Mycorrhiza (2008) 18:385–392
DOI 10.1007/s00572-008-0190-5

ORIGINAL PAPER

Freezing tolerance of ectomycorrhizal fungi in pure culture

Tarja Lehto · Arlena Brosinsky · Helvi Heinonen-Tanski · Tapani Repo

Received: 28 March 2008 / Accepted: 11 July 2008 / Published online: 8 August 2008

Abstract The ability to survive freezing and thawing is a key factor for the existence of life forms in large parts of the world. However, little is known about the freezing tolerance of mycorrhizal fungi and their role in the freezing tolerance of mycorrhizas. Threshold temperatures for the survival of these fungi have not been assessed experimentally. We grew isolates of *Suillus luteus*, *Suillus variegatus*, *Laccaria laccata*, and *Hebeloma* sp. in liquid culture at room temperature. Subsequently, we exposed samples to a series of temperatures between +5°C and −48°C. Relative electrolyte leakage (REL) and re-growth measurements were used to assess the damage. The REL test indicated that the lethal temperature for 50% of samples (LT_{50}) was between −8.3°C and −13.5°C. However, in the re-growth experiment, all isolates resumed growth after exposure to −8°C and higher temperatures. As many as 64% of *L. laccata* samples but only 11% in *S. variegatus* survived −48°C. There was no growth of *Hebeloma* and *S. luteus* after exposure to −48°C, but part of their samples survived −30°C. The fungi tolerated lower temperatures than was expected on the basis of earlier studies on fine roots of ectomycorrhizal trees. The most likely freezing tolerance mechanism here is tolerance to apoplastic freezing and the concomitant intracellular dehydration with consequent concentrating of cryoprotectant substances in cells. Studying the properties of fungi in isolation promotes the understanding of the role of the different partners of the mycorrhizal symbiosis in the freezing tolerance.

Keywords Ectomycorrhiza · Freezing · Hydrophilic · Hydrophobic · Temperature · Tolerance

T. Lehto (✉) · A. Brosinsky
Faculty of Forest Sciences, University of Joensuu,
P.O. Box 111, 80101 Joensuu, Finland
e-mail: tarja.lehto@joensuu.fi

H. Heinonen-Tanski
Department of Environmental Science, University of Kuopio,
P.O. Box 1627, 70211 Kuopio, Finland

T. Repo
Joensuu Research Unit, Finnish Forest Research Institute,
P.O. Box 68, 80101 Joensuu, Finland

Introduction

The ability to survive the winter is a key factor for the existence of life forms in cold climates. Although soil temperatures generally are not as extreme as air temperatures, the soil commonly freezes for several months each year in the boreal zone. Soil temperature may be one of the most limiting factors for the performance of trees at the tree line, particularly through the effects of low temperature on nutrient acquisition (Karlsson and Nordell 1996).

Fine roots of woody plants do not tolerate as low temperatures as the aboveground parts of the same plants (Lindström and Nyström 1987; Bigras and Dumais 2005). On the other hand, field studies on fine roots do not suggest massive dieback in spring in areas with regular soil frost (Makkonen and Helmisaari 2001). In a detailed study on *Pinus sylvestris* and *Picea abies* seedlings, Laiho and Mikola (1964) concluded that only a very small part of the mycorrhizas died during the winter in the nursery, and the usual reason for death was soil movement caused by ground frost, which physically broke long roots. In field studies in temperate southern Sweden, the amount of external mycelium in ingrowth bags increased between October and April, indicating more growth than mortality (Wallander et al. 2001). However, in sites with below-zero

soil temperatures, there is still uncertainty about the winter survival of the mycelia.

There are few previous studies on the freezing tolerance of mycorrhizal fungi, and in most, the temperatures have been mild. Moser (1958) found that many ectomycorrhizal isolates survived an extended period of −5°C. Tibbett et al. (2002) showed that both arctic and temperate isolates of *Hebeloma* survived −5°C. France et al. (1979) tested a number of ectomycorrhizal isolates for their tolerance to −10°C for 48h and found that almost all fungi survived this, but there were differences in the rate of recovery. Corbery and Le Tacon (1997) studied the freezing tolerance with the objective of testing long-term storage methods. In their study, some fungi survived −196°C in the presence of glycerol as an external cryoprotectant. In most studies, the cooling and warming rates have not been mentioned, although Corbery and Le Tacon (1997) found an effect of cooling rate on the survival. To the best of our knowledge, no studies have been published with exposure to a range of different subzero temperatures to assess the threshold temperatures for survival.

A classification of ectomycorrhizal species into hydrophobic and hydrophilic has been suggested by Unestam and Sun (1995) based on distinct properties of the cell wall. Hydrophilic species, such as *Laccaria* and *Hebeloma* species, are thought to be able to transport water in the apoplast. By contrast, hydrophobic species such as *Suillus* species form complex mycelial cords that transport water in the symplast. Only a small part of their mycelium is hydrophilic, with direct contact to soil water. We suggest that the difference in the wetting properties of the hyphal cell wall may make a difference for the freezing tolerance of hydrophobic and hydrophilic fungi. As the ice crystal formation is expected to start at the surfaces of cell walls, it may be affected by their wetting properties.

We hypothesise that (1) different species have different threshold temperatures for survival, (2) hydrophobic species tolerate lower temperatures than hydrophilic, and (3) abrupt changes in temperature are more detrimental than gradual. We exposed pieces of mycelium of two hydrophobic and two hydrophilic species to a range of low temperatures and afterwards assessed their performance by relative electrolyte leakage, survival, and re-growth tests.

Materials and methods

The fungal isolates used were *Laccaria laccata*, *Hebeloma* sp, *Suillus luteus*, and *Suillus variegatus* from the culture collection of University of Kuopio, culture numbers 93, 74, 20, 19, respectively. *L. laccata* and *Hebeloma* were originally isolated in Sweden, Lund and both *Suillus* strains in Sweden, Uppsala. In the culture collection, they had been stored in sterile water at 3–4°C, with reinoculation every 8–9months. Cultures were first grown at room temperature (23°C) on Hagem agar. Subsequently, pieces from the actively growing edges of 4-week-old cultures were transferred to 250-ml glass jars containing 100ml of liquid growth medium (modified Melin-Norkrans medium, Mason 1980) and grown for 5 or 3weeks for the REL or re-growth tests, respectively.

In the first experiment, relative electrolyte leakage (REL) was used to assess the damage caused by the exposure treatments (Radoglou et al. 2007). Prior to exposure, intact pieces of mycelium were taken from the culture medium and rinsed twice with deionised water (Milli-Q, Millipore) to remove the culture solution. The mycelium was placed in test tubes, which were closed, and subjected to different frost temperatures. Hence, during the exposure, the mycelia were covered by a thin layer of water. Any ion leakage from the tissue during the freezing and thawing remained in the test tube and was included in the electrical conductivity measurement.

The exposure temperatures were +5°C, −4°C, −6°C, −8°C, −12°C, −30°C, and −48°C. The temperature was first lowered to +5°C within 15min, and then the rate of decrease was 5°C h^{-1}. The destination temperature was maintained for 4h, and subsequently, the temperature was raised again at the rate 5°C h^{-1}. In the end, the temperature was kept at +5°C for 1h before opening the chamber.

After the exposure, 5ml of deionised (Milli-Q) water was added to each tube. After 2-h shaking on a bench top shaker (200rpm), the conductivity of solution in test tubes (L_1) was measured (CDM92-conductivity meter with CDC641T-electrode, Radiometer, Copenhagen, Denmark). After this, the samples were heat-killed by placing the tubes in a water bath at 92°C for 20min. The tubes were shaken for another 2h, and the conductivity was measured again (L_2). The number of replicate tubes varied from four to seven, the largest numbers being in the middle range of exposure temperatures where the most prominent changes in the ion leakage were expected to occur.

Relative electrolyte leakage was calculated as

$$\mathrm{REL} = \frac{L_1}{L_2}. \quad (1)$$

For an estimation of the lethal temperature for 50% of the samples (LT_{50}), a sigmoid function was fitted to the REL data (Repo and Lappi 1989). Freezing tolerance of each isolate was calculated as the inflection point, C, of the sigmoid function shown in Eq. 2.

$$y = \left(\frac{A}{1 + e^{B(C-x)}}\right) + D, \quad (2)$$

where y is REL, x is exposure temperature, A and D define the asymptotes, and B is slope at the inflection point C.

In the second experiment with re-growth test, the exposure temperatures were +5°C, −4°C, −8°C, −12°C, −30°C, and −48°C. The mycelia were rinsed in sterile deionised water and transferred to empty Petri dishes. The dishes were sealed with Parafilm to avoid drying and subjected to temperature treatments as before. Afterwards, the mycelia were transferred to fresh Hagem agar plates and incubated at room temperature for 4weeks. The mycelial growth was recorded weekly without magnification. The measure taken was the extension (millimeter) of the new growth from the edge of the original piece of mycelium on one side.

To test the effect of the slow cooling and warming rate as opposed to sudden changes in temperature, another set of samples was exposed to −12°C by placing the plates directly from room temperature to the exposure chamber. After 4-h exposure, they were removed immediately. Otherwise, this set was treated in the same way as the other ones in the second experiment.

The number of samples (pieces of fungal mycelium) in the re-growth experiments varied from 7 to 21. The median was 13. The lowest sample numbers were in *L. laccata* and *Hebeloma* sp. at −30°C and *Hebeloma* sp at −48°C. The reason for the variability was to concentrate the largest sample numbers in the middle temperatures, which were expected to show more variability than the lowest and highest temperatures (which were expected to show 0% and 100% survival, respectively).

The experimental designs of the experiments comprised either a range of exposure temperatures or rate of cooling and warming as a factor (treatment). In the re-growth experiments, time was another factor. In the re-growth experiments, the data were subjected to analysis of variance for repeated measures, and Tukey's test was used for pairwise comparisons of the response to temperature (over all time points). To explore the growth of the surviving colonies independent of the dead colonies, the analysis was done also excluding the colonies that did not show growth by the first week. Dunnett's T3 test was used for pairwise comparisons, as it does not assume equal variances. Results with $P < 0.05$ are taken as significant. The statistical analysis made use of the general linear model procedure, SPSS, v. 15.0.

Results

The estimated LT_{50} by REL was −8.3°C for *S. luteus*, −11.5°C for *L. laccata*, and −13.5°C for *Hebeloma* sp. (Fig. 1). The data for *S. variegatus* were too variable for curve fitting, although the REL was in the same range as that for the other species (data not shown).

Almost all samples of all isolates resumed growth after exposure to −8°C and higher temperatures (Table 1). All

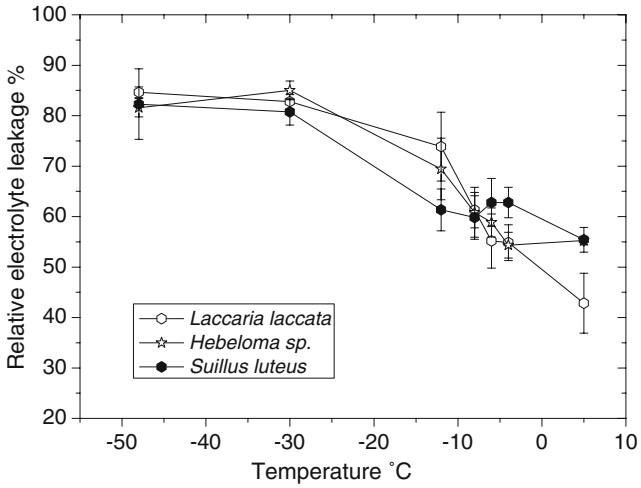

Fig. 1 REL % from fungal samples after exposure to different temperatures. Rate of cooling and warming during exposure was 5°C h^{-1}, and the duration of the target temperature was 4 h (n=4–7). *Bars* show two standard errors

Hebeloma sp. and *S. variegatus* samples survived −12°C, whilst the survival of *L. laccata* and *S. luteus* was over 80% at this temperature. As many as 64% of *L. laccata* samples survived −48°C and 11% in *S. variegatus*. No samples of *Hebeloma* sp. and *S. luteus* survived −48°C, but 67% of *Hebeloma* and 7% of *S. luteus* survived −30°C.

The coldest treatments also delayed the commencement of growth, as there was a larger part of the samples showing growth at 4weeks than after the first week (Table 1, Fig. 2). This phenomenon was seen in all species although at different temperatures. In *Laccaria*, the time lag was clear at the two lowest temperatures; in *Hebeloma* and *S. luteus*, it was seen at −30°C; and in *S. variegatus*, at the three lowest temperatures. After 4weeks, no further samples started growth.

Most of the samples did not grow at all after exposure to −48°C and −30°C, and the mean for these treatments was significantly lower than in all other treatments in all isolates (Fig. 2, Table 2). The growth was largest after +5°C in *Laccaria* and *S. luteus*, and the mean growth decreased with decreasing temperature. In *S. variegatus*, it was largest at −4°C; however, this did not significantly differ from +5°C. The growth of *S. variegatus* was significantly lower after −12°C than after −4°C and +5°C. By contrast, in *Hebeloma*, the growth was significantly larger at −12°C than at −4°C and +5°C.

The growth curves are shown also excluding those samples that had not started growth during the first week (Fig. 3, Table 3) in order to assess the potential growth of the fungi after the exposure treatments. In *Laccaria* and *Hebeloma*, the growth was largest at −30°C and lowest or nil at −48°C (not assessed by ANOVA because of low number of replicates). In the other treatments, the result is

Table 1 Percent of samples that showed growth 1 or 4 weeks after exposure to different temperatures

Temperature (°C)	*Laccaria laccata*		*Hebeloma* sp.		*Suillus luteus*		*Suillus variegatus*	
	Week 1	Week 4	Week 1	Week 4	Week 1	Week 4	Week1	Week 4
5	100	100	100	100	100	100	100	100
−4	100	100	90	100	95	100	100	100
−8	100	100	100	100	100	100	100	100
−12	83	83	100	100	71	86	58	100
−30	29	57	17	67	0	7	20	60
−48	18	64	0	0	0	0	11	11

The exposure consisted of gradual cooling and warming (5°C h^{-1}) with 4 h at the target temperature (n=7–21 with median 13).

mostly the same as for all data (Fig. 2). *S. variegatus*, the growth after −30°C, was as large as that at −12°C. By contrast, in *S. luteus*, the cultures exposed to lower temperatures consistently grew less, +5°C being significantly higher than −8°C and −12°C. There was no growth at −48°C or −30°C in the first week.

There were differences in the tolerance between isolates to the gradual (used in the other experiments) and abrupt change in temperature between room temperature and −12°C. *L. laccata* and *Hebeloma* showed no difference in survival, but *S. luteus* survived clearly better if the change was gradual. In *S. variegatus*, there was a time lag in the growth initiation after the gradual change in temperature but not in the case of abrupt temperature change (Table 4). The growth was larger after gradual exposure in all other species except *Hebeloma* (Fig. 4), but this was nearly significant only in *S. luteus* (P=0.068). If we excluded those samples that did not grow by the first week, the gradual exposure remained higher for *L. laccata* (P=0.053) and *S. variegatus* (P<0.001; data not shown). The growth of the surviving colonies of *S. luteus* was higher after the abrupt exposure than gradual (data not shown), but this difference was not significant.

Discussion

The ectomycorrhizal isolates tested here survived temperatures down to −30°C (*Hebeloma* sp. and, to some extent, *S. luteus*) or −48°C (*L. laccata* and *S. variegatus*). Tolerance to temperatures this low has not been shown earlier in ectomycorrhizal fungi in the absence of external

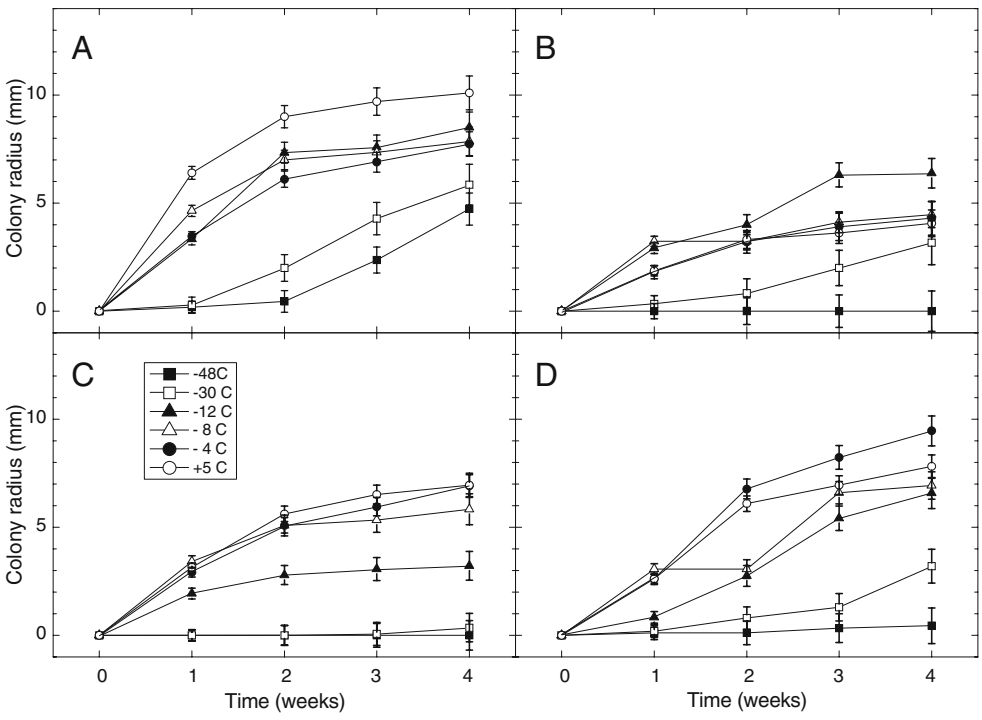

Fig. 2 Extension growth of fungi in pure culture after exposure to different temperatures. The rate of cooling and warming was 5°C h^{-1}, and the duration of the target temperature was 4 h. **a** *L. laccata*, **b** *Hebeloma* sp., **c** *S. luteus*, **d** *S. variegatus* (n=7–21, median 13). *Bars* show two standard errors

Table 2 Significance of differences between the temperature exposure treatments on the overall extension of the fungal colonies shown in Fig. 2

Comparison	*Laccaria laccata*	*Hebeloma* sp.	*Suillus luteus*	*S. variegatus*
−48/−30	0.927	0.123	1.000	0.492
−48/−12	<0.001	<0.001	<0.001	<0.001
−48/−8	<0.001	<0.001	<0.001	<0.001
−48/−4	0.001	<0.001	<0.001	<0.001
−48/+5	<0.001	<0.001	<0.001	<0.001
−30/−12	0.043	<0.001	<0.001	0.001
−30/−8	0.033	0.001	<0.001	<0.001
−30/−4	0.100	0.044	<0.001	<0.001
−30/+5	<0.001	0.035	<0.001	<0.001
−12/−8	1.000	0.074	<0.001	0.408
−12/−4	0.983	0.013	<0.001	<0.001
−12/+5	0.376	0.002	<0.001	0.002
−8/−4	0.975	0.899	0.985	0.009
−8/+5	0.352	0.721	0.710	0.333
−4/+5	0.071	1.000	0.944	0.446

Tukey's test following repeated-measures analysis of variance

cryoprotectants. Previously, France et al. (1979) showed that survival of −10°C was common in a range of ectomycorrhizal isolates, and Heinonen-Tanski and Holopainen (1991) found that the survival percentage of different ectomycorrhizal strains at −20°C was 36%.

The results from the REL test and the growth test both showed that the first signs of damage occurred in the temperature range from −8°C to −12°C. The LT_{50} values from the REL test were mostly in this region. However, according to the growth test, a much larger proportion than 50% of samples was alive after exposure to −8°C or −12°C. There was a time lag for the commencement of growth in all species at the lower temperatures (although this occurred at different temperatures in different species). These results suggest that the cold exposure damaged the cell membranes in a partially reversible way, causing increased REL and a time lag in the initiation of growth.

Those colonies that survived the lowest temperatures showed a high growth rate (particularly after −30°C in *Hebeloma*, *Laccaria*, and *S. variegatus*). This effect may

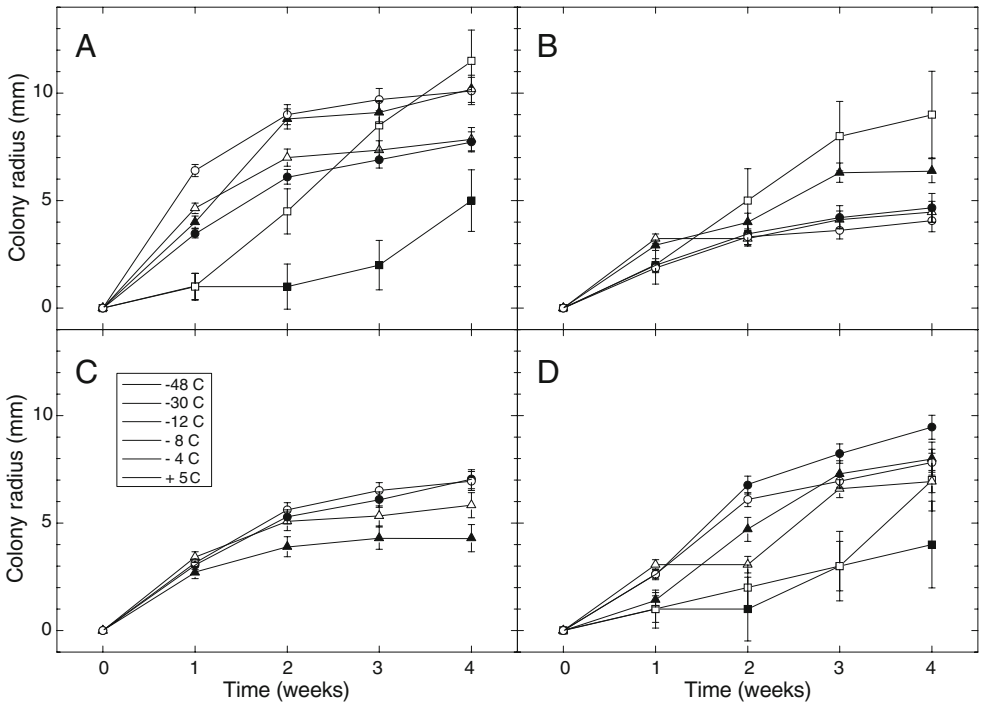

Fig. 3 Extension growth of fungi in pure culture after exposure to different temperatures. The rate of cooling and warming was 5°C h^{-1}, and the duration of the target temperature was 4 h. **a** *L. laccata*, **b** *Hebeloma* sp., **c** *S. luteus*, **d** *S. variegatus*. Colonies that started growth later than 1 week after the exposure are not included (n=7–21, median 13). *Bars* show two standard errors

Table 3 Significance of differences between the temperature exposure treatments on the overall extension of the fungal colonies shown in Fig. 3

Comparison	*Laccaria laccata*	*Hebeloma* sp.	*Suillus luteus*	*S. variegatus*
−48/−30	0.048	n.a.	n.a.	n.a.
−48/−12	<0.002	n.a.	n.a.	n.a.
−48/−8	<0.001	n.a.	n.a.	n.a.
−48/−4	0.001	n.a.	n.a.	n.a.
−48/+5	<0.001	n.a.	n.a.	n.a.
−30/−12	0.773	n.a.	n.a.	0.252
−30/−8	1.000	n.a.	n.a.	0.387
−30/−4	1.000	n.a.	n.a.	0.117
−30/+5	0.002	n.a.	n.a.	0.166
−12/−8	0.967	0.019	0.297	0.940
−12/−4	0.640	0.067	0.119	0.060
−12/+5	0.999	0.002	0.049	0.957
−8/−4	0.977	0.996	0.727	<0.000
−8/+5	0.070	0.353	0.042	0.214
−4/+5	0.001	0.995	0.996	0.401

Dunnett's T3 test, following repeated-measures analysis of variance
n.a. ANOVA was not possible because of too few replicates.

have been caused by reduced competition between colonies, as there were fewer growing colonies on each agar plate. Furthermore, the conditions were favourable for recovery, as the temperature was high, and carbohydrate, nutrient, and water availability was not limiting. Nevertheless, the time frame for recovery was several weeks in some cases. In the field, competition may also be reduced after extreme soil temperature regimes. It would be important to find out in further studies whether mycorrhizal fungi can resume growth also after an extended cool period following the exposure to low temperatures. Moreover, exposure to repeat freezing–thawing cycles in field could be particularly damaging for these fungi.

The cultures were grown at room temperature continuously for 4–5 months before the frost exposure. The cold tolerance of the fungi was high considering that they had not been preconditioned to cold treatments. The fungi tolerated lower temperatures than has been found for fine roots of their host plants. Fine roots of *P. sylvestris* and *P. abies* have been shown to tolerate only −4°C...−10°C when grown at above-zero temperatures (Lindström and Nyström 1987; Sutinen et al. 1998). The aboveground parts of woody plants of cold climates may tolerate temperatures lower than −80°C during dormancy, while in the active phase, the same organs may tolerate a few degrees below zero only.

Little is known about the acclimation of mycorrhizal fungi to variations in temperature. From the results of Tibbett et al. (2002), it appears that pure-cultured ectomycorrhizal *Hebeloma* species have an ability to adapt physiologically, as strains grew more after freezing to −5°C if they had been preconditioned at 2°C as opposed to 22°C. The freezing rate can also be important; Corbery and Le Tacon (1997) found that reducing the rate to 60°C h^{-1} was sufficient to reduce the damage, as opposed to an abrupt change in temperature. Here, the largest effect caused by the abrupt temperature change was the reduced survival of *S. luteus*. Further studies into both physiological and genetic adaptation are necessary.

The hardiness of these fungi can be due to several mechanisms. One possible tolerance mechanism might be the lack of ice nucleation centres. Air spaces separating hydrophobic fungal surfaces from water could in some conditions allow deep supercooling. This did not gain

Table 4 Percent of samples that showed growth 1 or 4 weeks after −12°C, either after gradual cooling and warming (5°C h^{-1}) or after abrupt change from and to 22°C (n=11–17 with median 13)

Temperature (°C)	*Laccaria laccata*		*Hebeloma* sp		*Suillus luteus*		*Suillus variegatus*	
	Week 1	Week 4	Week 1	Week 4	Week 1	Week 4	Week1	Week 4
−12 gradual	83	83	100	100	71	86	58	100
−12 abrupt	92	92	100	100	25	25	100	100

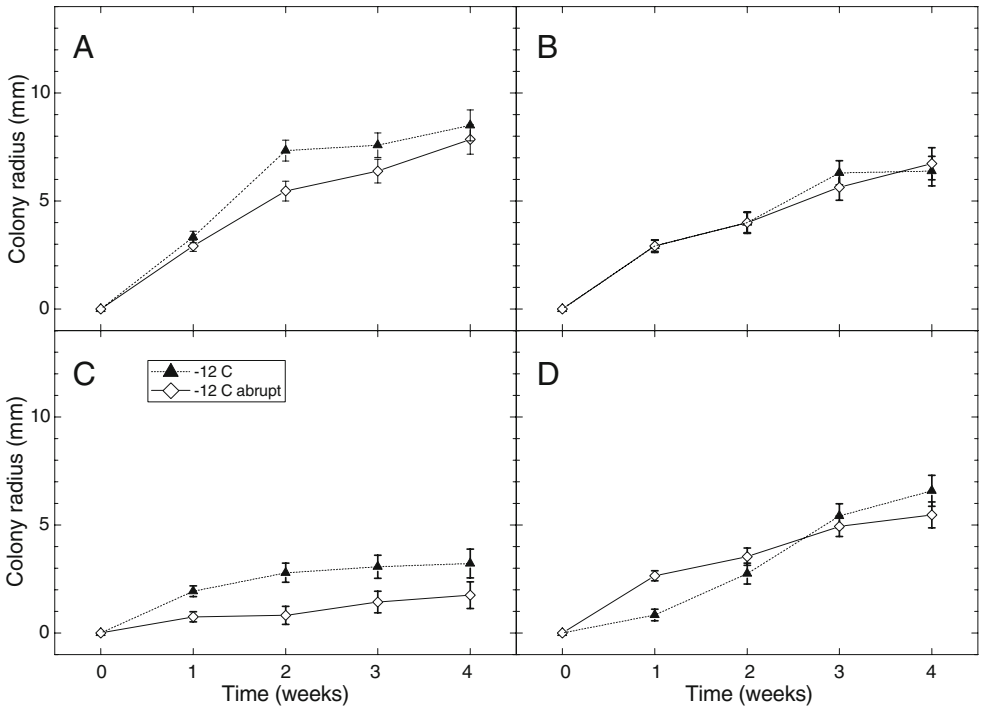

Fig. 4 Extension growth of fungi in pure culture after exposure to −12°C, either after gradual cooling and warming (5°C h^{-1}) or after abrupt change from and to 22°C. The duration at −12°C was 4 h in all cases. **a** *L. laccata*, **b** *Hebeloma* sp., **c** *S. luteus*, **d** *S. variegatus* (n=11–17, median 13). *Bars* show two standard errors. For statistics, see text

support from our results as the hydrophobic *Suillus* species did not perform better than the two hydrophilic species. Moreover, deep supercooling is not considered to function at temperatures much below −40°C (Quamme 1995). Therefore, this was not likely to be the mechanism for the survival of *S. variegatus* at −48°C. However, the hydrophobic and hydrophilic properties of the mycelia of different species may not be fully expressed in cultures grown in liquid media, and the differences between hydrophobic and hydrophilic species need to be further explored.

The most likely freezing tolerance mechanism here is tolerance to apoplastic freezing and the concomitant intracellular dehydration with consequent concentrating of cryoprotectant substances in cells. Earlier studies agree with this explanation. The concentration of trehalose, one of the major carbohydrates in many ectomycorrhizal fungi, doubled in alpine mycorrhizas when they were exposed to low temperatures (Niederer et al. 1992). Similarly, Tibbett et al. (2002) found an increase in trehalose and polyol concentrations in pure-cultured fungi in response to low temperatures. Trehalose, and possibly other carbohydrates, can have a particular role in the protection of cell membranes from dehydration (Crowe 2007).

Hebeloma and *S. variegatus* grew more after exposure to −12°C or −4°C, respectively, compared to +5°C. At these temperatures, the survival rate was 100% in both species. Therefore, the higher growth rates were not caused by reduced competition, as suggested for the high growth rates after −30°C (above). It is possible that the protective response to freezing was not solely expenditure for the fungus.

Obviously, the frost hardiness of mycorrhizal fungi in pure culture can be different from what it is in mycorrhizal symbiosis. The structure of the intercellular spaces is important for the spread of the ice crystallisation front in tissues, and this structure is different in mycorrhiza, compared to either mycelia or roots (Nylund 1987). Moreover, there may be factors in the field, which would initiate ice nucleation more readily than in the present experiment. One possibility is that mycorrhizosphere bacteria (cf. Frey-Klett et al. 2007) may function as ice nucleation centres, as certain bacteria are known to have this role in other environments (Szyrmer and Zawadzki 1997).

This study has demonstrated that ectomycorrhizal fungi have a high capacity to tolerate low temperatures. In further studies, it is necessary to compare mycorrhizal and non-mycorrhizal plants in terms of frost hardiness, as well as to study the winter survival of intact external mycelium in field soils subject to freezing–thawing cycles. Nevertheless, it is fruitful to study the stress tolerance of the fungi and trees also as individual organisms for understanding the function and the role of each partner in the symbiosis. On the basis of our results, we suggest that the relatively poor freezing tolerance of fine roots, including mycorrhizas (Räisänen et al. 2007), is not due to the fungal partner. The freezing tolerance of ectomycorrhizal fungi might be a key issue in the freezing tolerance of mycorrhizas and their host plants or in their recovery from repeated freezing and thawing.

Acknowledgements We thank Markus Eskelinen for help in the laboratory. The Erasmus student exchange programme made it possible for Arlena Brosinsky to participate in this work.

References

Bigras F, Dumais D (2005) Root-freezing damage in the containerized nursery: impact on plantation sites—a review. New For 30:167–184 doi:10.1007/s11056-005-4423-6

Corbery Y, Le Tacon F (1997) Storage of ectomycorrhizal fungi by freezing. Ann Sci For 54:211–217 doi:10.1051/forest:19970208

Crowe JH (2007) Trehalose as a "chemical chaperone": facts and fantasy. Adv Exp Med Biol 594:143–158 doi:10.1007/978-0-387-39975-1_13

France RC, Cline ML, Reid CPP (1979) Recovery of ectomycorrhizal fungi after exposure to subfreezing temperatures. Can J Bot 57:1845–1848 doi:10.1139/b79-231

Frey-Klett P, Garbaye J, Tarkka M (2007) The mycorrhiza helper bacteria revisited. New Phytol 176:22–36 doi:10.1111/j.1469-8137.2007.02191.x

Heinonen-Tanski H, Holopainen T (1991) Maintenance of ectomycorrhizal fungi. Methods Microbiol 23:413–422 doi:10.1016/S0580-9517(08)70188-0

Karlsson PS, Nordell KO (1996) Effects of soil temperature on the nitrogen economy and growth of mountain birch seedlings near its presumed low temperature distribution limit. Ecoscience 3:183–189

Laiho O, Mikola P (1964) Studies on the effect of some eradicants on mycorrhizal development in forest nurseries. Acta For Fenn 77:1–34

Lindström A, Nyström C (1987) Seasonal variation in root hardiness in container-grown Scots pine, Norway spruce, and lodgepole pine seedlings. Can J Res 8:787–793 doi:10.1139/x87-126

Makkonen K, Helmisaari H-S (2001) Fine-root biomass and production in Scots pine stands in relation to stand age. Tree Physiol 21:193–198

Mason PA (1980) Aseptic synthesis of sheathing (ecto-) mycorrhizas. In: Ingram DS, Helgeson JP (eds) Tissue culture methods for plant pathologists. Blackwell Scientific, Oxford, pp 173–178

Moser M (1958) Der Einfluss tiefer Temperaturen auf das Wachstum und die Lebenstätigkeir höherer Pilze mit speziell Berücksichtung von Mycorrhizapilzen. Sydowia 12:386–399

Niederer M, Pankow W, Wiemken A (1992) Seasonal changes of soluble carbohydrates in mycorrhizas of Norway spruce and changes induced by exposure to frost and desiccation. Eur J Forest Pathol 22:291–299 doi:10.1111/j.1439-0329.1992.tb00796.x

Nylund J-E (1987) The ectomycorrhizal infection zone and its relation to acid polysaccharides of cortical cell walls. New Phytol 106:505–516

Quamme HA (1995) Deep supercooling in buds of woody plants. In: Lee RE Jr, Warren ja GJ, Gusta LV (eds) Biological ice nucleation and its applications. The American Phytopathological Society, Minnesota

Radoglou K, Cabral R, Repo T, Hasanagas N, Sutinen M-L, Waisel Y (2007) Appraisal of root leakage as a method for estimation of root viability. Plant Biosyst 141(3):443–459 doi:10.1080/11263500701626143

Räisänen M, Repo T, Lehto T (2007) Cold acclimation was partially impaired in boron deficient Norway spruce seedlings. Plant Soil 292:271–282 doi:10.1007/s11104-007-9223-7

Repo T, Lappi J (1989) Estimation of standard error of impedance-estimated frost resistance. Scand J For Res 4:67–74

Sutinen M-L, Ritari A, Holappa T, Kujala K (1998) Seasonal changes in soil temperature and in the frost hardiness of Scots pine (*Pinus sylvestris*) roots under subarctic conditions. Can J Res 28:946–950 doi:10.1139/cjfr-28-6-946

Szyrmer W, Zawadzki I (1997) Biogenic and anthropogenic sources of ice-forming nuclei: a review. Bull Am Meteorol Soc 78:209–228 doi:10.1175/1520-0477(1997)078<0209:BAASOI>2.0.CO;2

Tibbett M, Sanders FE, Cairney JWG (2002) Low-temperature induced changes in trehalose, mannitol and arabitol associated with enhanced tolerance to freezing in ectomycorrhizal basidiomycetes (*Hebeloma* spp.). Mycorrhiza 12:249–255 doi:10.1007/s00572-002-0183-8

Unestam T, Sun YP (1995) Extramatrical structures of hydrophobic and hydrophilic ectomycorrhizal fungi. Mycorrhiza 5:301–311 doi:10.1007/BF00207402

Wallander H, Nilsson LO, Hagerberg D, Bååth E (2001) Estimation of the biomass and seasonal growth of external mycelium of ectomycorrhizal fungi in the field. New Phytol 151:753–760 doi:10.1046/j.0028-646x.2001.00199.x