Hemisphere. Important pathogens of agricultural crops are *Pythium iwayamai*, *Microdochium nivale* (syn.: *Fusarium nivale*), *Sclerotinia borealis* (syn.: *Myriosclerotinia borealis*), *Coprinus psychromorbidus*, *Typhula incarnata*, and *T. ishikariensis* (syn.: *T. idahoensis*). *Phacidium infestance*, *P. abietis*, and *Racodium therryanum* attack conifer seedlings.

The snow molds *S. borealis* and *T. ishikariensis* are widely distributed not only in the cool temperate zone and frigid zone but also in Arctic regions such as Alaska and the Yukon (Lebeau and Longston 1958), Greenland (Hoshino et al. 2006b), Finnmark (northern Norway: Årsvoll 1975; Matsumoto and Tronsmo 1995; Matsumoto et al. 1996), Iceland (*S. borealis* not found: Kristinsson and Guðleifsson 1976; Hoshino et al. 2004a), Lapland (northern Finland and Sweden: Ekstrand 1955; Jamalainen 1949, 1957), and Svalbard (Hoshino et al. 2003c). These investigations suggest that *S. borealis* and *T. ishikariensis* are highly adapted to the Arctic environment.

Many studies have been carried out on snow molds from the aspect of plant protection. New findings regarding their physiological and biochemical characteristics have been reported during the past decade by a few researchers, including the author's group. In this review, we show the biodiversity and physiological and biochemical characteristics of snow molds.

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Table 1. Major snow molds from the Northern Hemisphere

Snow molds	Common name	Growth temperature (°C)			Host	Reference
		Min.	Opt.	Max.		
Oomycetes						
Pythium iwayamai	Pythium snow rot	<0	18–22	25–30	Grasses	Hirane 1960
P. okanoganense	do	<0	22	30	do	Lipps & Bruehl 1978
P. paddicum	do	<0	22	30	do	Takamatsu 1989
Ascomycetes						
Microdochium nivale (teleomorph: *Monographella nivalis*, syn. *Fusarium nivale*)	Pink snow mold	>–5	10–20	30	do	Smith 1986
Phacidium infestans	Snow blight	<–5	14	21–24	Trees	Hanso 2000
Racodium therryanum	Racodium snow blight	<–5	15–20	ND	Trees	Sakamoto & Miyamoto 2005
Sclerotinia borealis (syn. *Myriosclerotinia borealis*) Sclerotia snow mold	Snow scald	<–7	10–15	<20	Grasses and trees	Smith 1986
S. nivalis	None	<0	20	ND	Grasses	Iriki et al. 2001
S. trifoliorum	Clover rot Sclerotinia steam rot	<0	15–19	ND	Forage Legumes	Iriki et al. 2001
S. kitajimana	Snow molding	7	20	31	Trees	Ito and Hosaka 1951
Basidiomycetes						
Coprinus psychromorbidus	Cottony snow mold, low-temperature basidiomycete	<–5	5–10	<25	Grasses Apple and pear fruits	Smith 1986
Typhula incarnata	Grey snow mold	<–7	10–15	<20	Grasses	Smith 1986
T. ishikariensis (syn. *T. idahoensis*)	Speckled snow mold	<–7	5–10	<20	Grasses and trees	Smith 1986

ND, not described

Biodiversity of snow molds

Oomycetes

Pythium snow rot occurs in Japan, the United States (Takamatsu and Takenaka 2001), and Kola Peninsula in the European part of Russia (Petrov 1983). One of the pathogens, i.e., *P. iwayamai*, has also been collected in Australia (van der Plaats-Niterink 1981). *Pythium* sp. from mosses in Signy Island, South Orkney Islands, Antarctica, had a high similarity of DNA sequence with *P. iwayamai* (Bridge et al. 2008). Rotted leaves of winter cereals and grasses are water soaked and dark green just after snow melt, then turn brownish green and finally become pale green to gray. A large number of oospores and sporangia are found in rotted leaves. Three *Pythium* species, *P. iwayamai* (Ito 1935), *P. okanoganense* (Lipps 1980a), and *P. paddicum* (Hirane 1960), are the major pathogens in Japan and the United States (Takamatsu and Takenaka 2001). *P. okanoganense* was isolated from Far Province in Iran (Mostowfizageh-Ghalamfarsa and Banihashemi 2005). Other *Pythium* spp. such as *P. aristosporum*, *P. graminicola*, *P. ultimum*, *P. vanterpoolii*, and *P. volutum* have also been isolated from rotted leaves under snow cover (Takamatsu and Takenaka 2001).

Similar symptoms were also found on mosses in the Arctic (Hoshino et al. 1999b, 2000, 2001c, 2006c) and Antarctica (Hoshino et al. 2001b; Bridge et al. 2008). *Pythium* sp., morphologically similar to *P. ultimum* var. *ultimum* and other *Pythium* spp., were isolated from moribund tissues of polar mosses.

Ascomycetes

Pink snow mold, *Microdochium nivale*, is the most widespread snow mold fungus. This fungus is distributed not only in the Northern Hemisphere but also in the Southern Hemisphere (Smith 1986; Hsiang et al. 1999; Tronsmo et al. 2001). Serious damage occurs on winter cereals under snow cover lasting for 2 months or more (Årsvoll 1973). In addition, this fungus can attack grasses in autumn and spring when it is cold and wet even in cool, wet summers. The disease, known as fusarium patch, occurs as small patches, which first appear as water-soaked lesions later becoming yellow, orange, or brown, sometimes with a fringe of pinkish-white mycelium (Smith 1986; Smith et al. 1989; Hsiang et al. 1999; Tronsmo et al. 2001).

The teleomorph of *Microdochium nivale* is *Monographella nivalis* (Tronsmo et al. 2001). Macroconidia are curved, falcate, and tapering toward each end, with a pointed apex and a round, wedge-shaped base. Based on conidial morphology, *M. nivale* is divided into two varieties: var. *majus*, which has large and predominantly three-septate cells, and var. *nivale*, which has one- to three-septate cells (Samuels and Hallett 1983). These varieties also exhibit some host specificity and even specialization to annual and perennial grasses (Smith 1986; Lees et al. 1995; Tronsmo et al. 2001).

Sclerotinia borealis causes snow mold of winter cereals and forage crops. *S. trifoliorum* incites stem and crown rot of forage legumes in winter (Purdy 1979), and *S. nivalis* causes snow mold of herbaceous dicots (Saito 1997). They belong to the family Sclerotiniaceae, which is an important family in the Discomycetes, commonly known as the cup fungi (Saito 2001). *S. borealis* has been found in cold regions in the Northern Hemisphere, such as northern Japan, Russia, northern Scandinavia, and North America. The southern distribution limit of this fungus is Iwate, northern Honshu, in Japan (N. Matsumoto, personal communication), the Altai in the central alpine part of Siberia (Hoshino et al. 2004c), and possibly Xingjian Province in China (Tai 1979). However, this fungus has not been found in temperate snowfall regions except for Japan.

On snow melt in spring, water-soaked leaves and sparse gray mycelia and sclerotia of *S. borealis* appear. Infected leaves are often bleached and wrinkled, becoming thread like when exposed to light, but later darken with the growth of saprophytic fungi. Sclerotia of this fungus are found in sheaths, crowns, and on the surface or within leaves. They are black, up to 7–8 mm in length and 3–4 mm in width when fresh. Sclerotia germinate in autumn to produce cup-shaped apothecia varying in color from pale yellow to pale brown. Apothecial disks range between about 1 and 6 mm in diameter, and their stalks vary from 1 to about 20 mm in height (Smith 1986; Smith et al. 1989).

Many ascomycetes can attack forest trees under snow cover (Boyce 1961). *Phacidium infestance* attacks *Pinus* spp. in Europe and Asia, and *Phacidium abietis* also incites snow mold of conifers in North America. *R. therryanum* causes snow blight to mainly conifer seedlings in Japan (Sakamoto and Miyamoto 2005) and Korea (Cho et al. 2007). Other fungi, such as *Lophophacidium hyperboreum*, *Nothophacidium abietinellum*, *Sarcotrochila balsameae*, and *S. piniperda*, also cause similar snow blight. *Sclerotinia borealis* has been reported to attack conifer seedlings in the Volga-Ural regions of Russia (Gulaev 1948; Hoshino et al. 2004c). *Sclerotinia kitajimana* causes snow blight of *Cryptomeria japonica* seedlings in Honshu, Japan (Ito and Hososaka 1951), but its taxonomic identity needs further investigation.

Basidiomycetes

Cottony snow mold caused by low-temperature basidiomycetes (LTB, = *Coprinus psychromorbidus*) attacks numerous garden perennials and wild species (Smith 1986; Smith et al. 1989; Hsiang et al. 1999; Gaudet 2001). The fruit rot low-temperature basidiomycete (FRLTB) causes storage rot of apples and pears in fruit-growing areas of Oregon and British Columbia (Gaudet 2001). Sclerotial strains of LTB (SLTB) have been found in the crowns of perennial grasses and winter cereals (Gaudet 2001). The disease is first seen on snow melt in spring as bleached patches of host plants. Abundant grayish-white mycelia are often present on the edges of patches. There are no or few sclerotia as with *Sclerotinia* spp. and *Typhula* spp. (Smith 1986; Smith et al. 1989; Gaudet 2001).

Coprinus psychromorbidus has only been found in regions of North America. di-mon mating experiments demonstrates that SLTB and FRLTB are conspecific with *C. psychromorbidus*. However, isolates from spores or hyphae from basidiocarps of *C. psychromorbidus* are mesophilic, with an optimal temperature of about 22°C. Mating experiments and DNA analyses of isolates previously lumped as a single taxon, *C. psychromorbidus*, were divided into four or more different taxa, consisting of LTB, SLTB, FRLTB, and *C. psychromorbidus* strains (Laroche et al. 1995).

Shimizu found a similar fungus causing snow mold on winter wheat in eastern Hokkaido, Japan. The fungus did not mate with monocaryons of known LTB isolates (Shimizu and Miyajima 1990). Isolates of this fungus form thin brown sclerotia, and its internal transcribed spacer (ITS) sequence suggested the affinity with *Athelia* spp. (A. Kawakami, personal communication). The fungus is considered to be a forest inhabitant (N. Matsumoto, personal communication).

The genus *Typhula* includes about 100 species, none of which occurs in tropical regions (Berthier 1976). Most species are saprophytic and low-temperature tolerant, and only five species, namely, *T. incarnata*, *T. ishikariensis*, *T. phacorrhiza*, *T. trifolii*, and *T. variabilis*, are known to cause disease of grasses and forage crops as well as winter cereals.

The gray snow mold fungus *T. incarnata* is widely distributed in snowy regions in Asia, Europe, and North America, and this fungus has not been found in high Arctic regions. The southern distribution limit of this fungus is Italy (Titone et al. 2003), Adygea Republic, the Caucuses in Russia (O.B. Tkachenko et al., unpublished results), East Azerbaijan, Northern Iran (Hoshino et al. 2007), to Tokushima, Shikoku, in Japan (Tasugi 1936). Although this fungus and *M. nivale* can cause injury in the absence of snow cover, more severe damage occurs under 2 to 3 months of snow cover (Årsvoll 1973; Smith 1986). On snow melt, sparse to dense white to grayish-white mycelia may mat together in patches. Globular to flattened-spherical, faintly pink sclerotia, up to 5 mm in diameter, are present in or on infected tissues of leaves and plant bases. Sclerotia darken from pink to brown to reddish brown, wrinkle on drying, and may be firmly attached to substrate. Sporophores (basidiocarps) up to about 20 mm in height with pale pink or white stipes and pink to rose-colored clubs may develop from sclerotia in moist autumn (Smith 1986; Smith et al. 1989).

The distribution limit of the speckled snow mold fungus *T. ishikariensis* is in higher latitudes than *T. incarnata*, such as Switzerland (Schmidt 1976), south Siberia (Hoshino et al. 2001a), to Mie, Honshu of Japan (Hoshino et al., unpublished results). Grassland stands in localities with more than 150 days of snow cover suffer recurrent damage from *T. ishikariensis* (Årsvoll 1973; Matsumoto et al. 2001). Dark-colored sclerotia give patches of disease a speckled appearance, although plant symptoms of the disease are similar to those caused by *T. incarnata*. Sclerotia of this fungus are never pink or red but turn dark amber to dark chestnut when fresh and dark brown to almost black when dry. They are not gelatinous. Sporophores have pale yellow to grayish-white clavulae shading into smoky-brown stipe bases (Smith 1986; Smith et al. 1989).

Typhula ishikariensis varies in morphology, physiology, and genetics (Matsumoto 1997; Matsumoto et al. 2001). Bruehl et al. examined isolates from the United States, Finland, and Japan (Bruehl and Cunfer 1975; Bruehl et al. 1975). They regarded *T. idahoensis* as a separate species from *T. ishikariensis* based on intersterility between the two as well as on morphological and ecological differences. Based on interfertility within the *Typhula ishikariensis* complex, Årsvoll and Smith (1978) divided this fungus into three varieties, i.e., var. *ishikariensis*, var. *idahoensis*, and var. *canadiensis*, which differ in morphology of basidiocarps and sclerotia (rind cell patterns). DNA sequences (ITS regions) suggest that North American varieties of *T. ishikariensis* retain high similarity to each other (Hsiang and Wu 2000). Matsumoto et al. (1982, 1983) found two intersterility groups (biotypes A and B) within Japanese isolates but included them in a single species, *T. ishikariensis*, because these biotypes could be genetically related through North American taxa. Matsumoto et al. (1995, 1996) also divided Norwegian isolates into three groups (groups I, II, and III) based on cultural characteristics and mating reactions with Japanese biotypes. Finally, Matsumoto (1997) classified this fungus into two biological species (biological species I and II) based on morphologies and mating reactions. Biological species I includes biotype A from Japan, var. *ishikariensis* and var. *idahoensis* from North America, and groups I and III from Norway, and attacks monocots, dicots, conifer seedlings, and hop roots in Russia (Hoshino et al. 2004c). Biological species II consists of biotype B from Japan, var. *canadiensis* from North America, and group II from Norway. They cause snow mold only on monocots.

Physiological characteristics of snow molds

Temperature sensitivity

Snow molds attack dormant plants at low temperatures under snow cover. Therefore, they can grow at subzero temperatures. Morita (1975) divided cold-adapted microorganisms into two groups, i.e., psychrophile (cold-loving microbes) and psychrotolerant (cold-tolerant microbes). Psychrophiles can grow at low temperatures from below 0°C to 10°C but cannot grow at temperatures higher than 20°C. On the other hand, psychrotolerants can grow at temperatures above 20°C. Many kinds of psychrophiles have been isolated from both polar oceans. However, there are few psychrophiles in terrestrial ecosystems because annual fluctuation in terrestrial ecosystems is much larger than that in marine ecosystems. Table 1 shows mycelial growth temperature relations of major snow molds. Snow molds, except *S. borealis* and *T. ishikariensis*, are psychrotolerants. Both species are psychrophiles and form sclerotia that are tolerant to drought and remain dormant during summer. However, *S. borealis* is not regarded as psychrophilic, considering the conditions for sclerotia germination to produce apothecia: optimal germination occurs when sclerotia are subjected to a daily thermal cycle at 20°C/5°C after incubation at 25°C/15°C for 4 weeks (Saito 2001).

Typhula ishikariensis is specialized as a parasite (Matsumoto 1992), whereas *T. incarnata* retains its saprophytic nature (Årsvoll 1973; Smith et al. 1989). *T. phacorrhiza* is known to be both saprophytic and pathogenic (Schneider and Seaman 1986; Burpee et al. 1987; Matsumoto et al. 2001). Mycelial growth rate at low temperatures is correlated with the extent of specialization as pathogens in *Typhula* spp. These facts suggest that cold-adapted fungi to gain aggressiveness become snow molds in Typhulaceae. Pathogenic species of *Typhula* spp. found the new resource of overwintering plants and evolved in a cold environment under snow cover (Matsumoto 1992).

Typhula incarnata is a versatile pathogen with different ecological capacities to be used in different environments (Matsumoto et al. 1995). In contrast, *T. ishikariensis* has evolved several infraspecific taxa adapted to different winter climates (Matsumoto 1992, 1994). Size variation of sclerotia in *T. ishikariensis* is as great as that in *T. incarnata*, but this is caused by genetic variability, and the character is stable within isolates (Matsumoto et al. 2001). According to Matsumoto and Tajimi (1990), there is a correlation between winter climate and sclerotium size in *T. ishikariensis* biotype B; populations from snowy localities tend to produce large sclerotia, and those from localities with ephemeral snow cover have small sclerotia. Sclerotium size is genetically determined and not dependent on environmental conditions in *T. ishikariensis* biotype B (Matsumoto and Tajimi 1991). On the other hand, sclerotium size of Polish isolates of *T. incarnata* was greatly affected by incubation temperature (Hoshino et al. 2004b). They formed many small sclerotia (<1 mm) at 0°C. Small sclerotia were not produced by other isolates from other localities. Polish isolates of *T. incarnata* have adapted to short periods of snow cover among populations from regions with a diverse winter climate (Prończuk and Zagdańska 1993). Small sclerotia is an adaptation to an unpredictable, less snowy habitat for both *T. incarnata* (Matsumoto et al. 1995) and *T. ishikariensis* (Matsumoto and Tajimi 1990).

Typhula ishikariensis group III is prevalent in Finnmark (the northernmost part of Norway), Greenland, and Svalbard (Matsumoto and Tronsmo 1995; Matsumoto et al. 1996; Hoshino et al. 2003c, 2006b). Isolates of groups I and II exist in the southern and middle parts of Norway. This distribution pattern indicates that group III is more adapted to low temperatures than the two other groups. Isolates of groups I and II grow normally at 10°C, whereas group III isolates showed irregular growth at this temperature (Matsumoto et al. 1996; Hoshino et al. 1997a), and hyphal growth stopped at 15°C. When *T. ishikariensis*, *T. incarnata*, and *T. trifolii* from Canada were first exposed to the maximum growth temperatures (20° or 25°C) and then incubated at their optimum growth temperatures (Dejardin and

Ward 1971), *T. ishikariensis* formed a "fan-shaped" irregular colony that was similar to the colony morphology of *T. ishikariensis* group III in Norway incubated at 10°C. Exposure of *S. borealis* to its maximum growth temperature (20°C) also resulted in irregular mycelial growth: the mycelia became compact and knotty (Ward 1968b). Experiments on oxygen uptake of psychrophilic snow molds indicated that these fungi would not grow above 20°C. Oxygen uptake by *T. ishikariensis* was optimal at 20°C (maximum growth temperature), about 15°C higher than its optimal growth temperature (5°–10°C) (Dejardin and Ward 1971). Similar results were obtained from *S. borealis* (Ward 1966a, 1968a) and *C. psychromorbidus* (Ward 1966b).

Growth temperature relationships of *P. iwayamai* suggest that this fungus is psychrotolerant. Zoospores of *P. iwayamai* and *P. okanoganense* are released between 1° and 15°C and between 1° and 10°C, respectively, but not at temperatures exceeding 20°C and 15°C, respectively (Lipps 1980b). *P. iwayamai* oospores are activated following disintegration of thick cell walls in water at 1° to 10°C. Breaking of dormancy was reduced at 15°C and was totally inhibited at temperatures higher than 20°C (Takamatsu 1989). These findings indicate that *P. iwayamai* and *P. okanoganense* are psychrophiles.

Cold tolerance

The ambient temperature under snow is about 0°C, but the temperature falls much lower than 0°C, often causing freezing damage to plants in the Canadian prairies (Smith 1986) and along the coastal regions of Norway (Årsvoll 1973). Ordinary phytopathogenic fungi survive severe winters in the form of spores and sclerotia, and they resume infection the following spring when plants start to grow. On the other hand, snow molds develop mycelia in winter (Matsumoto 1994). Freezing resistance of mycelia, sclerotia, and spores is considered critical to their survival, especially in areas with climatic fluctuation during winter.

Pythium spp. have often been found on moss colonies in polar regions, and they have pathogenic activity against mosses (Hoshino et al. 1999b, 2000, 2001b,c, 2006c). Isolates of *Pythium* sp., morphologically similar to *P. ultimum* var. *ultimum* from Svalbard (the Arctic), and isolates of *P. ultimum* var. *ultimum* from the temperate zone have similar optimum growth temperatures. However, isolates from Svalbard can grow and survive at 0° to 5°C. Chilling treatment at 0°C for 3 days or at 4°C for 1 week induced irregular cell morphology in Arctic isolates. On the other hand, isolates from Japan did not grow at temperatures below 5°C and were destroyed after the chilling stress (Hoshino et al. 2002).

Intracellular ice formation is lethal to living organisms because of the loss of cell integrity that occurs when growing ice crystals rupture cellular membranes (Levitt 1980). The strategies developed by fungi to protect themselves from freezing stress are based on the inhibition of intracellular freezing. Hoshino et al. (1998, 2001a) determined freezing tolerance of mycelia and sclerotia of *T. ishikariensis* from Norway and Russia. After freezing at –40°C, the regrowth of group I isolates from southern Norway was delayed at their optimal growth temperature (10°C), whereas group III isolates from Finnmark, northernmost Norway, readily resumed growth when returned to their optimal growth temperature at 4°C. Isolates from Moscow had lethal damage from the freezing stress, but mycelial regrowth of isolates from Novosibirsk, central Siberia (considered to be equivalent to Norwegian group III) was not affected by the freezing treatment. Mycelia of group III isolates froze at temperatures higher than –10°C. Therefore, the freezing resistance of group III isolates may be ascribed to extracellular ice formation (Hoshino et al. 1998). Sclerotia are the most important organs for the survival of *Typhula* spp.: survival rate of sclerotia from Norwegian group I isolates and Moscow isolates decreased by freeze–thaw cycles (–40°C for 8 h/2°C for 16 h), whereas sclerotial survival of Norwegian group III isolates and Siberian isolates was not affected by the freeze–thaw stress. These results suggest that freezing resistance is one of the important factors that determine geographic distribution of *T. ishikariensis* (Hoshino et al. 1998, 2001a). In addition, isolates of group III showed relatively normal growth on potato dextrose agar (PDA) at 10°C after freezing. Mycelia of group III isolates suspended in water poured over PDA plates showed normal growth at 10°C (Hoshino et al. 2008). These data suggested that isolates of group III are more resistant than isolates of group I to freezing, representing one of the mechanisms for adaptation to climatic condition in the northwest, coastal regions of Norway where freeze–thaw cycles also cause freezing stress to plants (Årsvoll 1973).

Nissinen (1996) showed a strong positive association between the incidence of *S. borealis* and depth of soil frost in November in Lapland, northern Finland. In years when the average depth of frozen soil was 21 cm or more by the middle of November, damage caused by *S. borealis* was severe. Conversely, when the soil was frozen to a depth of less than 5 cm, *Typhula* spp. caused more damage. *Typhula* spp. predominated when soil freezing was delayed by early establishment of a thick snow cover. Röed (1960) also reported that a thin snow cover and deep soil freezing promoted plant damage caused by *S. borealis* and that a thick snow cover and unfrozen or slightly frozen soil favored the development of *Typhula* spp. and *M. nivale*. Thus, freezing is critical to the incidence of *S. borealis*.

Tomiyama (1955) cultured *S. borealis* and *T. incarnata* on frozen and unfrozen PDA plates that were kept outside in Sapporo, Hokkaido, northern Japan, during winter. In his study, 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. We confirmed his results: *S. borealis* grew on frozen PDA under controlled condition (Table 2; Hoshino et al., unpublished results). *S. borealis* showed normal mycelial growth under the frozen condition, and mycelial growth rate on frozen PDA at –1°C was faster than that on unfrozen PDA at the optimal growth tempera-

Table 2. Mycelial growth of snow molds on frozen potato dextrose agar (PDA)

Snow mold	Mycelial growth rate (mm/month, at –1°C)		
	Frozen PDA (A)	Unfrozen PDA (B)	A/B
Oomycetes			
Pythium iwayamai	0.0 ± 0.0	15.2 ± 32	0.0
Ascomycetes			
Microdochium nivale var. *nivale*	0.0 ± 0.0	7.2 ± 0.5	0.0
Racodium therryanum	17.5 ± 3.5	14.5 ± 2.8	0.8
Sclerotinia borealis	13.2 ± 8.5	20.4 ± 9.4	1.5
S. kitajimana	0.1 ± 0.0	4.8 ± 0.2	>0.0
S. nivalis	0.2 ± 0.0	8.2 ± 0.2	>0.0
S. trifoliorum	0.1 ± 0.0	12.8 ± 2.5	>0.0
Basidiomycetes			
Typhula incarnata	7.5 ± 1.5	28.3 ± 3.1	0.3
T. ishikariensis biological species I	18.0 ± 1.2	36.9 ± 2.2	0.5
T. ishikariensis biological species II	11.9 ± 0.5	15.5 ± 0.5	0.8
Supponuke disease fungus, *Athelia* sp.	0.0 ± 0.0	36.9 ± 2.6	0.0

Mycelial discs of 5 mm diameter were cut from the margin of actively growing colonies on potato dextrose agar (PDA) plates of the tested fungi, inoculated 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 media were transferred to –1°C

Regrowth of mycelia was determined every week for 1 month

Linear mycelial growth rate in triplicate was calculated per day after the initial lag period

ture range from 4° to 10°C. Our results support the findings of Tomiyama and previous studies by others (Röed 1960; Nissinen 1996) showing that this fungus adapts to harsh winters with soil freezing.

Higher plants tolerate freezing stress by means of avoidance of extra- and/or intracellular freezing (Levitt 1980). The latter mechanism includes tolerance of freeze-induced cell dehydration through enhanced osmotic conditions (Kacperska 1993). Many ascomycetes survive and grow under high osmotic stress (Grant 2004). Osmophile (high osmotic condition loving) and osmotolerance (high osmotic condition tolerant) in fungi provide us with clues to elucidate the adaptation mechanism of *S. borealis* to freezing. *S. borealis* can grow under low water potential conditions on PDA containing twice the concentration of medium ingredients (Tomiyama 1955), sucrose, KCl (Bruehl and Cunfer 1971), and D-mannitol (Namikawa et al. 2004). An increase in intracellular osmosis enhanced mycelial growth and shifted the optimal mycelial growth temperature from 10°–15°C to 4°C (Hoshino et al., unpublished results). However, mycelial growth of other snow molds such as *T. ishikariensis* and *T. incarnata* was inhibited at low water potential (Bruehl and Cunfer 1971). 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 showed a considerable decrease in dry weight production when water potential of the medium was reduced from –0.7 to –3 MPa. These results suggest that *S. borealis* is specialized to adapt to soil freezing conditions. *S. borealis* also utilize nutrients in unfrozen water on frozen PDA under low water potential condition. These physiological characteristics are important features for *S. borealis* to grow on plants in frozen soil.

Biochemical characteristics of snow molds

Response to temperature

Because of their unique feature of growing under snow, snow molds are expected to produce proteins and lipids that are active at low temperatures. However, mechanisms to maintain high metabolic activity at low temperatures have rarely been found from snow molds, although all biochemical and physiological processes required for growth function at low temperatures. Mycelial growth of *M. nivalis* was impaired with mycelia at 12°C, but general protein synthesis increased up to 25°C, suggesting that protein synthesis per se is not responsible for the sensitivity to temperature above 12°C (Cairns et al. 1995a). At least one biochemical or physiological process is damaged above 12°C.

Survival of *M. nivale* under snow cover involves both qualitative and quantitative alterations in fatty acid composition (Tronsmo et al. 2001). Triacylglycerol is the sole major component of the neutral lipid fraction of *M. nivale* and is considered to be present in the form of storage lipids (Istokovics et al. 1998). Neutral lipids accounted for approximately 75% of total lipid in mycelia grown at 15°C; however, at 4°C, this increased to 90% (Okuyama et al. 1998). As temperatures decreased from 25° to 10°C, levels of linolenic acid (18:3) increased at the expense of linoleic (18:2) and oleic acid (18:1). A further drop in temperature, from 10° to 4°C, caused few notable differences in fatty acid composition; instead, fatty acids accumulated as triacylglycerol at 4°C, at the expense of biomass production (Okuyama et al. 1998). These results suggest that *M. nivale* preferentially accumulates triacylglycerol containing linolenic acids as storage lipid in response to low temperatures.

There is considerable evidence suggesting that membrane composition is critical to the ability of fungi to grow over specific temperature ranges (Robinson 2001). Because membrane fluidity varies with the degree of unsaturation of lipids, the abundance of polyunsaturated fatty acids (18:2 and 18:3) among the phospholipids of *M. nivale* would enhance the ability of the fungus to survive at low temperatures (Istokovics et al. 1998). Polar lipid fractions of *M. nivale*, *T. incarnata*, and *T. ishikariensis* contain betaine lipid (diacylglyceryl-trimethylhomoserine), which has not been found in nonphotosynthetic microorganisms. However, the physiological function of this lipid in *M. nivale* is still unknown and should be further investigated.

Major sclerotinial proteins of *S. borealis* and *C. psychromorbidus* were absent from vegetative hyphae during growth at 5°C, but significant accumulation occurred in hyphae upon prolonged exposure to a relatively high temperature, 10°C, and the lethal temperature at 25°C. In contrast, low levels of sclerotinial proteins were detected in the vegetative hyphae of *T. incarnata* and *T. ishikariensis* during growth at 5°C (Newsted and Hunter 1988). When *S. borealis*, *Coprinus* spp., *T. ishikariensis*, and *T. incarnata* were incubated at 4°C and then at the lethal temperature at 22° or 30°C, the number of protein bands in the soluble fraction of mycelia decreased significantly during incubation at the lethal temperatures (Newsted et al. 1985).

One reason for the inability of growth or irregular growth at high temperature between 10° and 20°C in *S. borealis* and *T. ishikariensis* may be ascribed to temperature-induced modification of proteins, causing the loss of vital properties of intracellular proteins. Two-dimensional native-gel electrophoresis at different temperatures revealed that some intracellular proteins of *T. ishikariensis* group III isolates were modified (probably denatured) at the lethal temperature of 15°C (Hoshino et al. 1997a). Oxygen uptake of snow molds at maximum growth to sublethal temperatures is higher than at its optimum growth temperatures (Ward 1966a,b, 1968a,b; Dejardin and Ward 1971). Excess respiration probably induces denaturing of intracellular proteins by oxidation. Isolates of *T. ishikariensis* group III showed relatively normal growth at 10°C on PDA containing exogenous free-radical scavengers (to inhibit protein oxidation) such as ascorbic acid or β-carotene (Hoshino et al., unpublished results). These results suggest that *T. ishikariensis* group III has the same temperature range for mycelial growth as those of other groups and that the fungus cancels oxidative stress at sublethal temperatures by free-radical scavengers obtained from host plants. Therefore, isolates of group III are probably most dependent on hosts among the isolates of *T. ishikariensis*.

Cold-active enzymes

Snow molds produce various cell wall-degrading enzymes, as is the case with other phytopathogens. When hyphae of *C. psychromorbidus* (Gaudet and Kokko 1985) and *T. ishikariensis* (Ohshiman et al. 1995) penetrate the intact cuticle of epidermal cells, the host cell cuticle seems to be dissolved enzymatically at the penetration site. Activity of cellulase and hemicellulase was detected in *C. psychromorbidus* (Inglis et al. 2000) and *T. ishikariensis* (Mulanax and Huber 1972). Polygalacturonase activity was found in *S. borealis* (Takasawa et al. 1997; Takahashi et al. 2002; Takeuchi et al. 2002), *S. nivalis* (Ikeura et al. 2003; Watanabe et al. 2005), *S. trifoliorum* (Watanabe et al. 2003), and *T. ishikariensis* (Tanaka et al. 2003). Xylanase was found in *C. psychromorbidus* (Inglis et al. 2000) and *T. ishikariensis* (Mulanax and Huber 1972). *M. nivale*, *T. incarnata*, and *T. ishikariensis* produced lipolytic enzymes (Mulanax and Huber 1970; Hoshino et al. 1996, 1997b; Ohgiya et al. 1999; Tronsmo et al. 2001).

Because snow molds infect host plants under snow cover, extracellular cell wall-degrading enzymes should be active at low temperatures. Polygalacturonase activity of the psychrotolerant snow mold *S. nivalis* from bran culture incubated at 5°C was twice higher than that of culture at 20°C (Ikeura et al. 2003). Relative activity of polygalacturonase from the 5°C culture extract was about 10% higher than that of the 20°C culture extract. These results indicate that *S. nivalis* produces polygalacturonase isozymes that differ according to incubation temperature.

Microdochium nivale (syn.: *Monographella nivalis*) grows well at 5°C on cellulose, starch, inulin, and polygalacturonic acid, showing that this fungus degrades these major classes of plant carbohydrate polymers at low temperatures (Cairns et al. 1995a). Acid invertase (195 kDa) was purified from culture filtrate of *M. nivale*, and its activity was shown to increase exponentially with increasing temperature from 7° to 55°C (Cairns et al. 1995b). Q_{10} of acid invertase from *M. nivale* fell to 1.96 with increasing temperature from 5° to 15°C and to 1.60 from 40° to 50°C. Q_{10} represents the factor by which the rate of a reaction increases for every 10° rise in temperature. The thermal stability and thermal kinetic properties of this invertase are similar to those of mesophilic invertase (Cairns et al. 1995b). Intracellular lipase activity was detected in *M. nivale* mycelia only during the early exponential phase (Tronsmo et al. 2001). The thermal dependency of intracellular lipase activity was the same as that from mesophiles.

On the other hand, extracellular lipase activity of *M. nivale* occurs at low temperatures (Hoshino et al. 1996). The activity was the highest at 20°C and retained 19% of its maximum activity at 0°C. The extracellular lipase activity of *M. nivale* is higher at low temperatures than that of mesophilic fungi such as *Fusarium oxysporum* f. sp. *lini*. Its lipase is not as thermostable as that of mesophiles. Purified polygalacturonase of *S. borealis* (40 kDa) showed maximum activity at temperatures between 40° and 50°C. The fungal polygalacturonase retained 30% of its maximum activity at 5°C (Takasawa et al. 1997). The enzyme remained active for more than 2 years at 5°C but was inactivated when kept overnight at room temperature or heated at 50°C for 30 min. The presence of a low molecular mass molecule (1.8–6 kDa) maintained the activity at low temperatures in crude extract from bran culture (Takahashi et al. 2003). The optimum temperatures of cellulases (CMCases) and xylanases of *C. psychromorbidus* in filtrates from straw medium ranged from 25° to 55°C, but significant activity was observed at 5°C. Zymogram pattern indicated that both cellulases and xylanases consisted of two isozymes, i.e., 25 and 31 kDa for cellulases and 24 and 34 kDa for xylanases for *C. psychromorbidus* and FRLTB, respectively (Inglis et al. 2000). Purified extracellular lipase of *T. ishikariensis* (83 kDa) was most active at 30°C, and 23.4% of maximum activity remained at 4°C (Hoshino et al. 1997b). These results indicate that activity of extracellular cell wall-degrading enzymes of snow molds is optimal at the same temperature range as that of other phytopathogens but that their enzymes retain considerable activity at low temperatures.

Antifreeze proteins

Many living organisms have various biochemical and ecological strategies to protect themselves from intracellular freezing. Antifreeze proteins (AFPs) have the unique ability to attach to hexagonal ice crystals to inhibit their growth, resulting in depression of the freezing point of water and leading to protection of cells from freezing injury (Duman et al. 1993). Such depression of the freezing point is 500 times greater than that of colligative salts on a molar basis and occurs noncolligatively because of AFP-induced thermal hysteresis: a disparity between the melting and freezing points of the solution. AFPs also affect the morphology of ice crystals, creating bipyramidal ice crystals (Fig. 1A,C) (Duman et al. 1993).

AFPs were first found in body fluids from different kinds of polar fish. Some cold-adapted organisms (Duman et al. 1993), such as plants and microorganisms including fungi, lichens, and bacteria, produce AFPs as well. The mechanisms by which AFPs in fish inhibit the growth of ice crystals have been elucidated by many authors (e.g., Hoshino et al. 1999a). Considering the divergent structures of moderate AFPs from various fish AFPs, it is surprising that the mechanisms by which they inhibit ice crystal growth are so similar. Figure 1A shows a schematic diagram of the interaction between fish AFPs and ice. AFP binds to the prism face of an ice crystal, but there have not been reports of AFP binding to the basal face of an ice crystal. As a results, ice crystals, when associated with AFP, grow along their c-axis. The ice crystals are hexagonal in shape during the first stage of their growth (Fig. 1C,D).

Duman and Olsen (1993) detected thermal hysteresis from fruit bodies of four kinds of mushrooms: *Trametes versicolor*, *Flammulina velutipes*, *Pleurotus ostreatus*, and *Stereum* sp. Newsted et al. (1994) found intracellular 3.5 kDa proteins exhibiting eitopic homology to the Atlantic winter flounder type I AFP from *S. borealis*, *C. psychromorbidus*, and *T. incarnata*. In the cytosol of *T. ishikariensis* group III, a 30 kDa protein that strongly immunoreacted with the anti-AFP type I antibody was found, and the 30 kDa protein level in group III isolates was higher than that in *T. ishikariensis* group I isolates (Hoshino et al. 1998). Snider et al. (2000) reported ice nucleation and extracellular antifreezing activities (thermal hysteresis and ice crystal

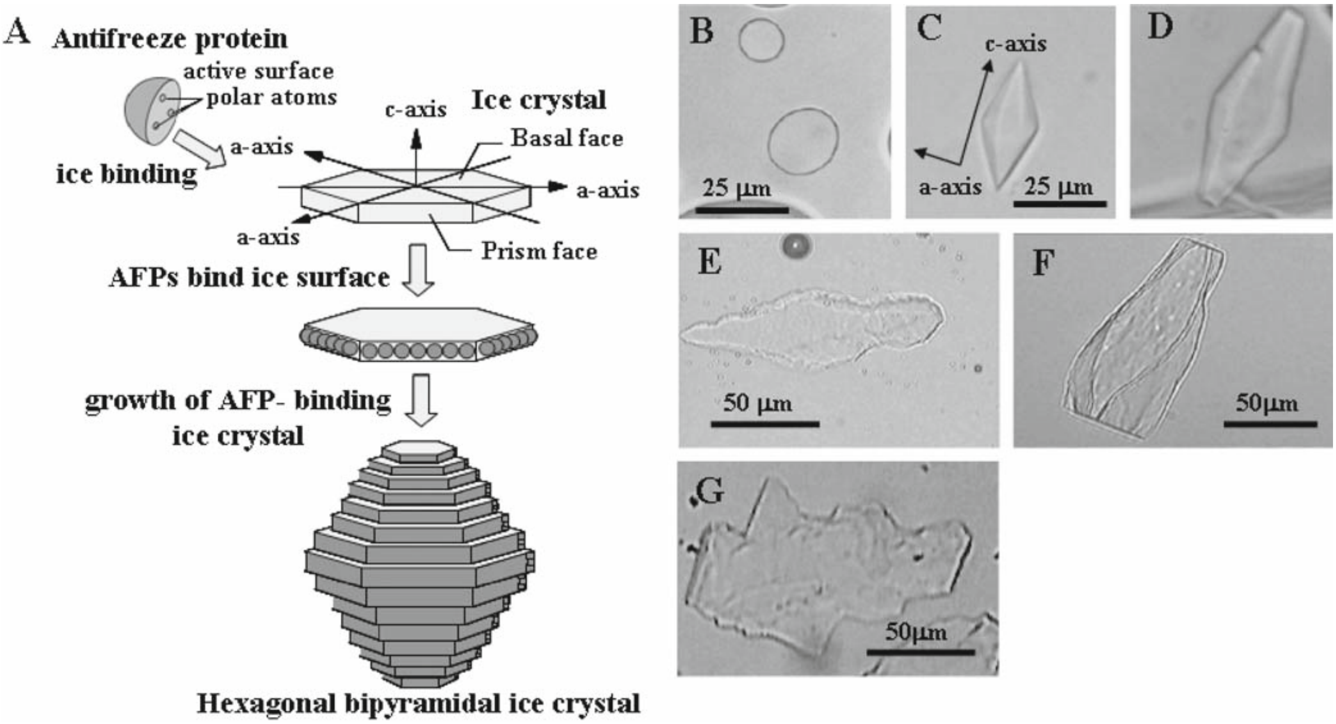

Fig. 1. Mechanisms of antifreeze protein (AFP)-induced ice growth inhibition and morphology of ice crystal. A Model of ice growth inhibition by moderate AFPs. AFPs bind preferentially to prism faces of the ice crystal by hydrogen bonds. **B** Control (25 mM Tris-HCl buffer, pH 7.5). **C** Type III AFP from Antarctic fish. **D** Apolast from cold-acclimated winter wheat. **E** AFP of *Typhula ishikariensis* (20 µM). **F** AFP of *Coprinus psychromorbidus* (20 µM). **G** Supponuke disease fungus, *Athelia* sp. (culture filtrate)

Table 3. Extracellular antifreeze activity of various snow molds

Snow mold	Thermal hysteresis (°C)	Modification of ice crystal shape
Oomycetes		
Pythium iwayamai	0.000	–
Pythium sp. HS group	0.000	–
Ascomycetes		
Microdochium nivale var. *nivale*	0.000	–
Racodium therryanum	0.000	–
Sclerotinia borealis	0.000	–
S. kitajimana	0.000	–
S. nivalis	0.000	–
S. trifoliorum	0.000	–
Basidiomycetes		
Coprinus psychromorbidus	0.014	+
Typhula incarnata	0.018	+
T. ishikariensis biological species I	0.115	+
T. ishikariensis biological species II	0.095	+
T. phacorrhiza	0.027	+
T. variabilis	0.015	+
Supponuke disease fungus, *Athelia* sp.	0.004	+

Potato dextrose broth cultures were grown for 3 months at –1°C to determine antifreeze activity

+, indicates modification of ice crystals shape from the circular form; –, indicates that the circular ice crystals were produced as in the control samples

From Hoshino et al. 2003b; Xiao et al., unpublished results

modification) in culture media from *C. psychromorbidus*, *T. incarnata*, *T. ishikariensis*, and *T. phacorrhiza*. Antifreezing activity was present only in culture filtrates of the basidiomycetous snow molds such as *C. psychromorbidus* and *Typhula* spp., but not in culture filtrates of other snow molds belonging to oomycetes and ascomycetes (Table 3; Xiao et al., unpublished results).

Antifreezing activity is based on the covering of AFP molecules on the ice crystal surface and the inhibition of ice crystal growth. Thus, diffusion of AFPs secreted in the extracellular environment does not support mycelial growth under subzero temperatures. The basidiomycetous snow molds *T. incarnata* and *T. ishikariensis* produce extracellular polysaccharides (H. Okuyama, personal communication), and they probably bind the AFP molecules they secreted. *M. nivale* does not produce AFPs but also produces extracellular polysaccharides such as cellulose (Schweiger-Hufnagel et al. 2001) and fructan (Cairns et al. 1995a), and extracellular polysaccharides of *M. nivale* bind plant antifungal peptides and reduce activities of these peptides (R. Imai et al., unpublished results).

We purified and cloned AFPs from *C. psychromorbidus* and *T. ishikariensis* (Hoshino et al. 2003a,b). Thermal hysteresis activity of AFPs from *C. psychromorbidus* and *T. ishikariensis* was higher than that reported for fish and plants (Duman et al. 1993). Purified fungal AFPs from culture filtrates of basidiomycetous snow molds form unique ice crystals resembling "Stone Age knives" (Fig. 1E–G). These AFPs do not form proper hexagonal ice crystals, implying that they can probably bind to both prisms and basal surfaces of ice crystals as do insect AFPs (Duman et al. 1993) to produce irregular crystals (Xiao et al., unpublished results); this is why fungal AFPs have higher activity than typical moderate AFPs from fish. The AFPs from basidiomycetous snow molds including *T. ishikariensis* were considered to be a new class of AFPs because of their dissimilarity in gene sequences (Hoshino et al. 2003b). Recently, ice-binding proteins were cloned from an ice diatom (Janech et al. 2005) and a bacterium in Antarctica (Raymond et al. 2007), and these proteins showed high similarities with fungal AFPs. These findings suggest that fungal AFP homologues are widely distributed in several kingdoms, implying the possibility of horizontal gene transfer between eukaryotic microbes and prokaryotes (Raymond et al. 2007).

Extracellular AFPs do not have any effects on freeze resistance of mycelia in *T. ishikariensis*. Mycelia of *T. ishikariensis* isolated from Moscow failed to survive in the medium containing endogenous AFPs after freeze–thaw cycles, but an extremely freeze-resistant isolate from Siberia survived the same condition (Hoshino et al. 2006a). We previously obtained the same results using a medium without fungal AFPs (Hoshino et al. 2001a). AFPs also accumulate in sporophores of *T. incarnata* and *T. ishikariensis* in nature (Hoshino et al., unpublished results). Cells of fruit bodies such as basidia are less freeze resistant than mycelia in *Typhula* spp. The volume of differentiated cells in basidiocarps is larger than that of mycelial cells, and the supercooling temperature of sporophore cells is lower than that of that of mycelial cells. Therefore, cell damage by freezing is more serious in the fruit body in *Typhula* spp. Other fruit bodies of autumn-collected mushrooms have thermal hysteresis activity (Duman and Olsen 1993). The primary significance of AFP in basidiomycetes is probably to protect their differentiated cells for reproduction. Basidiomycetous snow molds such as *Typhula* spp. can grow at temperatures less than –5°C (see Table 1). However, once the ambient environment freezes, mycelial growth of these fungi is

Table 4. Biochemical and physiological mechanisms for cold adaptation in snow molds

Snow mold	Mechanisms	Reference
Oomycetes		
Pythium iwayamai	Oospore maturation	Takamatsu 1989
	Freeze resistance of oospores	Tojo et al., unpublished results
Ascomycetes		
Microdochium nivale	Increasing membrane fluidity	Tronsmo et al. 2001
Sclerotinia borealis	Osmotic stress tolerance	Hoshino et al., unpublished results
Basidiomycetes		
Typhula spp.	Production of antifreeze proteins	Snider et al. 2000; Hoshino et al. 2003a,b

arrested (see Table 2; Tomiyama 1955; Hoshino et al., unpublished data): a frozen environment does not allow mycelial growth of basidiomycetous snow molds. Our hypothesis on the physiological significance of AFPs in basidiomycetous snow molds is that they extend the application range of AFP to mycelia so that mycelia may be able to grow at subzero temperatures.

Mechanisms for freeze resistance in snow molds

Many fungi belonging to diverse taxa developed their habitat under snow to establish their niche as snow molds. Snow molds differ in ecology, and this distinction applies to the mechanisms they require to survive subzero temperatures. Table 4 summarizes mechanisms of freeze resistance in various taxa of snow molds. *Pythium* spp. are less freeze resistant than other snow molds, and they do not produce AFPs (Hoshino et al. 2003b). Tojo et al. investigated the freeze resistance of *Pythium* spp. from both polar regions. Mycelia of all tested isolates were destroyed after freezing, and only oospores and hyphal swellings survived the freezing stress (Tojo et al., unpublished results). Low temperatures promote maturation of oospores in oomyceteous snow molds (Takamatsu 1989). *Pythium* spp. can grow under snow, but they are simply psychrotolerant. These fungi exploit low temperatures to break the dormancy of propagules.

Many fungi choose to keep membrane fluidity for cold adaptation (Robinson 2001). *M. nivale* changes fatty acid composition quantitatively and qualitatively at low temperatures (Okuyama et al. 1998) and accumulates unsaturated lipids; the amount of polyunsaturated fatty acids (18:2 and 18:3) increases as the phospholipids for membrane fluidity vary (Istokovics *et al.* 1998). Ascomycetes do not produce extracellular AFPs (Snider et al. 2000; Hoshino et al. 2003a). *S. borealis* can grow at temperatures below –7°C (Ward 1966a, 1968b), and its growth is accelerated on a frozen medium at subzero temperatures (Tomiyama 1955). Thus, *S. borealis* does not need extracellular AFPs for cold adaptation. Bacteria in permafrost soil can retain metabolic activity and continue to grow under subzero temperatures (Rivkina et al. 2000). Permafrost soil is known to contain unfrozen water (Ershov 1998), and cold-adapted bacteria grow in the unfrozen water that contains concentrated soluble substrates such as ions. *S. borealis* may also utilize nutrients in unfrozen water on frozen PDA at low water potential. These physiological characteristics are important features for *S. borealis* to grow on host plants in frozen soil.

Basidiomycetous snow molds, *Typhula* spp., can also grow at temperatures below –5°C (Smith 1986; Smith et al. 1989; Matsumoto et al. 1996; Hsiang et al. 1999), but mycelial growth of these fungi on a frozen medium is arrested. A frozen environment is not suitable for mycelial growth of basidiomycetous snow molds. AFPs are produced to prevent freezing of the extracellular environment in basidiomycetous snow molds. We hypothesize that the feature has evolved from AFPs in sporophores and that the role of AFPs shifted to protect their mycelia.

These findings suggested that snow molds have cold-adaptation mechanisms that differ at the phylum level. However, we do not know that this conclusion generally applies to all species in fungi and related kingdoms under snow: there must be exceptions. We should further investigate the various fungi adapted to cold environments.

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