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Short note

Storage of ectomycorrhizal fungi by freezing

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Summary – Ectomycorrhizal fungi are usually maintained by subculturing at about +25 °C. Ito and Yokohoma (1983) and Jong and Davis (1987) demonstrated that some ectomycorrhizal fungi could be preserved by freezing. We show that the survival of ectomycorrhizal fungi after freezing at −196 °C or −80 °C depends on the cooling rate and on the species or strains. The optimum rate of cooling is −1 °C per min. *Thelephora terrestris* and *Paxillus involutus* did not survive any freezing method. The resistance of *Cenococcum geophillum* to freezing may be related to its tolerance of water stress and of high salinity.

freezing / storage / ectomycorrhizal fungi

Résumé – La conservation de champignons ectomycorhiziens par congélation. Les champignons ectomycorhiziens sont habituellement conservés par repiquage successif à environ + 25 °C. Les travaux d'Ito et Yokohoma (1983) et ceux de Jong et Davis (1987) ont démontré qu'il était possible de conserver certaines espèces à très basse température. Le présent travail montre que la survie des champignons ectomycorhiziens dépend de la vitesse de congélation à − 196 °C ou − 80 °C ainsi que des espèces ou des souches. La vitesse optimale de congélation est de − 1 °C par minute. *Thelephora terrestris* et *Paxillus involutus* ne survivent à aucune méthode de conservation. La résistance de *Cenococcum geophillum* à la congélation est probablement à mettre en relation avec sa tolérance au stress hydrique et à la salinité.

congélation / conservation / champignons ectomycorhiziens

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INTRODUCTION

Ectomycorrhizal fungi are essential for the growth of forest trees. They enhance uptake of mineral nutrients from soil by increasing the absorbing surface (Harley and Smith, 1983) and by mobilizing insoluble forms of phosphates or other minerals (Bowen and Theodorou, 1967; Voigt, 1971; Lapeyrie et al, 1990). They act also by inhibiting root pathogens (Marx, 1972) and by producing growth regulators, auxin and cytokinins (Slankis, 1973; Miller, 1977; Gay et al, 1989).

Some ectomycorrhizal fungi can be cultivated in pure culture, and artificially introduced in forest nurseries. The use of selected and ecologically adapted strains enhances survival and growth of some forest trees after outplanting in adverse or routine reforestation sites (Marx and Bryan, 1975; Marx, 1980; Shaw et al, 1982; Le Tacon et al, 1992).

The genetic selection of very efficient and competitive strains is now in progress in several laboratories (Debaud et al, 1990). Genetic studies of ectomycorrhizal fungi require the manipulation of large numbers of monokaryotic or dikaryotic strains. These strains are maintained and propagated by subculturing on artificial media at +25 °C. This method is costly and time-consuming and can be accompanied by a loss of properties such as efficiency and infectivity (Laiho, 1970; Giltrap, 1981; Marx, 1981; Thomson et al, 1993; Di Battista et al, 1996).

One of the most frequently used methods of preserving microorganisms is freezing. Progress has been made in cryopreservation of living fungi in culture (Goos et al, 1967; Hwang, 1968; Butterfield et al, 1978; Smith, 1983; Jong and Atkins, 1985; Jong and Davis, 1986). The most commonly used methods of cryogenic storage are immersion in liquid nitrogen (–196 °C) or in liquid nitrogen vapour (–150 °C and below).

It is well known that during freezing and thawing injury to cells can occur. The formation of intracellular ice crystals and the effects of the concentration of solutes during the process are the most important factors responsible for freezing injury (ice damage or solution effect damage). The intensity of damage seems well correlated with the rapidity of cooling. A too slow cooling rate leads to overdehydration and excessive concentration of solute resulting in solution effect damage. A too rapid rate leads to inadequate dehydration and subsequent formation of many intracellular ice crystals which are lethal.

Although many fungi tolerate uncontrolled rapid cooling (direct immersion in liquid nitrogen), they survive better using a controlled slow cooling rate. To reduce injury during freezing and thawing, cryoprotectants are used in most successful methods of cryopreservation of living cells. Many compounds have been used as cryoprotectants either alone or in combination. There are two categories of cryoprotectants: permeating compounds (dimethyl sulphoxide [DMSO] and glycerol) and non-permeating additives (sugars, sugar alcohols, polyvinylpyrolidone, dextran, etc). DMSO and glycerol are the most successful protectants for the cryopreservation of fungi (Jong, 1981). Generally a concentration of 5% of DMSO and 10% of glycerol is adequate.

Some ectomycorrhizal fungi have been frozen at the Institute for Fermentation, Osaka, Japan and at the American Type Culture Collection (ATCC) and it has been found that not all can be cryopreserved by standard techniques. The aim of the present work was to find the optimum cooling rate for ectomycorrhizal strains having a range of physiological properties and particularly *Laccaria bicolor* (Maire) Orton in order to preserve the numerous strains needed for genetic improvement.

MATERIALS AND METHODS

We used a Nicool LM 10 apparatus which is employed for long-term maintenance and preservation of a wide variety of microorganisms (bacteria, fungi, virus) or cells. It possesses a programmable freezing unit.

The fungi were subcultured in petri dishes on malt medium. After 2 weeks of growth at +25 °C three agar disks from the advancing edge of the colony were placed in a screw-cap polypropylene vial. The size of each culture plug was uniform throughout the entire study (diameter 5 mm, thickness 4 mm). The vials were gamma ray sterilized and had a capacity of 1.8 mL. For each freezing experiment the cooling rate was registered by implanting a thermocouple directly in the vial containing a sample.

The samples were either directly plunged in liquid nitrogen (–196 °C), directly placed in a refrigerator at –80 °C, or slowly cooled before freezing at –196 °C or –80 °C.

We used a solution of glycerol in distilled water (15% v/v) as cryoprotectant. In a preliminary experiment we found that the immersion of the agar plugs in a solution of glycerol (15% v/v) had no effect on the further mycelium growth.

For recovery, the vials were always thawed during 60 min at +4 °C and then placed at +25 °C. After thawing the agar plugs were cultured on malt agar medium at +25 °C for 2 weeks. Then the diameter of the fungal colonies was measured and compared to a control (non-frozen culture) in order to estimate the rate of survival and the rate of cryoinjuries.

There were three replicates per treatment and per strain. For each experiment, analysis of variance was performed to check the overall significance of the different treatments on growth and survival of the different fungal species; tests were performed to examine the difference between two means (Fisher test).

Nine different strains of ectomycorrhizal fungi were used (table I). Three different experiments have been conducted as follows:

Experiment 1:
1. Cooling from +20 °C to –30 °C in 40 min and transfer to –196 °C for 5 min
2. Cooling from +20 °C to –30 °C in 40 min and transfer to –196 °C for 5 days
3. Uncontrolled freezing and direct transfer to –196 °C
4. Control (no freezing)

Experiment 2:
1. Cooling from +20 °C to –60 °C in 80 min and transfer to –196 °C for 15 min
2. Cooling from +20 °C to –60 °C in 80 min and transfer to –80 °C for 15 min
3. Cooling from +20 °C to –60 °C in 80 min and transfer to –80 °C for 7 days
4. Uncontrolled freezing and direct transfer to –80 °C for 7 days
5. Control (no freezing)

Experiment 3:
1. Cooling from +20 °C to –60 °C in 80 min and transfer to –196 °C for 1 month
2. Cooling from + 20 °C to –80 °C in 80 min and transfer to –196 °C for 1 month
3. Control (no freezing)

Table I. Isolates of ectomycorrhizal fungi used in the three different experiments of storage.

Isolate	*Genus*	*Species*	*Site of isolation*	*Host plant*	*Source*
SIV	*Hebeloma*	*crustuliniforme*	Nancy, France	*Picea abies*	1
S 238 N	*Laccaria*	*bicolor*	USA	*Tsuga mertensiana*	2
NAU	*Paxillus*	*involutus*	Leuglay, France	*Quercus robur*	1
441	*Pisolithus*	*tinctorius*	Sao-Paulo, Brazil	*Eucalyptus citriodora*	3
H 498	*Pisolithus*	*tinctorius*	Australia	*Eucalyptus* sp	4
S A 9	*Scleroderma*	*flavidum*	Australia	*Eucalyptus camaldulensis*	1
KTP	*Rhizopogon*	*luteolus*	Australia	*Eucalyptus* sp	4
Schmidt	*Thelephora*	*terrestris*	USA		3
SIV	*Cenococcum*	*geophillum*	Nancy, France	*Picea abies*	1

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In all experiments and treatments, except controls, the vials were thawed after freezing during 60 min at +4 °C and then placed at +25 °C.

RESULTS

Experiment 1 (table II)

Among the nine strains tested only one, *Cenococcum geophillum*, was unaffected by freezing, even with rapid cooling. *Rhizopogon luteolus, Scleroderma flavidum* and *Laccaria bicolor* did not tolerate an uncontrolled cooling, but survived freezing if the cooling rate was slow. Nevertheless, the mycelium, even if it had survived, was injured as indicated by its weak growth after freezing. *Hebeloma crustuliniforme* did not survive immersion in liquid nitrogen for 5 min, but did survive if thawing did not immediately follow freezing. *Pisolithus tinctorius, Paxillus involutus* and *Thelophora terrestris* did not tolerate freezing, even with a slow cooling rate.

Experiment 2 (table II)

The second experiment confirmed the first one and has underlined the importance of the initial period of cooling. Except for *Scleroderma flavidum*, a cooling rate of −1 °C in 60 s decreased the freezing injuries compared to a cooling rate of −1 °C in 48 s. *Pisolithus tinctorius*, strain 441, which did not survive freezing at −196 °C with an initial cooling rate of −1 °C in 48 s, did survive with a slower cooling rate. We may speculate that with a still slower cooling rate, it would be possible to protect the very sensitive strains, *Thelephora terrestris* and *Paxillus involutus*, from freezing injuries. Freezing to −80 °C could be an alternative. *Cenococcum geophillum* was not affected by this treatment even with an uncontrolled cooling. This method of cryopreservation at −80 °C associated with a slow cooling rate could be used also for *Rhizopogon luteolus, Laccaria bicolor* and *Scleroderma flavidum*. The other species did not survive freezing at −80 °C.

Experiment 3 (table II)

The third experiment confirmed the importance of the cooling rate. With a slow cooling rate (−1 °C per min) *Cenococcum geophillum, Rhizopogon luteolus* and *Laccaria bicolor* can be stored without any damage for at least 1 month in liquid nitrogen. An additional experiment, not described here, showed that these three species can be stored in these conditions for at least 1 year. *Hebeloma crustuliniforme* and *Scleroderma flavidum*, which were much more sensitive to freezing, were slightly injured at this rate of cooling (−1 °C per min) and were killed at a slightly faster cooling rate.

DISCUSSION

The survival of ectomycorrhizal fungi during freezing in glycerol depends on the species or strains and on the cooling rate.

The strains of *Thelephora terrestris* and *Paxillus involutus* which were used did not survive any freezing method tested. *Pisolithus tinctorius* reacted very similarly, although one strain survived when the cooling rate was slow, even then the mycelium was damaged as shown by the slow growth of the mycelium after treatment.

Hebeloma crustuliniforme seemed to be a little more resistant than *Pisolithus tinctorius*, but even with a slow cooling rate the mycelium was injured.

Laccaria bicolor and *Rhizopogon luteolus* tolerated freezing if the cooling rate was slow. Nevertheless, the mycelium was injured at a cooling rate faster than −1 °C per min. With a cooling rate of −1 °C per min the mycelium survived a freezing at

Table II. Effect of different methods of freezing on diameter growth in mm of nine ectomycorrhizal fungal strains on a solid malt medium after freezing and 15 days of culture at 25 °C.

Experiment 1

Treatments	*Thelephora terrestris* Schmidt	*Paxillus involutus* NAU	*Pisolithus tinctorius* 498	*Pisolithus tinctorius* 441	*Hebeloma crustuliniforme* SIV	*Scleroderma flavidum* SA9	*Laccaria bicolor* S 238	*Rhizopogon luteolus* KTP	*Cenococcum geophillum* SIV
1 from +20 °C to −30 °C in 40 min −196 °C	0 **a**	0 **a**	0 **a**	0 **a**	0 **a**	15.8 **a**	11.3 **a**	11.6 **a**	23.6 **a**
2 from +20 °C to −30 °C in 40 min −196 °C 5 days at −196 °C	0 **a**	0 **a**	0 **a**	0 **a**	23.6 **b**	-	0 **b**	12.0 **a**	18.6 **b**
3 uncontrolled freezing from +25 °C to −196 °C 5 min at 196 °C	0 **a**	0 **a**	0 **a**	0 **a**	0 **a**	-	0 **b**	0 **b**	16.3 **b**
4 control + 25 °C	17.3 **b**	34 **b**	18.6 **b**	27.6 **b**	27.6 **c**	42.6 **b**	28.3 **c**	43.6 **c**	20.6 **a**

Experiment 2

Treatments	*Thelephora terrestris* Schmidt	*Paxillus involutus* NAU	*Pisolithus tinctorius* 498	*Pisolithus tinctorius* 441	*Hebeloma crustuliniforme* SIV	*Scleroderma flavidum* SA9	*Laccaria bicolor* S 238	*Rhizopogon luteolus* KTP	*Cenococcum geophillum* SIV
1 from +20 °C to −60 °C in 80 min 15 min at −196 °C	0 **a**	0 **a**	0 **a**	4.0 **a**	13.3 **a**	17.0 **a**	20.6 **a**	31.0 **a**	21.3 **a**
2 from +20 °C to −60 °C in 60 min 15 min at −196 °C	0 **a**	0 **a**	0 **a**	0 **b**	0 **b**	28.3 **b**	18.3 **a**	21.6 **b**	13.6 **b**
3 from +20 °C to −60 °C in 60 min 7 days at −80 °C	0 **a**	0 **a**	0 **a**	0 **b**	3.6 **c**	20.0 **a**	25.3 **b**	20.6 **b**	15.3 **b**
4 uncontrolled freezing from +20 °C to −80 °C 7 days at −80 °C	0 **a**	0 **a**	0 **a**	0 **b**	0 **b**	0 **c**	-	5.6 **c**	18.3 **a**
5 control +25 °C	22.3 **b**	22.6 **b**	20.0 **b**	13.3 **c**	21.0 **d**	22.3 **a**	29.3 **c**	26.3 **a**	20.3 **a**

Experiment 3

Treatments	*Pisolithus tinctorius* 441	*Hebeloma crustuliniforme* SIV	*Scleroderma flavidum* SA9	*Laccaria bicolor* S 238	*Rhizopogon luteolus* KTP	*Cenococcum geophillum* SIV
1 from +20 °C to −60 °C in 80 min 1 month at −196 °C	1.4 **a**	18.50 **a**	33.86 **a**	27.96 **a**	33.3 **a**	18.3 **a**
2 from +20 °C to −80 °C in 80 min 1 month at −196 °C	0 **b**	0 **b**	0 **b**	8.33 **b**	0 **b**	16.3 **a**
3 control +25 °C	14.3 **c**	23.0 **c**	29.6 **c**	29.6 **a**	28.3 **a**	17.3 **a**

–196 °C for at least 1 month. After thawing, the mycelium was not injured, and its growth was not significantly different from that of the control.

We assume that this *Laccaria* strain might be stored at –196 °C for several years, agreeing with the work of Ito and Yokoyamo (1983). However, according to these two authors, *Laccaria proxima* appeared to be more sensitive to freezing than *Laccaria laccata*. Jong and Davis (1987) also successfully preserved 12 strains of *Laccaria laccata* for 90 months.

Cenococcum geophillum was not affected by freezing, whether the cooling rate was slow, rapid or uncontrolled.

These results suggest that, among the different species of ectomycorrhizal fungi, there are fundamental differences in physiology and water relationships of the mycelium. We therefore suggest that every specie or strain will have an optimum cooling rate that could avoid cell injury.

All the isolates of *Cenococcum geophillum* studied by Mexal and Reid (1973) and by Coleman et al (1989), were found to be drought tolerant compared to *Pisolithus tinctorius* or *Laccaria laccata* or *L bicolor* isolates. Tolerance to water stress may result from the ability of the fungus to adjust osmotically during stress (Coleman et al, 1989). We know that freezing and thawing processes involve the separation of pure water as ice, and this concentrates any solutes present in the remaining liquid phase. The sites of cryoinjury are in the cellular membranes. The transport of water across the cell membrane during freezing plays a major role in the mechanism of freezing injury (Merymann, 1966; Pegg, 1976).

We also know that *Cenococcum geophillum* is very resistant to high salinity. It can grow on media with more than 11 g NaCl per L (Saleh-Rastin, 1976).

We may assume that there is a strong correlation between the resistance to water stress of *Cenococcum geophillum*, its tolerance to high salinity and its resistance to freezing and thawing.

Poor survival of *Thelephora terrestris, Paxillus involutus* and *Pisolithus tinctorius* could probably be improved by modifying cooling rate and thawing conditions.

CONCLUSION

The main objective of this study was to find a method of cryopreservation of *Laccaria bicolor*. The good survival of *L bicolor* and the absence of mycelium injury after slow cooling followed by freezing at –196 °C or –80 °C show that this species could be preserved without loss of viability. This is of particular importance for the genetic work which is now in progress in different laboratories. Additional experiments show that freezing *L bicolor* S 238 at –196 °C or –80 °C did not affect its ability to form mycorrhizas.

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