Proc NIPR Symp. Polar Biol., 11, 112–118, 1998

FREEZING RESISTANCE AMONG ISOLATES OF A PSYCHROPHILIC FUNGUS, *TYPHULA ISHIKARIENSIS*, FROM NORWAY

Tamotsu HOSHINO[1], Anne Marte TRONSMO[2], Naoyuki MATSUMOTO[3], Tadashi ARAKI[4], Fawzy GEORGES[5], Takako GODA[1], Satoru OHGIYA[1] and Kozo ISHIZAKI[1]

[1] *Hokkaido National Industrial Research Institute, 17–2–1, Tsukisamu-higashi 2-jo, Toyohira-ku, Sapporo 062-8517*

[2] *Department of Plant Pathology, Plant Protection Centre, Norwegian Crop Research Institute, Fellsbygget, N-1432 Ås, Norway*

[3] *Department of Environmental Biology, National Institute of Agro-Environmental Sciences, 1–1, Kannon-dai 3-chome, Tsukuba 305-0856*

[4] *Institute of Low Temperature Science, Hokkaido University, Kita-19, Nishi-8, Kita-ku, Sapporo 060-0019*

[5] *Plant Biotechnology Institute, National Research Council, 100 Gymnasium Road, Saskatoon, SK, S7N 0W9, Canada*

Abstract: Frost tolerance was determined in Norwegian varieties of groups I and III of the psychrophilic fungus *Typhula ishikariensis*. When mycelia of group I and III strains were rapidly frozen to −40°C in a program freezer at a cooling rate of 20°C/h, the regrowth of group I strains was delayed at their optimal growth temperature (10°C), whereas the regrowth of group III strains was not affected at their optimal growth temperature (2°C). Furthermore, when group I and III strains were rapidly frozen and regrew at 0°C, the average temperature during snowfall in the northernmost area of Norway, group I strains, in contrast to group III strains, did not regrow at all. These results suggest that group III strains are more resistant than group I strains to rapid freezing. Group III strains are well adapted to climatic conditions in northernmost Norway. Freezing points of mycelia of group III strains were determined by calorimeteric analysis. Mycelia of group III strains froze at temperatures higher than −10°C. Therefore, the freezing resistance of group III strains might be based on extracellular ice formation. In the cytosol of group III, a 30 kDa protein that strongly immunoreacted with the anti-antifreeze protein (AFP) I antibody was found, and the 30 kDa protein level in group III strains was higher than that in group I strains. These results suggest that the difference in the distribution pattern of both groups can be ascribed, at least partially, to the difference in freezing resistance of mycelia.

key words: snow mold, psychrophile, *Typhula ishikariensis*, freezing resistance, Norway

Introduction

Snow mold fungi are psychrophilic fungal pathogens that infect winter cereals

grown in Nordic countries, North America, Russia and Japan. Among these snow molds, *Typhula ishikariensis* S. Imai has evolved several infraspecific taxa adapted to different winter climates (MATSUMOTO, 1992, 1995). *Typhula ishikariensis* in Norway has been classified into three groups (groups I, II and III) by MATSUMOTO and TRONSMO (1995) according to genetic relationships and cultural characteristics. These three groups also have a different distribution pattern: group I and II are predominant in the southern and middle parts of Norway, while group III is most prevalent in the north (MATSUMOTO *et al.*, 1996), where grasses and wheat are killed due to severe subzero temperatures after intermittent snow melt during winter (ÅRSVOLL, 1973). This distribution pattern indicates that group III seems to be more adapted than the other two groups to lower temperatures.

Phytopathogenic fungi survive the severe winter in the form of spores and sclerotia, and they resume infection the following season (SMITH, 1993). On the other hand, snow mold fungi develop mycelia in winter. Freezing resistance of the mycelia of snow mold is considered critical in determining ecological distribution. Freezing seriously affects the winter survival of sclerotia in *Rhizoctinia solani* (HYAKUMACHI, 1991). However, there have been very few biochemical studies on the freezing resistance of fungal mycelia, including *Typhula* spp. In this study, we attempt to elucidate the low temperature adaptation of group III *T. ishikariensis*.

Materials and Methods

Fungal strains and media

Six strains each of group I (1-1-1, 1-2-1, 1-2-3, 2-2-5, 2-5A-8 and 5-5-24) and group III (4-3-3, 5-5-22, 6-1-2, 6-1-9, 6-3-1 and Tana) were used in the experiments. These strains were collected in Norway in 1992 (MATSUMOTO and TRONSMO, 1995). Cultures were maintained on potato-dextrose agar (PDA) slants at 0°C.

Determination of the mycelial freezing resistance of *T. ishikariensis*

Mycelial freezing resistance was determined by the regrowth of mycelia after freezing stress. A mycelial disc 5 mm in diameter, cut from the margin of an active growing colony, was placed on a PDA plate 2.5 cm in diameter and frozen to −40°C in a program freezer (custom-made instrument, Ebara Co. Ltd., Tokyo) at a cooling rate of 1°C/h or 20°C/h. After freezing, the mycelial discs were thawed at 2°C for 16 h, transferred to fresh PDA plates, and incubated at respective optimal growth temperatures (10°C for group I and 2°C for group III). Mycelial growth was observed daily for up to 30 days.

We carried out the following experiments on the regrowth of groups I and III after freezing stress under simulated natural temperature conditions in which snow mold fungi were subjected to temperatures of −2°C to +2°C under snow cover. After rapid freezing (−40°C, 20°C/h), the mycelial discs were thawed at 2°C for 16 h, transferred to fresh PDA plates, and incubated at 0°C. Mycelial growth was observed over a period of 30 days.

Determination of the mycelial freezing point of *T. ishikariensis*

Freezing points of mycelia were determined by the calorimeterical method (SAKAI, 1982). Group III strains produced more aerial mycelia than did group I strains. Original mycelia of three strains of group III (4-3-3, 5-5-22 and Tana), which were cultured on PDA plates at 0°C, were used as the materials. Twenty mg (wet weight) of mycelia covered with thermocouples (I.D. 0.1 mm, Rika Denki Co. Ltd., Tokyo) were frozen to −80°C in the program freezer at a cooling rate of 10°C/h. The release of heat caused by mycelial freezing was observed.

Immunological detection of an antifreeze protein (AFP)-like protein in *T. ishikariensis*

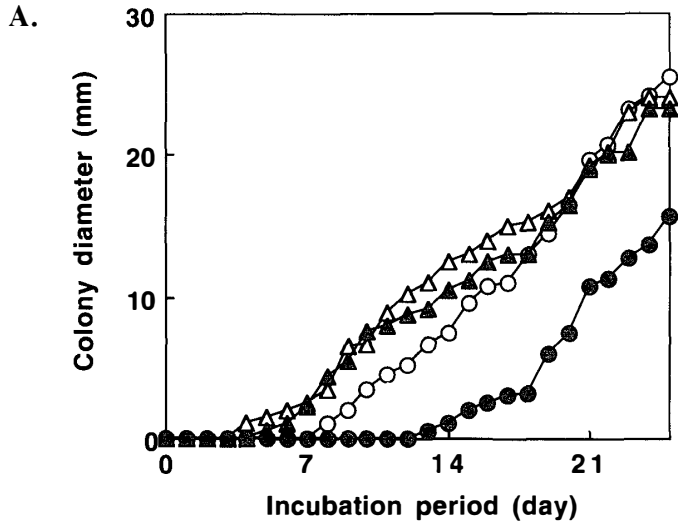
Mycelia were cultured in 100 m*l* of potato-dextrose broth for 3 weeks at 0, 5 or 10°C with vigorous shaking. They were collected by filtration and washed 3 times in cold water. Approximately 5 g (wet weight) of mycelia was ground using a mortar and pestle, together with 1 g of sea sand and 5 m*l* of 200 mM Tris-HCl buffer (pH 6.8) containing 10 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride and 30% (v/v) glycerol. The extract was obtained by centrifugation at 100000 g for 60 min at 4°C. Protein concentration of the extract was measured with the BCA protein assay reagent (Pierce, IL, USA).

One-dimensional tricine-SDS-PAGE of the soluble protein sample was performed according to SCHÄGGER and VON JAGOW (1987). AFP-like proteins in group I and III strains were detected by immunoblotting using the anti-Atlantic winter flounder AFP I antibody. The preparation of anti-AFP I immunoserum has been described previously (GEORGES *et al.*, 1990).

Results and Discussion

Strains of groups I and III were cultured at 0°C in order to simulate conditions under snow cover. Both groups reacted differently to the freezing stress. Examples of selected strains (strain 1-2-3 for group I and strain 4-3-3 for group III) are shown in Fig. 1A. Both strains survived temperatures lower than −40°C at slow (1°C/h) and rapid (20°C/h) cooling rates. Slow freezing (1°C/h) did not affect hyphal growth in either group (data not shown). However, after rapid freezing, regrowth of group I strains started 1 week later than that of the control at the optimal growth temperature (Fig. 1A). This was not the case with group III strains. Moreover, group III strains produced many aerial mycelia after rapid freezing stress (Fig. 1B). These results indicate that rapid freezing stress significantly inhibits hyphal cell growth of group I strains and that group III strains are frost-resistant. Group III strains grow in Finnmark, in the northernmost part of Norway, where the air temperature in winter decreases at the same rate as in our rapid freezing and the soil temperature also rapidly decreases due to shallow snowfall (SUNDHEIM, 1996). In contrast, group I strains predominate in the southern mountainous region of Norway, where the soil temperature does not decrease so rapidly due to the deep snow cover. Thus, it is thought that group III strains, which have acquired the ability of frost resistance, are adapted to the severe winter environment in Finnmark.

Figure 2 shows the regrowths of group I and III strains at 0°C at 30 days after



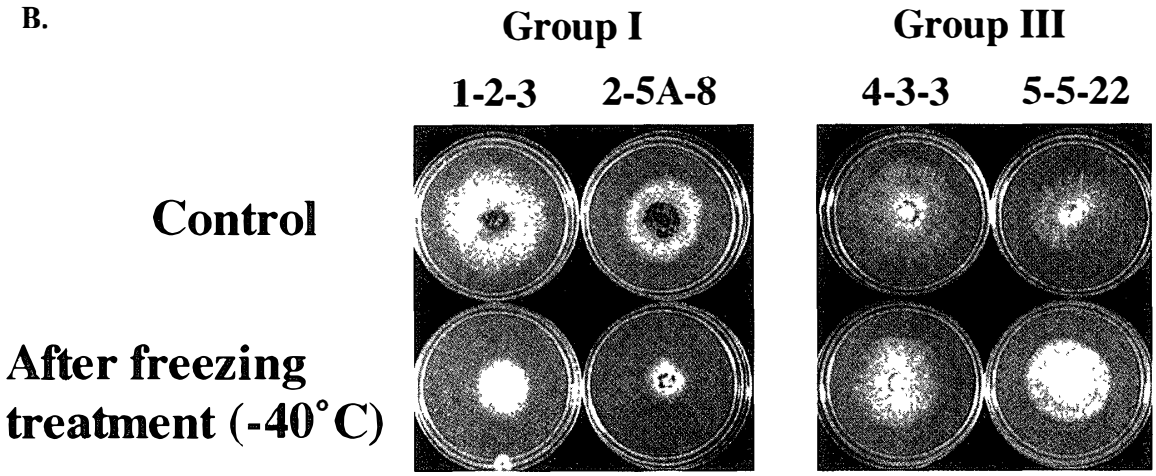


Fig. 1 Effects of freezing stress on mycelial regrowth and macromorphology of Norwegian *T ishikariensis*. Mycelial discs were frozen at −40°C. Cooling rate was 20°C/h Regrowth temperatures of groups I and III were 10 and 2°C, respectively.
A. Mycelial growth after freezing stress. ○: group I (strain 1-2-3), control; ●: group I (strain 1-2-3), frozen, △: group III (strain 4-3-3), control; ▲: group III (strain 4-3-3), frozen
B Macromorphology (growth for 30 days).

rapid freezing. Group III resumed growth after freezing. Group I, however, did not regrow under this temperature condition. Group I strains lost their ability to grow under snow cover by rapid freezing. These results suggest that resistance to freezing stress is an important factor in the predominance of group III strains in the northernmost region of Norway, since fungi under snow cover are subject to a temperature of around 0°C following a rapid temperature change in Finnmark.

As shown in Table 1, mycelia of all the group III strains tested froze at temperatures higher than −10°C. However, these strains survived even at −80°C and grew again after thawing on a new medium at 2°C (data not shown). These results suggest that freezing resistance of group III strains is based on extracellular ice formation, because

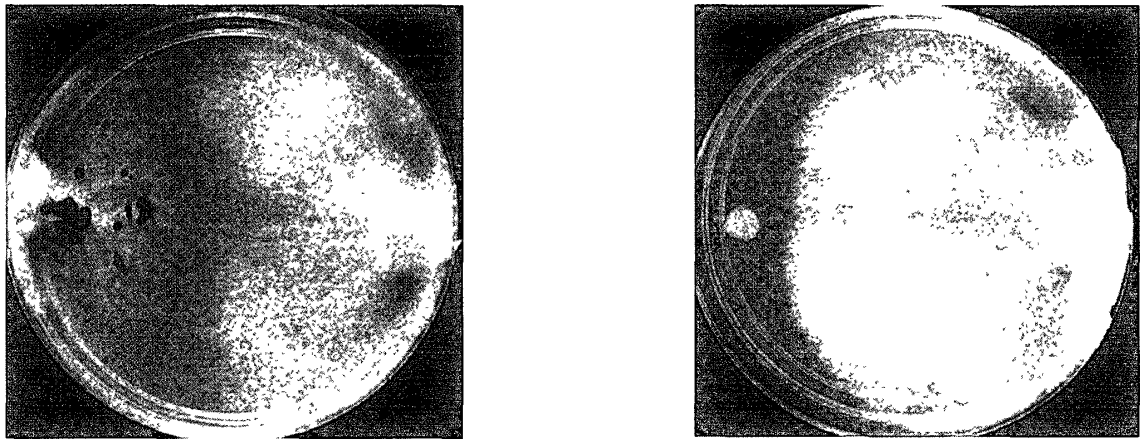


Fig 2. Effect of freezing stress on the macromorphology of Norwegian *T ishikariensis* Mycelial discs were frozen at −40°C Cooling rate was 20°C/h Regrowth temperature was 0°C. Group I strain (1-2-3) is on the left and group III strain (4-3-3) is on the right on each plate
A Control (grew for 30 days)
B. Frozen (grew for 30 days).

Table 1 Freezing temperature of mycelia of Norwegian *T ishikariensis* group III strains

Strain name (group III)	Freezing point (°C)
4-3-3	−6 1
5-5-22	−9 8
Tana	−8 6

extracellular ice formation prevents intracellular ice formation, which causes cryoinjury (SMITH, 1993). Similar results have also been reported in plants such as winter rye seedlings or azalea buds, which freeze at −40°C but can survive the freezing stress. It has been suggested that the frost-resistance mechanism in these plants is extracellular ice formation, which inhibits the growth of the ice crystals into cytosolic fractions (SAKAI, 1982).

Snow mold fungi can grow at subzero temperatures (SMITH, 1986). Under these conditions, the cytosol remains unfrozen and plays an important role in supercooling. A small molecular mass (3.5 kDa) protein, which is epitopically homologous to the Atlantic winter flounder AFP I, was found in the snow mold fungi *Coprinus psychromorbidus*, *Myriosclerotinia borealis* and *T. incarnata* (NEWSTEAD *et al.*, 1994). The AFP-like protein found in *C. psychromorbidus* is thought to be a cold-induced protein. However, AFP-like proteins with a similar molecular mass were not detected in *T. ishikariensis* (Fig. 3A), in contrast to the case in *T. idahoensis* (NEWSTEAD *et al.*,

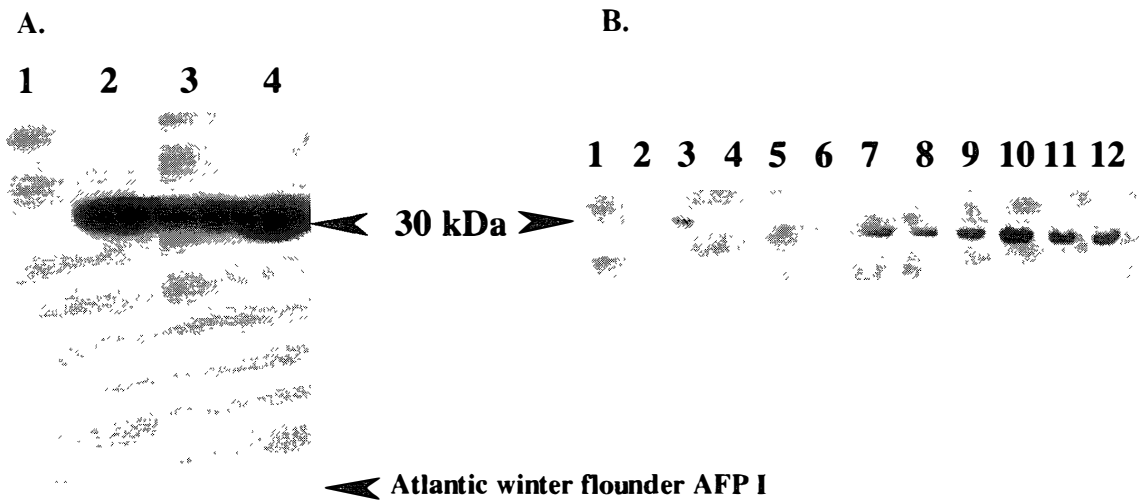


Fig. 3. Immunological detection of antifreeze proteins in Norwegian *T. ishikariensis*.
A. Effect of cultivation temperature on expression of AFP-like proteins in the cytosolic fraction of a group III strain (4-3-3). Lane 1, *ca.* 2 μg of Atlantic winter flounder AFP I (3.5 kDa). Lanes 2, 3 and 4, total soluble protein (*ca.* 25 μg each) extracted from hyphae grown at 0, 5 and 10°C, respectively.
B. Distribution of 30 kDa, an AFP-like protein, in strains of groups I and III. Lanes 1-6, total soluble proteins (*ca.* 10 μg each) extracted from group I strains 1-1-1, 1-2-1, 1-2-3, 2-2-5, 2-5A-8 and 5-5-24, respectively. Lanes 7-12, total soluble proteins (*ca.* 10 μg each) extracted from group III strains 4-3-3, 5-5-22, 6-1-2, 6-1-9, 6-3-1 and Tana, respectively. All group I and III strains were cultivated at 0°C.

1994). Interestingly, a 30 kDa protein of *T. ishikariensis* strongly immunoreacted with anti-AFP I antibody, and its level was not changed by the growth temperature (Fig. 3A), indicating that this protein is not cold-inducible. The 30 kDa AFP-like protein was also found in *C. psychromorbidus*, and its level was not changed by the growth temperature (NEWSTEAD *et al.*, 1994). As shown in Fig. 3B, levels of the 30 kDa AFP-like protein were higher in group III strains than in group I strains. These results suggest that the expression level of this protein seems to be critical in determining the freezing tolerance of Norwegian *T. ishikariensis*, although the biological function of the 30 kDa AFP-like protein is not known. Constitutive and common occurrence of the 30 kDa AFP-like protein in group III strains suggests that this protein plays an important role in freezing resistance of these organisms. Further studies are needed to obtain direct evidence of the involvement of this AFP-like protein in the freezing resistance of Norwegian *T. ishikariensis*.

Acknowledgments

We wish to thank Dr. K. TANNO, Institute of Low Temperature Science, Hokkaido University, for his technical advice and valuable comments.

References

ÅRSVOLL, K. (1973). Winter damage in Norwegian grassland. 1968–1971 Meld. Norg. LangbrHøgsk., **52** (3) 21p.

GEORGES, F., SALEEM, M. and CULTLER, A. J (1990): Design and cloning of a synthetic gene for the flounder antifreeze protein and its expression in plant cells Gene, **91**, 159–165

HYAKUMACHI, M. (1991). Cold adaptation of sugar beet root-rot fungus by freezing-thawing treatment. Shokubutsu Bôeki (Plant Protection), **45**, 144–147 (in Japanese).

MATSUMOTO, N. (1992). Evolutionary ecology of the pathogenic species of *Typhula*. Trans Mycol Soc Jpn., **33**, 269–285

MATSUMOTO, N (1995) Ecological adaptations of low temperature plant pathogenic fungi to diverse winter climates Can. J. Plant Pathol., **16**, 237–240

MATSUMOTO, N and TRONSMO, A M (1995) Population structure of *Typhula ishikariensis* in meadows and pastures in Norway Acta Agric Scand., **45**, 197–201

MATSUMOTO, N., TRONSMO, A M and SHIMANUKI, T (1996) Genetic and biological characteristics of *Typhula ishikariensis* isolates from Norway. Eur J Plant Pathol., **102**, 431–439

NEWSTEAD, W J., POLVI, S., PAPISH, B., KENDALL, E., SALLEM, M., KOCH, M., HUSSAIN, A., CULTLER, A J and GEORGES, F (1994) A low molecular weight peptide from snow mold with epitopic homology to the winter flounder antifreeze protein Biochem Cell Biol., **72**, 152–156

SAKAI, A (1982) Frost resistance and cold adaptation of plants-physiology and ecology in winter Tokyo, Gakkai Shupan Senta, 21–28 (in Japanese)

SCHAGGER, H and VON JAGOW (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa Anal Biochem., **166**, 368–379

SMITH, D (1993) Tolerance to freezing and thawing Stress Tolerance of Fungi, ed by D H JENNINGS New York, Marcel Dekker Inc., 147–171

SMITH, J D. (1986) Winter-hardiness and overwintering diseases of amenity turgrass with special reference to the Canadian Prairies Tech. Bull. 1987-12E, Agriculture Canada, 81

SUNDHEIM, E (1996) Historic climatological data from 52 stations in Norway http://spvntpre.nlh.no/weather.

(Received January 22, 1997, Revised manuscript accepted July 22, 1997)