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Low-temperature-induced changes in trehalose, mannitol and arabitol associated with enhanced tolerance to freezing in ectomycorrhizal basidiomycetes (*Hebeloma* spp.)

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Abstract Ectomycorrhizal fungi have been shown to survive sub-zero temperatures in axenic culture and in the field. However, the physiological basis for resistance to freezing is poorly understood. In order to survive freezing, mycelia must synthesise compounds that protect the cells from frost damage, and certain fungal-specific soluble carbohydrates have been implicated in this role. Tissue concentrations of arabitol, mannitol and trehalose were measured in axenic cultures of eight *Hebeloma* strains of arctic and temperate origin grown at 22, 12, 6 and 2°C. In a separate experiment, mycelia were frozen to –5°C after pre-conditioning at either 2°C or 22°C. For some, especially temperate strains, there was a clear increase in specific soluble carbohydrates at lower growth temperatures. Trehalose and mannitol were present in all strains and the highest concentrations (close to 2.5% and 0.5% dry wt.) were recorded only after a cold period. Arabitol was found in four strains only when grown at low temperature. Cold pre-conditioning enhanced recovery of mycelia following freezing. In four out of eight strains, this was paralleled by increases in mannitol and trehalose concentration at low temperature that presumably contribute towards cryoprotection. The results are discussed in an ecological context with regard to mycelial overwintering in soil.

Keywords Cryoprotection · Polyols · Arctic fungi · Carbohydrates · Ectomycorrhiza

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Introduction

In order to survive winter temperatures in arctic and cool temperate soils, ectomycorrhizal fungi might retreat into either vegetative propagules (spores or sclerotia) or the roots of the host plant. This would entail the loss of extraradical mycelium, which would then need to be re-grown during the warmer seasons. At high latitudes, this period can be short and it would, therefore, be of advantage for perennial mycelia to be associated with perennial plants (there are almost no arctic and alpine annuals). Ectomycorrhizal fungi have been shown to survive sub-zero temperatures (Moser 1958; France et al. 1979), but the physiological basis of resistance to freezing is poorly understood. Extraradical mycelia might be able to survive the winter freeze if able to accumulate substances that impart cold hardening and cryoprotection. A recent review of arctic and antarctic fungi concluded that the longevity of basidiomycete mycelium through periods of cold remains unknown (Robinson 2001).

The ability of ectomycorrhizal fungi to grow at low temperatures has been shown in strains isolated across a wide geographic range and it has been shown consistently that low temperature (<12°C) greatly reduces growth of the fungi grown in axenic culture (Melin 1925; Mikola 1948; Moser 1956, 1958; Hacskaylo et al. 1965; Cline et al. 1987; McInnes and Chilvers 1994; Tibbett et al. 1998); they may survive in these conditions for a number of years (Tibbett et al. 1999). However, the ability to survive freezing in axenic culture has been documented in only a few studies (Moser 1958; France et al. 1979).

The ability of ectomycorrhizas to survive winter months in the field has also been reported. Alexander and Fairley (1983) found that up to 75% of mycorrhizal colonisation was retained in a mature Scottish spruce plantation between September and December, although autumn soil freezing was not established. Coutts and Nicoll (1990) reported the winter survival of spruce mycorrhizas and mycelium of *Thelephora terrestris* in experimental soil columns, although the soil did not freeze during the experiment.

In studies where freezing is known to have occurred, the findings are consistent with a freeze-resistant strategy. Of 73 strains of ectomycorrhizal fungi frozen to –10°C in vitro, 71 were shown to have a survival rate of 100%; however, in some cases, sub-cultures of the defrosted mycelia took over 5 weeks to show any signs of growth (France et al. 1979). Under field conditions, an alpine basidiomycete *Clitocybe lateritia* has been observed to fruit at the same place for a number of years in soils which fell to –7°C at 5 cm depth. Fungal biomass measurements indicated that the same mycelial density existed at the beginning and end of the cold season, suggesting that the carpophores arose from the same mycelium (Debaud 1983, 1987). This apparent mycelial survival was qualified by reduced sporocarp production after the severest winter.

Perhaps the most convincing evidence of overwintering through a period of sub-zero temperatures was reported by Marx and Bryan (1975). They found that mycelium of *Pisolithus tinctorius* survived winter in the absence of a host and successfully colonised the roots of pine seedlings after 4 months in the soil. During this time, the soil was subject to sub-zero temperatures with a minimum of –8°C. Similar results have been reported more recently for arbuscular mycorrhizal (AM) fungi (Addy et al. 1997), although in this instance soil temperatures did not fall much below 2°C. Thus, it seems likely that ectomycorrhizal mycelia can tolerate freezing under appropriate conditions. However, mycelia may experience some hyphal damage and impairment to growth, presumably related to minimum incident temperature. The extent of injury and the rate of subsequent recovery and re-growth probably contribute towards the ecological fitness of ectomycorrhizal fungi subjected to low-temperature stress. Determining their ability to survive and recover from freezing, together with the physiological basis for cryoprotection, are important elements in understanding the physiological ecology of ectomycorrhizal fungi.

Mechanisms that confer protection against freezing injury may include the accumulation of cryoprotective compounds such as sugars and sugar alcohols (Meryman 1966; Crawford 1989; Robinson 2001). Acclimatisation at low temperatures has been shown to be important in enhancing the survival of the hyphae of *Fusarium oxysporum* f.sp. *lycopersici* (Robinson and Morris 1984) and AM fungi after freezing (Addy et al. 1998) and might be related to elevated levels of specific solutes. In mycorrhizas these may be in the form of fungal carbohydrates and sugar alcohols such as trehalose, mannitol and arabitol, which have been found previously in mycorrhizal roots and mycelium (Söderström et al. 1988; Cairney and Alexander 1992; Wallenda et al. 1996). Each of these three compounds is thought to act as compatible solutes under water stress (Jennings 1990) and might, therefore, also play a role in cryoprotection. Trehalose concentration in particular has been shown to double in excised mycorrhizas of Norway spruce (*Picea abies*) when exposed to low temperature (Niederer et al. 1992). Peaks in seasonal trehalose concentration also seem to reflect soil temperature minima in a range of mycorrhizas in spruce (Niederer et al. 1992) and red pines (*Pinus Resinosa*) (Koide et al. 2000). The accumulation of these compounds may, thus, confer enhanced freezing tolerance on ectomycorrhizal fungi.

The purpose of the current work was to test two related hypotheses for ectomycorrhizal fungi from the geographically widespread genus *Hebeloma*. First, that growth temperature experienced by mycelium affects the accumulation of specific carbohydrates that may have a role in freeze tolerance. Second, that low temperature pre-conditioning of mycelium increases tolerance to freezing. Eight strains of *Hebeloma* spp. of arctic and temperate origin were tested for improved freezing survival after pre-exposure to low temperature and the accumulation of the potential cryoprotectant compounds arabitol, mannitol and trehalose over a range of growth temperatures.

Materials and methods

Strains and media

Of the eight strains of *Hebeloma* used in this study, four were of arctic origin (A, B, C, H), and four of temperate origin (J, K, L, M) (Table 1). Seven of the strains used were of four known species: *Hebeloma marginatulum* (Bruchet), *H. polare* (Vesterholt), *H. crustuliniforme* (Bull. Ex. Fr) Quél., and *H. cylindrosporum* (Romagn), with one unidentified *Hebeloma* sp. (Table 1). Stock cultures were maintained on potato dextrose agar (PDA). Experimental cultures were grown in 9-cm-diameter Petri dishes containing 25 ml liquid medium of the following composition: (l^{-1}) 500 mg $(NH_4)_2HPO_4$, 300 mg KH_2PO_4, 140 mg $MgSO_4.7H_2O$, 50 mg $CaCl_2.6H_2O$, 25 mg NaCl, 3 mg $ZnSO_4$, 12 mg FeEDTA, 0.1 mg thiamine, 0.1 mg biotin and 10 g glucose.

Experiment 1. The effect of growth temperature on soluble carbohydrate accumulation

Each Petri dish was inoculated with ten 4-mm-diameter plugs of fungus cut from the actively growing edge of mycelia on PDA and incubated in the dark at 22°C ($_{\pm1}$°C), 12°C (±1°C), 6°C (±0.1°C), or 2°C (±0.1°C). Mycelium was harvested at each incubation temperature after ca. 33, 78, 125, 176 days, respectively. Three replicate cultures were incubated at each temperature for each strain. Each replicate (independent) culture was used for carbohydrate determination.

Carbohydrate determination

At harvest, mycelia were gently removed from Petri dishes and blotted on Whatman No. 1 filter papers prior to washing in modified universal buffer (Skujins et al. 1962). This was prepared by titrating 50 ml of a stock buffer containing 3.025 g Tris (hydroxymethyl) aminomethane, 2.9 g maleic acid, 3.5 g citric acid, 1.57 g boric acid; 122 ml 1 M NaOH solution made up to 250 ml with distilled H_2O and 0.1 M HCl to pH 4. Mycelia were re-blotted and the procedure repeated. Soluble carbohydrates were extracted by homogenising mycelia with a motorised Teflon pestle in pre-weighed Eppendorf tubes to which 200 µl of 80% ethanol had been added. Samples were pelleted in a microfuge at 15,000 rpm and the supernatant decanted. The pellets were re-suspended in a further 200 µl of 80% ethanol and the procedure repeated. Pooled supernatants were stored at –20°C prior to analysis. The remaining pellets were dried at 80°C and weighed.

Prior to analysis, extracts were dried in a vacuum desiccating centrifuge in order to remove all ethanol prior to injection into the chromatograph. Samples were re-suspended in 1 ml distilled water

Table 1 Strains of *Hebeloma* used in a freezing tolerance study

Strain	Species	Supplied coding (source)	Isolated from/under	Origin (habitat)
A	*H. marginatulum*	9331 (I.J. Alexander)	*Salix Dryas* mix	Svalbard, Norway (arctic tundra)
B	*H. polare*	9301 (I.J. Alexander)	*Salix Dryas* mix	Svalbard, Norway (arctic tundra)
C	*H. crustuliniforme*	VT 1925 (O.K. Miller)	*Alnus tenuifolia*	Near Fairbanks, Alaska (taiga)
H	*Hebeloma* sp.	VT 1930 (O.K. Miller)	*Alnus* sp.	Near Fairbanks, Alaska (taiga)
J	*H. crustuliniforme*	89.001 (R.D. Finlay)	*Pinus sylvestris*	Nancy, France (temperate forest)
K	*H. cylindrosporum*	90.005 (R.D. Finlay)	*Pinus pinaster*	Landres, France (temperate forest)
L	*H. crustuliniforme*	073 (D.J. Read)	*Picea abies*	Bush Estate, Scotland (cool temperate forest)
M	*H. crustuliniforme*	(D.J. Read)	*Picea abies*	Bush Estate, Scotland (cool temperate forest)

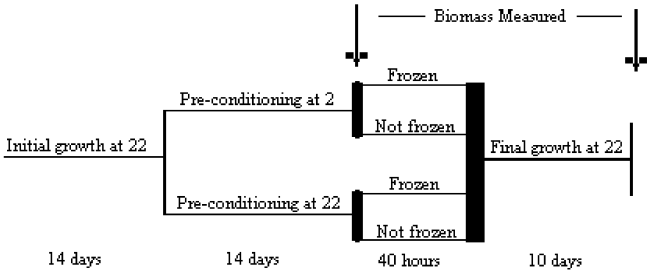

Fig. 1 Protocol for determining the effect of low temperature pre-conditioning on the tolerance to subsequent freezing in mycelia of eight *Hebeloma* spp.

10 min prior to injection. Analysis of carbohydrates was by anion exchange chromatography, with pulsed amperometric detection on a Dionex 4000i chromatograph. Carbohydrates were separated on a CarboPac PA1 column with isocratic 75 mM NaOH at 0.8 ml min^{-1} flow rate. Post-column addition of 0.3 M NaOH assured a high pH at the detectors.

A range of single sugar standards (10–200 μM) were run after every sixth fungal extract. Mixed standards were also introduced to account for interference or drift occurring when multiple sugars were present.

Experiment 2. Freezing tolerance of mycelia

From each strain, 16 Petri dishes were inoculated with three 4-mm-diameter plugs cut from the actively growing edge of mycelia on PDA. All dishes were incubated in the dark at 22°C for 14 days, after which they were either transferred to a pre-conditioning temperature of 2°C or maintained at 22°C for a further 14 days (Fig. 1). From each pre-conditioned temperature treatment, three Petri dishes of each strain were either frozen for 40 h at –5°C (± 2°C) or maintained at 2°C. All Petri dishes were randomised within the freezer or incubator. The remaining Petri dishes were harvested at the start of the freezing treatment by filtration through 0.8-μm membrane filters, dried (80°C) and weighed for pre-freezing treatment biomass estimate. After 40 h, the frozen cultures were returned to 22°C for 10 days for further growth prior to harvesting (as above). Differences between post- and pre-treatment biomass were calculated and a two-way analysis of variance performed on the data.

Results

Experiment 1. The effect of growth temperature on soluble carbohydrate accumulation

Trehalose concentration varied with *Hebeloma* strain and growth temperature. For strains A, B, and C, trehalose concentration was highest at 22°C and decreased at lower

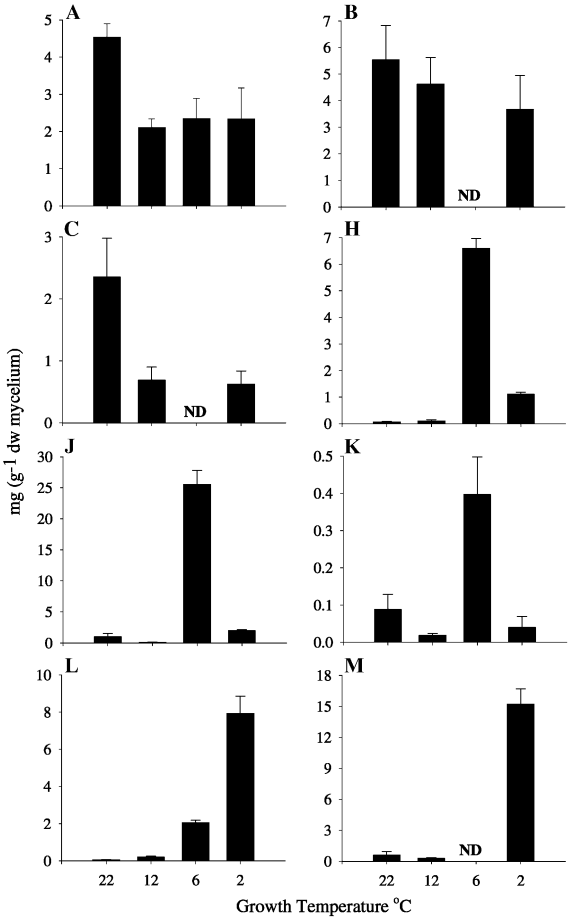

Fig. 2 Changes in trehalose concentration in mycelia of eight strains of *Hebeloma* spp. (A, B, C, H, J, K, L, M) when grown at temperatures in the range 22–2°C. *Bars* represent 2 standard errors of the means (*ND* not determined)

temperatures (Fig. 2). For strains H, J, and K, trehalose concentration was highest at 6°C, with only relatively small amounts produced at other growth temperatures. For strains L and M, trehalose concentration was highest at lower growth temperatures (2–6°C). Only strains H, J, L, and M had concentrations of trehalose in excess of 6 mg g^{-1} dry wt. and this only at low temperatures. Relative to other strains, only traces of trehalose were found in strain K at any temperature.

Overall patterns of change in mannitol concentration were similar to those of trehalose (Fig. 3). Strains A and

Table 2 The effect of temperature pre-conditioning on biomass production (mg dry wt.) and increase in soluble carbohydrates after freezing of eight *Hebeloma* strains. The freezing effect is the difference in growth between not frozen (*NF*) and frozen (*F*) treatments. The carbohydrate data represent a doubling in arabitol (*Ara*), mannitol (*Man*) or trehalose (*Tre*) levels between growth at warm (22° or 12°C) and low temperatures (6° or 2°C), where concentrations were at least 1 mg g^{-1} dry wt. (*NG* no growth)

Strain	Pre-conditioning at 22°C			Pre-conditioning at 2°C			Carbohydrates
	F	NF	Freezing effect	F	NF	Freezing effect	
A	3.6	4.5	+0.9***	5.0	5.1	+0.1	None
B	2.1	1.7	–0.4	2.4	2.3	–0.1	None
C	1.2	2.1	+0.9***	2.2	2.6	+0.4	None
H	2.8	2.9	+0.1	2.6	2.5	–0.1	Tre
J	1.1	2.4	+1.3***	1.5	2.1	+0.6*	Man, Tre
K	NG	NG	–	1.5	2.4	+0.9***	None
L	0.9	3.0	+2.1***	1.5	2.3	+0.8**	Ara, Man, Tre
M	1.7	2.4	+0.7**	2.1	1.6	–0.5	Ara, Man, Tre

*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$

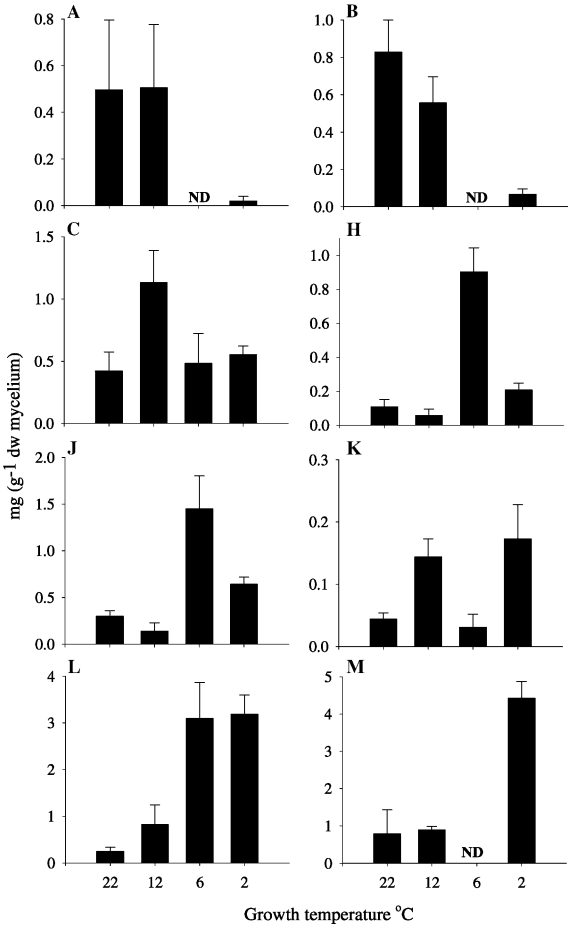

Fig. 3 Changes in mannitol concentration in mycelia of eight strains of *Hebeloma* spp. (A, B, C, H, J, K, L, M) when grown at temperatures in the range 22–2°C. *Bars* represent 2 standard errors of the means (*ND* not determined)

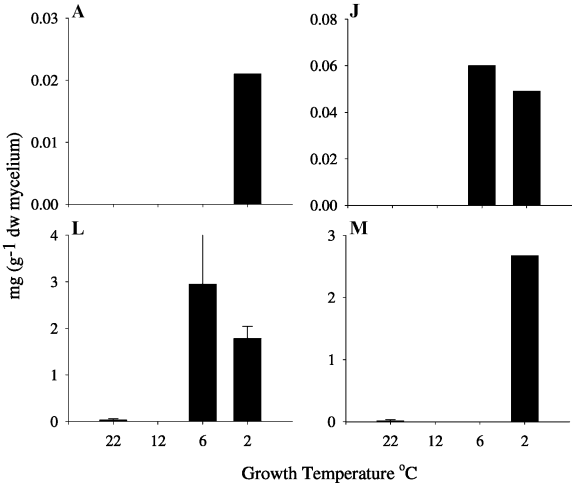

Fig. 4 Changes in arabitol concentration in mycelia of four strains of *Hebeloma* spp. (A, J, L, M) when grown at temperatures in the range 22–2°C. *Bars* represent 2 standard errors of the means (*ND* not determined)

B had highest concentrations at the warmer growth temperatures but only trace amounts at 2°C. Strain C had approximately twice the concentration of mannitol at 12°C compared with any other growth temperature, whereas this was the case for strains H and J at 6°C. Very little mannitol accumulated in strain K at any temperature. For strains L and M, mannitol concentration was highest at lower growth temperatures. Only strains J, L and M produced tissue concentrations of mannitol in excess of 1 mg g^{-1} dry wt. and then only at low temperature.

Arabitol was detected in only four strains (Fig. 4). Trace concentrations were found in strains A and J at low temperature. In strains L and M, up to 3 mg g^{-1} dry wt. was found at low temperatures and only trace amounts at 22°C.

Experiment 2. Freezing tolerance of mycelia

With the exception of strain K, which did not grow after pre-conditioning at 22°C, all strains survived freezing for 40 h at –5°C (Table 2). The 'freezing effect', the difference in growth between not frozen (NF) and frozen (F) treatments, was positive in nearly all cases. Exceptions were strains H and M, both pre-conditioned at 2°C, and strain B, regardless of pre-conditioning, where there was no significant effect of freezing.

Low temperature pre-conditioning tended to reduce the freezing effect. The difference in the freezing effects after pre-conditioning at low or higher temperatures (2–22°C) indicated a beneficial effect of low-temperature pre-conditioning in all strains except B (Table 2). Strain K was excluded from this analysis, as it did not grow after the warmer pre-conditioning treatment.

Of the fungi pre-conditioned at 22°C, only strains B and H showed no significant difference in growth after freezing (Table 2). All other differences were highly significant ($P \leq 0.01$–0.001), with post-freezing growth reduced to as much as one-third of that of unfrozen cultures (in the case of strain L). Strain K did not grow further after pre-conditioning at this temperature.

Of the fungi pre-conditioned at 2°C, only strains J, K and L showed a significant difference in growth after freezing. Differences in growth between pre-treatments in J and L were smaller than when pre-conditioned at 22°C and no longer significant at the 0.1% level. In all other strains, there was no significant difference in growth between frozen and unfrozen cultures.

Discussion

The ability to survive mild freezing was inherent in all *Hebeloma* strains tested, in agreement with the findings of France et al. (1979) that most strains of ectomycorrhizal fungi tested, including *H. crustuliniforme*, were able to survive freezing to –10°C without low-temperature pre-conditioning., The only strain (K) that did not continue to grow after pre-conditioning in the present study (only after warm pre-conditioning) is an extremely fast-growing strain in axenic culture (Tibbett et al. 1998, 1999). The observed failure to grow is almost certainly because the biomass gained during the initial growth phase plus the warm pre-conditioning phase (see Fig. 1) was the maximal biomass attainable by this strain in the batch culture.

While some strains were little affected by freezing (B and H), freezing had a marked impact on the subsequent growth of others (A, C, J and L) (Table 2). For these strains, the negative effect of freezing was alleviated by pre-conditioning at 2°C. This is the first clear demonstration that exposure to low temperatures can harden the mycelia of ectomycorrhizal fungi to a subsequent freeze. Curiously, this is also the first time this has been demonstrated for arctic fungi (Robinson 2001). Cold pre-conditioning reduced injury in all strains except B, which had a negative response. Strains H, J, K and L accumulated the highest concentrations of at least one of the three carbohydrates (trehalose, mannitol or arabitol) at 6°C rather than at 2°C, which may be too cold for acclimatisation. Some strains may have accumulated more cryoprotectant carbohydrates with slightly warmer pre-conditioning or a graduated chilling period.

Low temperature pre-conditioning had a further, unexpected, effect in some strains, that of stimulating growth of some not-frozen treatments. Subsequently, a spurt in growth of many arctic *Hebeloma* spp. removed from cold to warmer conditions has been observed (M. Tibbett, unpublished observation). We hypothesise that, under favourable conditions, carbohydrates such as mannitol and trehalose may act as storage compounds and/or cryoprotective agents (Thevelein 1984; Söderström et al. 1988; Van Laere 1989; Cooke and Whipps 1993) and be rapidly re-metabolised into energy and carbon for growth. Active growth from translocatable reserve carbohydrates may be important early in the growing season, at a time when plant photosynthetic organs are immature and host carbon is directed towards shoot growth.

In experiment 1, mycelia from colder growth temperatures were chronologically older than mycelia from the warmer treatments, since they grew for longer periods to achieve the same biomass. Nevertheless, the cultures incubated at low temperature may be physiologically younger than those at warmer temperatures. Earlier carbohydrate analysis of ectomycorrhizas of different morphological age has shown changes in levels of trehalose (Ineichen and Wiemken 1992; Wallenda et al. 1996) and mannitol (Cairney and Alexander 1992). In contrast, however, these studies also showed no effect of chronological ageing on levels of mannitol (Wallenda et al. 1996) or arabitol and trehalose (Cairney and Alexander 1992). Arabitol, mannitol and trehalose levels in axenic cultures of the ericoid mycorrhizal fungus *Hymenoscyphus ericae* declined with longer incubation periods (chronological age) (Hughes and Mitchel 1995). Similarly, Hallsworth and Magan (1996) reported polyol concentrations to be generally lower or unchanged in older cultures of entomopathogenic fungi. Although not entirely consistent, the evidence suggests that a high level of polyols in chronologically older (low temperature) cultures is unlikely to be solely an effect of ageing.

Changes in soluble carbohydrate concentration were variable across strains but clear patterns emerged in relation to growth temperature. In strains A and B from the high arctic, trehalose and mannitol concentrations were lowest at low temperatures. This may be because they are re-metabolised, presumably into other preferred cryoprotectants not measured here, e.g. amino sugars or other polyols. Such a mechanism would require enhanced enzymatic breakdown of mannitol and trehalose at low temperatures; this requires further investigation. Indeed, both strains survived freezing markedly well (Table 2), strongly implying the existence of an alternative form of cryoprotection. This may apply also to strain C, which behaved similarly, except that it produced most mannitol at 12°C.

In several strains, including H, J, K, L and M (three of which are from the species complex *H. crustuliniforme*), concentrations of all three carbohydrates increased at the lower growth temperatures (2°C and 6°C). In particular, strains L and M accumulated greater concentrations of all three compounds at low temperatures and exhibited the greatest increase in freezing tolerance when pre-conditioned at 2°C. This relationship is supported by evidence from Gélinas et al. (1989), who

found a correlation between trehalose concentration and freezing tolerance in yeast. Trehalose, in particular, is known to be important in stabilising desiccated membranes as would occur in freezing (Crowe et al. 1984), possibly with trehalose replacing water between the head groups of phospholipids and maintaining membrane integrity (Crowe and Clegg 1973).

The highest concentrations of all three carbohydrates were measured after low growth temperatures. Trehalose accounted for up to 2.5% of total dry weight in strain J when grown at 6°C and mannitol for almost 0.5% in strain M when grown at 2°C. These concentrations are low compared with some other ectomycorrhizal taxa (Koide et al. 2000) but in keeping with concentrations reported for *Hebeloma* (Wallenda et al. 1996). At these concentrations, a role in membrane protection seems more likely than a contribution to cytoplasmic supercooling.

Arabitol appeared to play no significant part in the carbon physiology of most of these *Hebeloma*, with the exception of strains L and M. When grown at low temperature, arabitol accounted for less than 0.3% of total dry weight in these two strains. It may be that the reported absence of arabitol (e.g. Wallenda et al. 1996 for *H. crustuliniforme* and others) is a consequence of growth at typical laboratory incubation temperatures. This may also apply to trehalose, mannitol and other soluble carbohydrates synthesised in response to environmental stress in situ but not present when cultured in favourable conditions in vitro. The growth medium, not considered here, may also have an important bearing in these concentrations.

While the present study may have identified some of the compounds involved in the cryoprotection of temperate *Hebeloma* strains (especially *H. crustuliniforme* strains J, L and M), the arctic strains A, B and C are clearly dependent on another mechanism that allows them to survive freezing. Nonetheless, it seems quite clear that mycelia can survive freezing and this ability is probably in proportion to levels of specific carbohydrates such as arabitol, mannitol and trehalose (and other compounds) that are able to offer cryoprotection. Such cryoprotectants accumulate in the fungi if acclimatised to low temperature. This has been shown to occur in ectomycorrhizal roots (e.g. Niederer et al. 1992) and it follows from this study that similar forms of cryoprotection occur in mycelia of a range of *Hebeloma* taxa.

In nature, the winter survival of extraradical mycelium would surely benefit both symbionts at the beginning of the growing season, especially in deep frozen arctic soils. However, it should be borne in mind that soils that freeze are subject to cryoturbation and, thus, permafrost soils may exert cryostatic pressure and compromise intact mycelial systems. Nonetheless, surviving mycelia able to rapidly re-metabolise cryoprotectants into growth carbon, would allow a more rapid exploitation of nutrients made available in the spring flush. This would serve to optimise nutrient capture and moderate plant carbon drain early in the growing season.

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