Freeze tolerance of soil chytrids from temperate climates in Australia

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Article history:
Received 3 December 2007
Received in revised form 14 January 2008
Accepted 24 January 2008
Corresponding Editor: Nicholas P. Money

Keywords:
Blastocladiomycota
Chytridiomycota
Conditioning
Extreme environments
Tolerance to drying
Tolerance to freezing

Abstract

Very little is known about the capacity of soil chytrids to withstand freezing in the field. Tolerance to freezing was tested in 21 chytrids isolated from cropping and undisturbed soils in temperate Australia. Samples of thalli grown on peptone–yeast–glucose (PYG) agar were incubated for seven days at \(-15^\circ\text{C}\). Recovery of growth after thawing and transferring to fresh medium at \(20^\circ\text{C}\) indicated survival. All isolates in the Blastocladiales and Spizellomycetales survived freezing in all tests. All isolates in the Chytridiales also survived freezing in some tests. None of the isolates in the Rhizophydiales survived freezing in any of the tests. However, some isolates in the Rhizophydiales recovered growth after freezing if they were grown on PYG agar supplemented with either 1 % sodium chloride or 1 % glycerol prior to freezing. After freezing, the morphology of the thalli of all isolates was observed under LM. In those isolates that recovered growth after transfer to fresh media, mature zoosporangia were observed in the monocentric isolates and resistant sporangia or resting spores in the polycentric isolates. Encysted zoospores in some monocentric isolates also survived freezing. In some of the experiments the freezing and thawing process caused visible structural damage to the thalli. The production of zoospores after freezing and thawing was also used as an indicator of freeze tolerance. The chytrids in this study responded differently to freezing. These data add significantly to our limited knowledge of freeze tolerance in chytrids but leave many questions unanswered.

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Introduction

Chytrids (phylum Blastocladiomycota and phylum Chytridiomycota; kingdom Fungi) (James et al. 2007; Letcher et al. 2006) have been isolated from soil samples collected in many parts of the world. In the soil they can be either saprophytes, which decompose a large number of substrates, or parasites of algae, fungi, animals, and plants (Sparrow 1960; Barr 2001). Many chytrids are found in environments where the winter is long and harsh (Barr 2001; Sparrow 1960). Soils in cold climates can repeatedly freeze and thaw or can remain frozen for long periods. Therefore, freeze-tolerant ecotypes must be important adaptations in cold climates. Very little is known about the capacity of any of the chytrids to withstand freezing in the field.

Some chytrids, such as the plant parasite Synchytrium endobioticum, can persist in cropping soil for years in cold climates (Laidlaw 1985) indicating that at least some ecotypes have the capacity to tolerate freezing, perhaps existing as survival structures. However, many other chytrids may be able to recover from freezing in the natural environment, given appropriate conditions. Cultures of some species of chytrids...
can recover from freezing with carefully controlled rates of freezing and thawing in the presence of cryoprotectants (Boyle et al. 2003; Gleason et al. 2007).

Many isolates in other groups of fungi can tolerate freezing (Mazur 1968). For example, most of 400 zygomycetes and ascomycetes stored on agar slants recovered after nine months at -20 °C in a freezer (Carmichael 1956). Some of these fungi were isolated from cold climates (Northern Alberta, Canada) and would be expected to be freeze tolerant. Isolates of arbuscular mycorrhizal fungi (Addy et al. 1997) and ectomycorrhizal basidiomycetes (Tibbett et al. 2002) also tolerated freezing. In a related phylum of zoosporic fungi, anaerobic rumen fungi remained viable in cowpats after several frosts and they could be isolated from faeces that had been stored in a freezer at -20 °C (McGranaghan et al. 1999). These data indicate widespread distribution of freeze tolerance among the fungi, but many of the fungi tested may be ecotypes adapted to freeze tolerance. We would expect that isolates of all groups of fungi from the colder environments would have survival structures that enable the fungus to over-winter.

Chytrids that produce freeze-tolerant survival structures must have mechanisms that prevent the formation or reduce the damaging effects of ice crystals within the cytoplasm of the cell. During winter drying can occur in soil because much of the soil water is held in ice crystals. It is possible for dehydration to occur within a cell when water outside the cell is frozen (Meryman et al. 1977). Thus freeze tolerance may involve tolerance of drying and high osmotic potentials. The capacity to tolerate drying varies among chytrids (Couch 1945; Johnson et al. 2003; Gleason et al. 2004) and freeze tolerance may have a similar distribution.

It is possible that resistance to environmental extremes, such as freezing and drying, involve physiological changes that occur during the maturation of resistant structures. Possible resistant structures include the zoospore cyst, the thick-walled mature zoosporangium or the resting spore in some species. We would expect that thin-walled structures, such as rhizoids, linking hyphae and flagellate zoospores, probably collapse as water rapidly moves out of the cell. Thick-walled structures have been seen in many genera of chytrids and are thought to be resistant to environmental extremes (Karling 1977; Sparrow 1960). However, ‘resistant’ structures have not been clearly identified in most chytrids and little is known about their physiological properties.

As freezing takes a significant period of time in soil and its frequency of occurrence is clearly seasonal, the production of survival structures may be a conditioned response within tolerant isolates. The processes of maturation and germination of resistant sporangia have been studied in three members of the Blastocladiomycota: Allomyces (Machlis & Ossia 1953), Blastocladiella (Lovett & Cantino 1960; Cantino 1969), and Catenaria (Couch 1945). In these three genera the maturation of resistant sporangia is a function of time and growth conditions. However, the particular environmental extremes to which these structures are resistant have not been clearly and thoroughly documented.

The purpose of this investigation was to examine the distribution of freeze tolerance among soil chytrids isolated from temperate regions of Australia. (1) Those chytrids that are subjected to freezing in their natural habitat are expected to be freeze tolerant, whereas those from warm climates are not. (2) Freeze tolerance is expected to be more common among fungi that readily recover from drying. (3) Survival may be more common among fungi that have been subject to conditioning prior to freezing as winter approaches. (4) The structures that enable survival are expected to be thick walled.

Materials and methods

Identity of isolates and culture conditions

The putative identities and origins of the isolates listed in Table 1 have been described previously (Commandeur et al. 2005; Gleason et al. 2004, 2005, 2006; Letcher et al. 2004a, b, 2006). Species descriptions and details of the life-cycles of these fungi can be found in Karling (1977) and Sparrow (1960). All of the fungi were isolated into pure culture from cropping and natural soils in temperate south eastern Australia. All cultures were maintained on peptone–yeast–glucose (PYG; glucose 3.0, peptone 1.25, yeast extract 1.25 and agar 20 g l⁻¹) agar medium (Fuller & Jaworski 1987, p. 297; Gleason et al. 2006). Voucher specimens have been deposited in at the University of Alabama.

Freeze tolerance in thalli

Procedures for freezing. Cultures of all chytrids were grown on PYG agar at 20 °C for 7 d prior to experimental use as inocula. More than 30 clumps of moist thalli from the monocolonnic species or ten small blocks of agar with mycelia from the polycentric species were placed into each 50 ml sterile polypropylene screw cap centrifuge tubes (Iwaki). Two different conditions were used for freezing the fungi: (1) no additional liquid was added (both types of inoculum) or (2) 1 ml de-ionized water was added to clumps of monocolonnic species. The tubes were then placed into the freezer and maintained at -15 °C for 7 d. After 7 d the contents were removed, spread onto the surface of PYG agar in Petri dishes (recovery medium) and incubated at 20 °C for 7 d. With the slower growing Cladocychruim sp. AUS 11 the incubation time on the recovery medium was extended to 14 d. Freezing was repeated three times with moist thalli and twice with thalli resuspended in de-ionized water in different experiments with each isolate. For each experiment a control without freezing for each isolate was incubated on PYG agar at 20 °C for 7 d to test for viability.

Assessment of survival. Three methods were selected to evaluate the effects of the freezing and thawing process on survival. (1) Resumption of growth. Survival after freezing was assessed first by monitoring the resumption of growth visually on the recovery medium at 20 °C. The appearance of new clumps of thalli, increase in the volume of clumps of thalli or increase in the diameter of the mycelium during the incubation period was taken to indicate growth. (2) Zoospore production. For those isolates that survived freezing, a separate set of experiments were conducted. After freezing moist thalli the discharge of zoospores was induced in the monocolonnic species by placing thalli in de-ionized water on a microscope slide at 20 °C. The zoosporangia were subsequently
observed microscopically over a 2 h period. (3) Freeze damage. The morphology of the thalli was examined microscopically for freeze damage immediately after thawing. The latter method only suggests the potential for survival because the structures that were observed in the microscope were not tested for viability.

**Response to osmotic stress.** The effects of two different growth conditions prior to freezing were examined in the nine isolates in the Rhizophydiales. These isolates were grown for 7 d on solid PYG agar supplemented with either 1 % NaCl or 1 % glycerol prior to freezing moist thalli. After 7 d at −15 °C the thalli were transferred to PYG recovery medium and incubated at 20 °C for 7 d. The cultures were then examined visually for growth and microscopically for freeze damage. These experiments were completed three times with all isolates. For each experiment a control without freezing for each isolate was incubated on PYG agar supplemented with either 1 % NaCl or 1 % glycerol prior to freezing moist thalli. After 7 d at −15 °C for 7 d to test for viability.

**Tolerance of freezing in zoospores**

Catenaria sp. Dec CC 4-10Z, Spizellomyces sp. Mar Ad 2-0, Gaertneriomycetes sp., Mar CC 2, Rhizophyctis rosea AUS 13, Pouellomyces sp. AUS 16 and AUS 17 and Chytridiomyces sp. AUS 14 and Ob 8-3 were grown on PYG agar for 7 d. Suspensions of zoospores were obtained by flooding the cultures in Petri dishes with 5 ml de-ionized water and incubating at 20 °C for 2 h. First, a few drops of the suspension of zoospores were placed on a microscope slide and frozen at −15 °C for 1 h in the freezer, thawed and then examined with the light microscope in order to ascertain the effect of freezing and thawing on zoospore morphology.

In order to test zoospores for survival, 1 ml suspensions of zoospores in de-ionized water were placed in sterile centrifuge tubes in the freezer at −15 °C for 7 d. Three centrifuge tubes containing zoospores of each isolate were frozen. After 7 d the suspensions were thawed and poured onto PYG agar to test for viability. The appearance of colonies on the recovery media after 3 d indicated survival. The suspensions of zoospores without freezing were also placed on PYG agar to test for viability.

**Tolerance of freezing in young thalli of AUS 16**

Zoospores of Pouellomyces sp. AUS 16 were incubated in screw cap test tubes with 5 ml liquid PYG growth medium at 20 °C for 22 and for 48 h. Three 1 ml samples of each culture were placed into the freezer at −15 °C for 7 d, then poured onto PYG agar and incubated at 20 °C for 14 d. Samples of the cultures were checked for composition with the light microscope before freezing. The formation of colonies on the recovery medium was followed during the incubation period.

**Results**

**Tolerance of freezing in thalli**

**Resumption of growth.** The results of the tests for survival of thalli after freezing are listed in Table 1. All isolates in the

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**Table 1 – Survival of chytrids isolated from soils in Australia after incubation at −15 °C for 7 d**

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Blastocladiales and Spizellomycetales resumed growth after freezing. The three isolates in the Chytridiales (Chytrium sp. Ob 3-8 and AUS 14 and Cladochytrium sp. AUS 11) survived freezing in some tests, whereas none of the Rhizophydiales survived freezing in any tests. Addition of de-ionized water before freezing made no difference to the results. The controls without freezing continued to grow normally. There was some inconsistency in the results for Chytriomyces sp. Ob 3-8 and AUS 14, because most but not all thalli in all experiments recovered. In one experiment with Cladochytrium sp. AUS 11 all of the mycelia resumed growth on the recovery medium after 14 d but in a second experiment none of the mycelia recovered. In the third experiment only some of the mycelia (six out of 13) of Cladochytrium sp. AUS 11 recovered.

**Zoospore production.** The release of zoospores from zoosporangia immediately after freezing and thawing moist thalli was tested in 11 of these isolates. Powellomyces sp. AUS 16 and AUS 17 produced lots of zoospores within 1 h after return to 20°C. Zoosporangia with discharge tubes were present in both isolates. No zoospores were produced by Chytriomyces sp. AUS 14 and Ob 3-8, Spizellomycetes sp. Mar Ad 2-0 and Dec CC 4-10F, Gaertneriomyces sp. Mar C/C2, Catenaria anguililae Dec 4-10Z that appeared to be broken by the formation of ice crystals during the freezing and thawing process, but the morphology of most thalli appeared unchanged. Massive damage due to ice crystal formation, resulting in many distorted, broken, and collapsed cells, was observed in samples of thalli of Bothiomyces sp. AUS 6 and AUS 7. There was also evidence of damage to the cell membranes and cell walls as these structures were distorted or broken. Broken cells of Bothiomyces sp. AUS 6 after freezing are shown in Fig 1C. The morphology of the other members of the Rhizophydiales was not observed after freezing.

**Freeze damage of polycentric isolates.** The three polycentric isolates were examined in the light microscope after freezing for 1 h at −15°C. In Catenaria sp. Poly Ad 2-0 the morphology of the resisting sporangia remained unchanged, whereas the isthmus hyphae collapsed during the freezing process (Fig 1E). In Allomyces arbuscula Allo Mar CW16 the resistant sporangia remained intact while the zoosporangia and gametangia were damaged and most hyphae collapsed. In Cladochytrium sp. AUS 11 the resting spores remained intact while all other structures, including spindle bodies, either collapsed or lost their cytoplasm.

**Response to osmotic stress.** The results of the tests for survival after growth on PYG agar supplemented with 1% NaCl or 1% glycerol followed by freezing for 7 d are given in Table 2. When grown on medium supplemented with 1% NaCl prior to freezing and thawing process. An intact zoosporangium of Powellomyces sp. AUS 16 after freezing is shown in Fig 1A. There were some thalli from the isolates of Chytriomyces sp. AUS 14 and Ob 3-8, Gaertneriomyces sp. Mar C/C2, Spizellomyces sp. Mar Ad 2-0 and Dec CC4-10F and Catenaria anguililae Dec 4-10Z that appeared to be broken by the formation of ice crystals during the freezing and thawing process, but the morphology of most thalli appeared unchanged. Massive damage due to ice crystal formation, resulting in many distorted, broken, and collapsed cells, was observed in samples of thalli of Bothiomyces sp. AUS 6 and AUS 7. There was also evidence of damage to the cell membranes and cell walls as these structures were distorted or broken. Broken cells of Bothiomyces sp. AUS 6 after freezing are shown in Fig 1C. The morphology of the other members of the Rhizophydiales was not observed after freezing.
freezing Boothiomyces sp. AUS 2 and Terramyces sp. AUS 3, Kap-
pamycyes laurelensis sp. AUS 15 and Rhizophydia sp. Mar Ad 14
recovered. When grown on medium supplemented with 1 %
glycerol prior to freezing Boothiomyces AUS 9 and Rhizophydia
sp. Mar Ad 14 recovered.

When observed in the light microscope there was no
evidence of freeze damage in Boothiomyces sp. AUS 2 and
Terramyces sp. AUS 3 after growth on media supplemented
with NaCl and in Boothiomyces sp. AUS 9 after growth on media
supplemented with glycerol prior to freezing for 7 d. Extensive
freeze damage was observed in the other isolates. This indi-
cates that osmotic stress prior to freezing was only partially
successful in preventing death of the cells.

Tolerance of freezing in zoospores

Suspensions of zoospores of Catenaria sp. Dec CC 4-10Z, Spizel-
lomyces sp. Mar Ad 2-0, Gaertneriomyces sp. Mar CC 2, Rhizo-
phytis rosea AUS 13, Powellomyces sp. AUS 16 and AUS 17
and Chytromyces hyalinus AUS 14 and Ob 8-3 were placed in
the freezer for 7 d. After transfer to PYG agar many colonies
Powellomyces sp. AUS 16 and AUS 17 appeared within 2 d indic-
ating survival. No growth was observed in the other isolates.

Suspensions of zoospores of the same isolates were placed
on microscope slides, frozen for 1 h and then thawed. The
process of cooling caused the zoospores to lose motility and
to encyst. After return to 20 °C only zoospore cysts were
observed with the light microscope. Flagella were still
attached to some of the cysts.

Tolerance of freezing in young thalli

After freezing and thawing, 22 and 48 h old thalli of Powellomy-
ces sp. AUS 16 released zoospores, and colonies appeared on
the PYG agar within 4 d.

Discussion

Twenty-one soil chytrids were grown for 7 d on PYG agar at
20 °C and then were incubated at −15 °C for 7 d (Table 1). All
of the chytrids in the orders Chytridiales, Blastocladiales, and
Spizellomyctales survived growth when returned to 20 °C,
whereas none of the chytrids in the order Rhizophydia re-
sumed growth. Therefore, some chytrids are more tolerant
to freezing than others.

Is tolerance to freeze tolerance an ancestral trait that
has been lost in some of the Rhizophydiales?

Although chytrids have been placed into orders by molecular
techniques (James et al. 2006; Letcher et al. 2006), the evolu-
tionary relationships between these groups of fungi are not
well understood at present. When more 18s and 28s rDNA
sequences for chytrids in all of the orders have been pub-
lished, we will have a better idea whether freeze tolerance is
an ancestral trait.

Are those chytrids that are subjected to freezing in cold
climates freeze tolerant, whereas those from warm climates
are not?

It is difficult to see a relationship between the temperature
range at the collection site in Australia and the tolerance of
the fungus to freezing. Most of the chytrids tested in this study
were isolated from soils in temperate climates that rarely
experience temperatures below 0 °C. Except for the Rhizophy-
diales they all have some degree of freeze tolerance.

However, the temperature at night in the winter can drop
below 0 °C at three of the sites. Chytridiales hyalinus Ob 3-8
was isolated from soil beside Ruby Creek in the Blue Moun-
tains near Oberon, NSW, Cladochrymium sp. AUS 11 was isolated
from soil in the mountains in southwestern Tasmania and
Boothiomyces sp. AUS 7 was isolated from under moss near
a spring on Bushwalker Mountain near Milton, NSW. Chytri-
omyces hyalinus Ob 3-8 and Cladochrymium sp. AUS 11 but not
Boothiomyces sp. AUS 7 survived freezing, although the results
for Chytridiales hyalinus Ob 3-8 and Cladochrymium sp. AUS 11
were inconsistent. We would expect all three of these chytrids
to be freeze tolerant but Boothiomyces sp. AUS 7 was not. The
freeze tolerance of Boothiomyces isolates from soil in cold
climates, such as the arctic regions, needs to be tested.

Is freeze tolerance more common among chytrids that
readily recover from drying?

Isolates in the orders Blastocladiales and Spizellomyctales sur-
vived drying at 20 °C for 7 d whereas isolates in the orders Chy-
tridiales or Rhizophydiales did not (Gleason et al. 2004, 2007).
Cycles of wetting and drying are common in the soil in temper-
ate climates. Both freezing and drying occur simultaneously in
cold climates during the winter. It can be concluded that the ca-
pacities to survive both freezing (in present study) and lack of
moisture (Gleason et al. 2004) tends to follow taxonomic lines,
although only a small number of chytrids have been tested.
The three isolates in the Chytridiales survived freezing in
some experiments but not drying. More isolates in all of these
orders need to be tested for tolerance to both freezing and dry-
ing. Also some of the Rhizophydiales, which are parasites in
aquatic environments, need to be included.
It is possible that these fungi can dehydrate in the centrifuge tubes during the processes of freezing, storage, and thawing. In order to reduce dehydration during the freezing and thawing process, the fungi were also frozen in water. However, the results for each fungus frozen with and without water were identical. The addition of water outside the cell does not necessarily prevent dehydration inside the cell during freezing (Meryman et al. 1977).

Is survival more common among chytrids that have been subject to conditioning prior to freezing as winter approaches?

Chytrids in the order Rhizophydiales persist in the soil in extremely cold climates where freezing occurs in the winter, such as the Canadian arctic (Booth & Barrett 1971, 1976). The reason that members of the Rhizophydiales did not survive freezing after growth on PYG agar in the present study is not known. Recovery of some Rhizophydiales cultures grown on media supplemented with either 1% NaCl or glycerol prior to freezing suggests that freeze tolerance can be induced by osmotic stress. The osmotic potential increases in soil solutions when drying occurs. It is not known whether incubation for long periods at low temperatures can also induce freeze tolerance in the Rhizophydiales.

Are the structures that enable resistance to freezing and lack of moisture thick walled?

The stages of the life-cycle that are resistant to freezing and lack of moisture are not known. Observation in the light microscope indicated that the process of freezing and thawing damages the structure of the thallus in isolates that are not freeze tolerant. It is possible that only specific resistant structures, such as zoosporangia, cysts, resistant sporangia, or resting spores, are freeze tolerant. Furthermore, the capacity to survive freezing and lack of moisture may increase during the maturation of resistant structures. Couch (1945) suggested that maturation is necessary for resting spores to survive drying in Catenaria. Therefore, only some thalli in the frozen or dried samples might resume growth. It was not possible to determine quantitatively how many thalli on the surface of solid media resumed growth after freezing using the techniques in the present study. Quantitative studies are necessary to determine the degree of freeze tolerance of these structures.

The experiment with discharge of zoospores following freezing provides some evidence as to which structures might be resistant to freezing. In Powellomyces hyalinus AUS 16 and AUS 17 some zoosporangia discharged zoospores within 1 h after removal from the freezer and return to 20 °C. In these fungi at least some of the zoosporangia must be resistant to freezing. As no zoospores were produced by Chytriomyces hyalinus AUS 14 and Ob 3-8, Gaertneriomyces sp. Mar CC 2, Spizellomyces sp. Mar Ad 2-0, and Dec CC 4-10F, Catarnia sp. Dec CC 4-10Z or Rhizophlyctis rosea AUS 13 within 2 h although zoosporangia were present, the zoosporangia in these isolates must go through a recovery process before discharge can begin. Eventually discharge must have occurred because these isolates resumed growth after transfer to the recovery media. The fact that in some chytrids zoospore cysts are resistant to freezing suggests that the zoospore cyst may also be a survival structure. Young thalli were tested for survival only in Powellomyces AUS 16. Some young thalli of Powellomyces sp. AUS 16 also survived freezing. It appears that almost all of the stages in the life cycle of Powellomyces sp. AUS 16 are freeze tolerant. The structures that possibly can survive freezing in each isolate are listed in Table 3. With further research more freeze-tolerant structures may be discovered.

The nature of the mechanisms for tolerance to freezing and drying is unknown in chytrids. In those chytrids that have been studied in the laboratory, the resistant sporangia are fully permeable to water (Machlis & Ossia 1953; Gleason et al. 2004). The volume of the protoplasm of the resistant sporangia will decrease (plasmolysis) when water leaves the cell. This occurs when resistant sporangia are placed in environments that lack moisture or in hypertonic solutions (Machlis & Ossia 1953; Gleason et al. 2004; Gleason et al. 2006). The effects of both freezing and lack of moisture on cell physiology and rates of survival need to be carefully studied with quantitative experiments in chytrids. A high rate of survival after freezing and drying might be important in maintenance of high population numbers in the soil in cold climates. Chytrids must begin to grow quickly at the onset of spring.

**Acknowledgements**

We authors thank Martha J. Powell and Sharon Standridge for their advice and encouragement and Malcomi Ricketts for assistance with photography. This research was supported in part by NSF-PEET grant no. DEB-9978094.

**References**
