CHAPTER 7-9
WATER RELATIONS: WINTER PHYSIOLOGY

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Freezing tolerance must necessarily be coupled with drought tolerance and therefore this chapter would be incomplete without a discussion of winter effects. While other plants are dormant and have either lost their leaves, died back to ground level, or adapted in other ways to prevent damage from heavy snow and loss of water due to ice crystals, many bryophytes remain exposed, ready to have photosynthesis whenever light, water, and temperatures permit (Figure 1). Here we will examine the conditions related to their winter water relations. Temperature relations will be covered in a different chapter.

Problems in Winter

Bryophytes do have problems to deal with in winter. These include damage to their DNA and photosynthetic tissue (chlorophyll) from the UV light, temperature stress, cellular freezing and structural damage, and desiccation damage due to ice crystals. Alberdi et al. (2002) consider that adaptations to cold include high resistance to light stress, high freezing resistance, and high photosynthetic capacity at low temperatures. To this list I must add the ability to regain hydration quickly upon thawing. Most of these topics will be discussed elsewhere in chapters that deal with that particular physiological parameter. This chapter will examine the winter water relations.

The all-important water, whether as fog or rain or dew, is suddenly no longer liquid, but solid. Not only does this present problems for obtaining water, but it also means that hygroscopic ice crystals can draw water from the bryophyte cells.

But not all bryophytes suffer from the problem of ice damage. The thallose liverwort Ricciocarpos natans (Figure 2-Figure 4) can spend the winter encased in ice and can tolerate temperatures to -30°C (Frahm 2006). Frahm suggested that it was able to survive this frozen condition because it has no water vacuoles, thus providing no free internal water to form crystals that could destroy its
membranes. Rod Seppelt (Bryonet discussion 14 November 1997) also noted an absence of vacuoles in Antarctic mosses. Both *Ricciocarpos natans* and *Riccia fluitans* (Figure 5-Figure 6) are common in Arctic streams, so we might expect them to have this absence of vacuoles. I find it interesting that no one seems to have reported either presence or absence of vacuoles in *R. natans* (based on literature search and question posed on Bryonet in April 2015). Rather, lipids and starch bodies may help in their winter tolerance (Rod Seppelt, Bryonet discussion 14 November 1997).

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**Figure 2.** *Ricciocarpos natans* in ice. Photo by Jan-Peter Frahm, with permission.

**Figure 3.** *Ricciocarpos natans* thallus, a species with lipids and starch bodies that may help it survive winter. Photo by Norbert Stapper, with permission.

**Figure 4.** *Ricciocarpos natans* section showing the many chambers packed with small chlorophyllose cells. Photo by Norbert Stapper, with permission.

**Figure 5.** *Riccia fluitans* with pearling, a species that survives freezing. Photo by Christian Fischer, with permission.

**Figure 6.** *Riccia fluitans* cross section showing large air chambers that help it to float. Photo by Ralf Wagner <www.dr-ralf-wagner.de>, with permission.

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**Frost Damage**

Those venues of green in the spring attest to the survival of bryophytes through the winter, subjected to frost before snow cover arrives and subsisting at near 0°C under the snow. But few studies give us specifics on what species survive and which ones are damaged.

Fletcher (1982) had the opportunity to document the frost responses of a number of species in cultivation. Among the winter survivors, reaching temperatures as low as -3°C, are species from New Zealand [*Papillaria crocea* (Figure 7), *Hypopterygium* spp. (Figure 8), *Rhizogonium bifarium* (see Figure 9), *Cyathophorum bulbosum* (Figure 10), *Eriopus brownii*], South Africa [*Hypopterygium* sp.], Australia [*Gigaspermum repens* (Figure 11), *Goniomitrium acuminatum* subsp. *enerve* (= *Goniobryum enerve*; Figure 12), and Florida, USA [*Rhizogonium spiniforme* (Figure 13)]. Even the delicate-looking *Takakia lepidozioides* (Figure 14-Figure 15) remains healthy. As we might expect, the widespread mosses *Sphagnum* spp. (Figure 16) and *Mnium* spp. [probably *Plagiobryum* since no *Mnium* species are present in New Zealand (NZOR 2015); Figure 17] survive the frost. On the other hand, *Haplomitrium hookeri* (Figure 18) from New Zealand and *H. mnioides* (Figure 19) from Japan had no healthy plants remaining after an exposure to -3°C, despite their ability to survive and grow in the winters in their native habitats.
Figure 7. *Papillaria crocea*, a winter survivor in NZ. Photo by Janice Glime.

Figure 8. *Hypopterygium didictyon*, a genus that can withstand temperatures to -3°C. Photo by Juan Larrain, with permission.

Figure 9. *Rhizogonium novae-hollandiae*. *Rhizogonium bifarium* survives temperatures as low as -3°C in New Zealand. Photo by Niels Klazenga, with permission.

Figure 10. *Cyathophorum bulbosum* from Tasmania, a moss that survives freezing. Photo by Vita Plasek, with permission.

Figure 11. *Gigaspermum repens* with capsules, a moss that tolerates freezing in Australia. Photo by David Tng, with permission.
Figure 12. *Goniumtrium acuminatum* subsp. *enerve* with capsules, a moss that tolerates freezing in Australia. Photo by David Tng, with permission.

Figure 13. *Rhizogonium spiniforme* with capsule, a moss that tolerates frost. Photo by Janice Glime.

Figure 14. *Takakia lepidozioides* in its native habitat in Japan. This moss species remains healthy through the Hokkaido winters. Photo from the Digital Herbarium of the University of Hiroshima, with permission.

Figure 15. *Takakia lepidozioides*, a winter survivor. Photo from the Digital Herbarium of University of Hiroshima, with permission.

Figure 16. *Sphagnum cristatum*, a New Zealand species that survives in winter there. Photo by Janice Glime.
Experiences with freezing in *Plagiomnium undulatum* (Figure 20) may help us to understand some of these differential responses (Hudson & Brustkern 1965). If this moss is cooled slowly, it experiences extracellular freezing; this prevents the intracellular freezing that could be fatal. Following that experience, the leaves can be cooled down to -30°C without injury. The slow freezing prevents the formation of extensive extracellular ice. Young shoots, however, cannot withstand temperatures below -12°C.

Among the thallose liverworts, *Lunularia* (Figure 21), *Pellia* (Figure 22), *Preissia* (Figure 23-Figure 24), *Riccardia* (Figure 25), *Riccia* (Figure 26), and *Marchantia polymorpha* (Figure 27), all survive frost (Fletcher 1982) and remain healthy. On the other hand, the thallose liverworts *Moerckia blyttii* (Figure 28-Figure 29), *Symphogyna* sp. (Figure 30), *Corsinia coreandra* (Figure 31-Figure 32), and *Asterella* (Figure 33) all can become severely bleached when subjected to frost. *Dumortiera hirsuta* (Figure 34-Figure 35) doesn't die, but it becomes blackened. Similarly, *Fossombronia* (Figure 36) and *Anthocerotophyta* experience decay, but for them the decay is a normal winter occurrence; growth resumes in the spring. In the greenhouse, which reaches -5.5°C, *Asterella* and *Monoclea forsteri* (Figure 37) are blackened by frost, whereas *Marchantia* spp, *Dumortiera hirsuta, Anthoceros punctatus* (Figure 38), and *Phaeoceros laevis* (Figure 39) remain healthy in the same greenhouse.
Figure 22. *Pellia endiviifolia* males with reddish antheridal cavities & females in center, a species that survives freezing. Photo by David Holyoak, with permission.

Figure 23. *Preissia quadrata* with archegoniophore, member of a genus that survives freezing. Photo by Michael Lüth, with permission.

Figure 24. *Preissia quadrata* thallus section showing several globose oil bodies that may help it to survive desiccation and freezing. Photo by Kristian Peters, with permission.

Figure 25. *Riccardia* sp, a thallus that survives freezing. Photo by Niels Klazenga, with permission.

Figure 26. *Riccia beyrichiana*, a genus that is able to survive frost – and desiccation. Photo by Jan-Peter Frahm, with permission.

Figure 27. *Marchantia polymorpha* with gemmae cups, a species that survives frost. Photo by Michael Lüth, with permission.
Figure 28. *Moerckia blyttii*, a liverwort that is sensitive to frost, becoming bleached. Photo by Michael Lüth, with permission.

Figure 29. *Moerckia blyttii* habitat. Photo by Michael Lüth, with permission.

Figure 30. *Symphyogyna podophylla*, a liverwort genus in which one species is sensitive to frost and becomes bleached. Photo by Andras Keszei, with permission.

Figure 31. *Corsinia coriandrina*, a thallose liverwort that is sensitive to frost under some conditions. Note bleached tissues, especially in the bottom center. Photo by Michael Lüth, with permission.

Figure 32. *Corsinia coriandrina* in its habitat on a ledge, a thallose liverwort that is sensitive to frost under some conditions. Photo by Michael Lüth, with permission.

Figure 33. *Asterella lindenbergiana*, a thallose liverwort that is sensitive to frost. Photo by Michael Lüth, with permission.
Figure 34. *Dumortiera hirsuta*, a thallose liverwort that is sensitive to frost and becomes blackened, but doesn't die. Photo by Michael Lüth, with permission.

Figure 35. *Dumortiera hirsuta* habitat. Photo by Michael Lüth, with permission.

Figure 36. *Fossombronia angustata*, a species in which frost causes decay, a normal winter occurrence. Note the patches of colorless plants. Photo by Michael Lüth, with permission.

Figure 37. *Monoclea forsteri*, a species that is blackened by frost. Photo by Jan-Peter Frahm, with permission.

Figure 38. *Anthoceros punctatus*, a species that survives to -5.5°C. Photo by Jonathan Sleath, with permission.

Figure 39. *Phaeoceros laevis* with capsules, a species that remains healthy to -5.5°C. Photo by Michael Lüth, with permission.

*Corsinia coreandra* (Figure 31-Figure 32) is a puzzle. It is a xerophyte, but in cultivation frost causes it to become bleached (Fletcher 1982). At the same time in the same garden as the cultivation containers, it remains healthy on an exposed wall top and likewise remains healthy in the greenhouse that goes down to -5.5°C.

Much of what we know about cold tolerance has come from Antarctic studies. The Antarctic continent has only
2% of its land free from ice (Seppelt & Ochyra 2008). These areas are dominated by bryophytes (24 species of mosses; 1 liverwort), lichens, and algae.

We have learned that macromolecular substances (ice-active substances or IASs) can modify the shape of the growing ice crystals (Raymond & Fritsen 2001). These semipurified substances from *Bryum* sp. (Figure 40) from the Antarctic contain both protein and carbohydrate. The substances lose most of their recrystallization ability by heat treatment. Raymond and Fritsen suggest that these substances might increase freezing tolerance by preventing ice recrystallization.

![Figure 40. Bryum pseudotriquetrum in Antarctica, a species in which protein and carbohydrate might increase freezing tolerance by preventing ice recrystallization. Photo by Catherine Beard, with permission.](image)

Some bryophytes thrive in habitats where they regularly get exposed to sub-zero temperatures. *Bryoxiphium norvegicum* (Figure 41) is such a species (Shirasaki 1984). In Japan, *B. norvegicum* subsp. *japonicum* lives in an altitudinal range of 80-2350 m, being most abundant in districts where deep snow covers the ground for a long period. But it does not grow where the snow is, but rather grows on the vertical sides of overhanging rocks in ravines. Hence, it survives winter without the protection of snow, but it is sheltered by the rocks from the cold, desiccating winds.

![Figure 41. Bryoxiphium norvegicum, a species that grows on vertical surfaces where it is exposed to sub-zero temperatures without snow cover in winter. Photo by Bob Klips, with permission.](image)

**Ice Crystals**

Ice crystals can cause plant tissues to dry out. Ice crystals are very hygroscopic and thus their presence can result in water being drawn out of tissues. But they also gather water from the atmosphere. Moffett *et al.* (2009) suggest that these ice crystals can sequester water that becomes available when they melt. Because bryophytes are able to absorb water through their leaves, this water can be immediately available and provide rapid rehydration.

Rod Seppelt (pers. comm. 7 April 2015) does not consider it to be unusual that *Ricciocarpos natans* (Figure 2-Figure 4) and *Riccia fluitans* (Figure 5) can be encased in ice or survive under a layer of snow. As he points out, temperatures within the ice are not typically very cold. As an example, he cites putting a pot of water 80 cm under the Alaskan snow overnight. The air temperature that night dipped to -22°C, but the pot of water remained unfrozen.

Of course dehydration caused by freezing can have other consequences. Dependence on the symbiont *Nostoc* is interrupted and nitrogen fixation is significantly reduced in winter due to dehydration resulting from freezing in the epiphytic leafy liverwort *Porella* (Figure 42-Figure 43) in Oregon, USA (De Gezelle 2003).

![Figure 42. Porella cordeana in one of its vertical habitats where the symbiotic Cyanobacterium Nostoc provides it with needed nitrogen. Photo by Michael Lüth, with permission.](image)

![Figure 43. Porella cordeana on a vertical substrate, a species that suffers in winter from diminished nitrogen fixation by its symbiont. Photo by Michael Lüth, with permission.](image)
Desiccating Conditions

If you have ever gone out in early spring in areas where there is snow cover all winter, bryophytes provide a refreshing green cover on the newly emergent ground. This fresh green color requires the presence of water to rehydrate the tissues. But where does it come from?

In many temperate regions, spring brings rain, hence making rehydration an easy task. But in some regions, my own home in the Keweenaw Peninsula of Michigan included, snowmelt is followed by drought, and this is exacerbated along roads by the sand and salt that was used to provide traction for vehicles during winter ice and snow.

Nevertheless, in northern habitats, snowmelt can provide water for a considerable time. In the Cairngorm Mountains, Scotland, Kiaeria starkei (Figure 44) is immediately ready for photosynthetic activity when its own snow cover disappears (Woolgrove & Woodin 1996). It has just spent its winter at temperatures of 0°C to slightly above, but with no light penetration while the snow depth is greater than 50 cm. When the snow disappears from it, its tissue chlorophyll recovers rapidly to 250% of its winter low and within two weeks its carbohydrate concentrations increase by 60%. This moss has nitrate reductase activity and is able to take advantage of pollutant nitrate, accumulated by the snow, that becomes available as the snow melts.

One advantage for bryophytes is that they have a low temperature compensation point. Hence, snowbed bryophytes such as Anthelia juratzkana (Figure 45-Figure 47) and Polytrastrum sexangulare (Figure 48-Figure 49) can maintain photosynthesis at low temperatures with a lower temperature compensation point of about -4 to -5°C. Furthermore, A. juratzkana can survive in the dark under cold, wet conditions for nine months with no effect on its photosynthetic capability. This makes A. juratzkana well adapted to grow in the border zone along permanent snow patches. However, the net photosynthesis is reduced due to an increase in respiration rate. Polytrastrum sexangulare, on the other hand, does not tolerate this border regime as well as does A. juratzkana.
In tracheophytes, freezing can cause ice to form within cells, potentially causing membrane damage and subsequent loss of cell constituents. Lenne et al. (2010) write "A dehydrating moss gathers no ice." Using the widespread moss Ceratodon purpureus (Figure 50-Figure 51), they demonstrated that no ice accumulates in the cells during freezing. But external ice does induce desiccation. The effects of this desiccation depend on the cell type. Water-filled hydroid cells cavitate like tracheophyte xylem cells, becoming embolized (blocked, in this case by ice) at -4°C. Parenchyma cells of the inner cortex of the stem exhibit cytorrhysis (complete and irreversible collapse of a plant cell wall due to loss of water through osmosis), losing 20% of their original volume at -20°C nadir temperature (lowest temperature of a cycle). It is puzzling that chlorophyll fluorescence shows no evidence of damage after thawing from a -20°C event, especially since the sugar concentrations are insufficient to confer freeze tolerance in these conditions (see below). Furthermore, ice nucleation occurs in hydrated tissues at ~-12°C. The answer to this puzzle seems to lie in the desiccation itself. No damage occurs to those desiccated mosses at -20°C. The very desiccating nature of ice crystals appears to be the mechanism that prepares the moss for the low temperatures.

This desiccation relationship is supported in the Antarctic moss Polytrichum juniperinum (Figure 52) wherein repeated freeze-thaw cycles cause a greater reduction in photosynthesis than constant freezing for the same time period (Kennedy 1993). This is much like the effect of repeated dehydration/rehydration that causes a net carbon loss. This is supported by the observation that freeze-thaw cycles every 12 hours cause more damage than those every 24 or 48 hours. Most of the damage occurs during the first cycle with little occurring during subsequent cycles. Kennedy found that at 10°C the gross CO₂ flux is directly proportional to moss water content between 0.3 and 3.5 g g⁻¹ dry mass. Mosses with a low water content withstand freeze-thaw cycles to sub-zero temperatures better than do samples with a high water content. Kennedy suggests that on Signy Island in the Antarctic the populations of Polytrichum juniperinum may be limited in distribution by sub-zero temperatures and freeze-thaw cycles at times when snow cover is insufficient to provide insulation.
Davey (1997) examined Antarctic bryophytes and demonstrated the importance of water. The photosynthetic rate decreased as the length of the dehydration period increased in all bryophytes examined. The photosynthetic capacity is affected by stress, and Davey found that both desiccation and winter freezing caused a loss of photosynthetic capacity. But the base level of photosynthetic capacity is able to survive both. Furthermore, frequent dehydration and rehydration cycles cause a loss of photosynthetic rate that is greater than that in continuous dehydration. Davey hypothesized that water availability is an important contributor to the distribution of bryophytes in the Antarctic, where winter-like weather can occur on almost any day of the year.

Barker et al. (2005) found bleaching in *Syntrichia caninervis* (Figure 53) during winter in the Mojave Desert, USA. They attributed this loss of green color to frequent rain events during warmer months that year, citing appearance of chlorosis just after that. This is consistent with the effects of frequent dehydration-rehydration events seen by Davey (1997). Under this regime, particularly for short, light rainfall events, the plants do not have enough time to repair membranes before they become dehydrated again, thus losing energy with each mild rainfall event. This leaves them with diminished color for the winter, a condition hopefully to be repaired in the spring.

**Desiccation Tolerance**

Desiccation tolerance is seasonal, probably in most bryophytes. Only the moss *Andreaea rothii* (Figure 55-Figure 56) failed to show seasonal variation in net assimilation following 24 hours of remoistening, compared to clear seasonal differences in the leafy liverwort *Plagiochila spinulosa* (Figure 57-Figure 58) and mosses *Hylocomium splendens* (Figure 59), *Scorpiurium circinatum* (Figure 60), *Syntrichia ruralis* (Figure 61-Figure 62), and *Racomitrium aquaticum* (Figure 63-Figure 64) (Dilks & Proctor 1976). Those with seasonal variation usually had low desiccation tolerance in autumn and winter and greater tolerance in spring and summer. *Hylocomium splendens* differed in having relatively high tolerance in January (winter), with little change from then until July.
Figure 56. *Andreaea Rothii* in a typical vertical rock habitat where snow does not accumulate. Photo by Michael Lüth, with permission.

Figure 57. *Plagiochila spinulosa* in a soil bank habitat where it exhibits seasonal differences in photosynthesis. Photo by Michael Lüth, with permission.

Figure 58. *Plagiochila spinulosa*, a leafy liverwort that has seasonal differences in its hydrated photosynthetic rate. Photo by Michael Lüth, with permission.

Figure 59. *Hylocomium splendens* with clinging snow, a moss that exhibits seasonal differences in photosynthesis. Photo by Michael Lüth, with permission.

Figure 60. *Scorpiurium cirratinum*, a moss that exhibits seasonal differences in photosynthesis. Photo by Michael Lüth, with permission.

Figure 61. *Syntrichia ruralis* habitat in a cliff splash zone. Photo courtesy of Betsy St Pierre.
their natural desiccation tolerance already gave them adequate protection during cryopreservation. What is it about freezing that actually kills or damages the bryophytes? Crystals can damage the membranes, but isn't the real damage ultimately desiccation damage? For example, 90-100% of the protonemata of the desiccation-tolerant *Bryum rubens* (Figure 65) survived freezing, whereas only 30% of those encapsulated and 20% non-encapsulated *Ditrichum cornubicum* (Figure 66) protonemata, with limited desiccation tolerance, survived freezing. These two species each had slightly better survival numbers after 18 days of desiccation with no freezing. *Cyclodictyon laete-vires* (Figure 67), a desiccation-intolerant species, did not survive desiccation or freezing. In *D. cornubicum*, pretreatment with sucrose or ABA in the medium caused a reduction in growth rate of the protonemata, but these compounds resulted in a high level of protection against tissue damage in both dehydration and freezing – 100% regeneration of pretreated plants after thawing compared to 53% of controls (Burch & Wilkinson 2002). Sucrose plus ABA gave the best results.

Burch (2003) noted that some mosses are able to survive cryopreservation (preservation at low temperatures) with no prior treatment. She suggested that...
to accomplish desiccation protection by out-competing the larger nucleating bacteria. These small species are sprayed on oranges to protect them. Such proteins or bacteria form centers for ice formation on the outsides of cells, providing a protective covering (Zachariassen & Kristiansen 2000).

Clouds use nucleation centers to create their precipitation (Ahern et al. 2007). Bacteria have been known from clouds for a long time. Clouds may be an ideal habitat for these bacteria to live and thrive. Ahern and coworkers found 100 OTUs (operational taxonomic units – used when species cannot be named) among 256 clones from clouds. Half of these were identified as bacteria from psychrophilic terrestrial habitats (habitats where low-temperature-tolerant organisms can live). Among these bacteria, a mix of fluorescent Pseudomonas species dominate and some are known ice nucleators. But none of the cultures demonstrated the ice-nucleation gene. Rather, 55% of the isolates from cloud and rain samples had significant biosurfactant activity. Surfactants influence droplet size and are important in lowering the critical supersaturations necessary for activating aerosols into cloud condensation nuclei. Such bacteria facilitate water scavenging and counteract desiccation. Could they perform such functions in some bryophytes?
In *Sphagnum capillifolium* (Figure 70-Figure 71) the chlorophyllous (containing chlorophyll) cells exhibit extended freezing cytorrhysis immediately after ice nucleation at -1.1°C in water (Buchner & Neuner 2010). This cytorrhysis is exhibited as cell shrinkage that appears within only 2 seconds. And the shrinkage is significant – 82%, with chloroplast diameter reduction from 8.9 to 3.8 μm. This is accompanied by a sudden rise in chlorophyll fluorescence. On the other hand, frost damage occurs at a much lower temperature (LT50 at -16.1°C) (LT50 = median time until death after exposure of organism to toxic substance or stressful condition). The ice-nucleation temperature of -1.1°C is likewise the temperature threshold of PS II. Surprisingly, the LT50 for freezing in *S. capillifolium* is higher than that in most tracheophytes in the European Alps in the summer.

**Atmospheric Source**

One big question in this story is the source of the nucleating proteins. Until recently, bacteria seemed to be the only organic source of nucleating proteins (Möhler *et al.* 2008). But only a few bacteria, the pseudomonads, seem capable of this role (Lindow 1983; Ahern *et al.* 2007). This notion has been challenged by the research of Kieft and coworkers (Kieft 1988; Kieft & Ahmadjian 1989; Kieft & Ruscetti 1990) and more recently by Moffett *et al.* (2009).

Bauer *et al.* (2002) supported their challenge and reported that both bacteria and fungal spores contribute to the organic content of cloud water. In fact, the fungal spores in clouds of the Austrian Alps contribute 1.5% of the organic content, whereas the bacteria contribute only 0.01%. Although Pouleur and coworkers did not discuss the roles of these groups in nucleation, their study (Pouleur *et al.* 1992) suggests that slime molds might also provide nucleating proteins.

**Hyphomycetous fungi** (*Fusarium* spp.; Figure 72) were also added to the list of organisms providing nucleating proteins to clouds (Pouleur *et al.* 1992). We also know that the fungal partner of at least some lichens contribute nucleating proteins (Kieft 1988; Kieft & Ahmadjian 1989; Kieft & Ruscetti 1990) and that the *Fusarium* proteins are more similar to those of lichens than to those of bacteria (Pouleur *et al.* 1992).

Despres *et al.* (2007) determined aerosol particles in the air by using DNA sequencing. They found that most of the bacteria were *Proteobacteria*, with some *Actinobacteria* and *Firmicutes*. Fungal DNA came from *Ascomycota* and *Basidiomycota*, most likely from spores. Two different DNA sequences came from moss spores.

Christner *et al.* (2008) reported that ice nucleators are widespread in snowfall and the most active ones are biological. Most of these are bacteria. Many of these nucleators, therefore, are likely to be added to the mosses during snowfall and may contribute their survival of low temperatures and winter desiccation.

Fukuta (1966) found that more than 20 organic compounds out of 329 were able to nucleate ice at temperatures >-5°C. Hence, it is possible that even pollutants may contribute to nucleation of water on bryophytes.
Nucleating Proteins in Lichens

Our knowledge of lichens may help us to understand the nucleation relationship in bryophytes. Like bryophytes, lichens are able to survive year round and renew their photosynthetic activity when suitable temperatures and hydration resume. Perhaps the response of lichens can give us some insight into moss behavior relative to nucleation. Most of the epilithic (rock-dwelling) lichens (Rhizoplaca, Xanthoparmelia, and Xanthoria) tested by Kieft (1988) had ice nucleation at temperatures above -8°C, whereas their substrates showed negligible nucleation above that temperature. The nucleation activity in the lichen appears to be non-biological. No nucleation-active bacteria could be isolated, and the activity did not cease when the lichen was heated to 70°C or subjected to sonication. An axenic culture of the fungal part of the lichen Rhizoplaca chrysoleuca showed nucleation activity at -1.9°C. Kieft hypothesized that these frost-tolerant lichens benefit from increased moisture deposition that results from ice nucleation.

Henderson-Begg et al. (2009) remind us that for water to freeze above -36.5°C requires the activity of an ice nucleator. Bacteria are the best known of these, inducing freezing at temperatures up to -1.8°C, but seem to be of little importance in the lichens. The nucleators are common in lichens and can become airborne. Many of these are non-bacterial, but are biological, probably fungal and lichen.

There are several studies that support the presence of lichen fragments in the atmosphere (Tormo et al. 2001; Ahern et al. 2007). Marshall (1996) demonstrated that lichen soredia (asexual reproductive structures) were the most abundant of the airborne propagules of lichens, with peaks occurring after the winter snowmelt while subzero temperatures continued.

Kieft and Ahmadjian (1989) found that of 14 species of mycobionts (fungal partners) in lichens, five have nuclei active at -5°C. However none of the 13 photobionts (algae & Cyanobacteria) have ice-nucleating activity at -5°C or warmer. Hence, the ice-nucleating nuclei are produced by the fungal partner of the lichen. Kieft and Ahmadjian suggested that these ice-nucleating proteins are involved in moisture uptake and frost protection.

Kieft and Ruscetti (1990) found that biological ice nuclei in the lichen Rhizoplaca chrysoleuca were active at ~-4°C. Their sensitivity to various substances indicated that they were proteinaceous, and they were relatively heat stable and active without lipids, demonstrating that they were significantly different from bacterial ice nuclei.

Nucleating Proteins as a Source of Water

Lindow (1983) found that ice-nucleation activity occurs primarily in the outer membrane of the cells of Pseudomonas syringae and Escherichia coli into which it has been inserted. It does not occur in soluble components of these cells. The ability of the ice-nucleating bacteria to operate depends on incubation temperature, growth medium composition, culture age, and genotype (Lindow et al. 1982). Their optimum conditions for nucleation in culture occur on nutrient agar containing glycerol at 20-24°C. Their ability to mitigate ice injury on corn seedlings depends on the bacterial population size and the number of ice nuclei active at that temperature.

Compounds for Winter?

Bryophytes produce record numbers of secondary compounds. These are best known for their antibiotic effects, but they can also play a role in both drought tolerance and freezing survival (Xie & Lou 2009). Among these, bibenzyls and bis(bibenzyls) have desiccation tolerance activity; phenylpropanoids have freeze tolerance activity. But the nature of these activities is unknown.

We know from several studies that the proportions of various fatty acids change with temperature (Saruwatari et al. 1999). Among these, linolenic acid and eicosapentaeenoic acid might increase freezing-tolerance, as suggested by Marchantia polymorpha (Figure 27). Xie and Lou (2009) likewise reported the freeze tolerance activity of fatty acid derivatives in bryophytes.

Sugars

Sugar concentrations have a role in frost tolerance. Sucrose can increase the ability of bryophytes to tolerate rapid drying (Stark & Brinda 2015). Among the bryophytes tested by Rütten and Santarius (1992), only Mnium hornum (Figure 73-Figure 74) among seven Bryidae and one of Marchantiidae lack an increase in sucrose concentration concomitant with an increase in frost hardiness. Insignificant changes in glucose and fructose contents accompany these frost hardiness events.

Figure 73. Mnium hornum forest floor habitat. Photo by Michael Lüth, with permission.

Brachythecium rutabulum (Figure 75-Figure 76) and Hypnum cupressiforme (Figure 77-Figure 78) have high sucrose concentrations in summer, similar to those of other species in winter, and thus are frost tolerant even in summer (Stark & Brinda 2015). Those mosses that are highly frost-resistant have a total sugar concentration of ~90-140 mM. Of this sugar, 80-90% is sucrose. Artificial degradation of the sucrose during higher temperatures causes a decline in cold hardiness, supporting the hypothesis that it is important in frost hardiness in these species.
Some species [Polytrichastrum formosum (Figure 79-Figure 81), Atrichum undulatum (Figure 82), Plagiomnium affine (Figure 83-Figure 84), Mnium hornum (Figure 73-Figure 74), Pellia epiphylla (Figure 85-Figure 86)] exhibit a distinct increase in cold tolerance from summer to winter (Rütten & Santarius 1992). Mosses have significant differences in frost resistance between summer and winter (15–25°C), but the thallose liverwort Pellia epiphylla experiences relatively little winter hardening capacity.
Figure 80. *Polytrichastrum formosum* with frost, a species that has a distinct increase in cold tolerance from summer to winter. Photo by Aimon Niklasson, with permission.

Figure 81. *Polytrichastrum formosum* leaf lamellae. The role of lamellae in frost protection is unknown. Photo by Michael Lüth, with permission.

Figure 82. *Atrichum undulatum*, a species that has a distinct increase in cold tolerance from summer to winter. Photo by David T. Holyoak, with permission.

Figure 83. *Plagiomnium affine* forest floor habitat. Photo by Michael Lüth, with permission.

Figure 84. *Plagiomnium affine*, a species that has a distinct increase in cold tolerance from summer to winter. Photo by Janice Glime.

Figure 85. *Pellia epiphylla* protected habitat under grass bank of flush in Wales. Photo by Janice Glime.
Using the bryological lab rat *Physcomitrella patens* (Figure 87), Oldenhof et al. (2006) demonstrated that sucrose helps to protect cells during freezing and drying, but accumulation of sucrose alone is not sufficient for survival. ABA serves to cause this sucrose accumulation, up to 22% of dry weight, but only 3.7% occurs in non-ABA-treated tissues. A combination of ABA treatment and the cryoprotectant DMSO permit the tissues to survive a freeze-thaw cycle down to -80°C. DMSO-mediated changes involved in the membranes are important and may be relevant to the essential desiccation tolerance.

Polyols may contribute to cold hardness as well. Tearle (1987) found that Antarctic lichens contained up to three times the amount of polyols when compared to temperate lichens, endowing them with extra freezing protection. The soluble sugars and polyols from mosses and lichens leach into the fellfield soils in the spring.

### ABA

ABA is the stress hormone, and it plays a role in freezing tolerance of plants as well (Minami et al. 2003; Takezawa et al. 2011). Nevertheless, slow freezing of the protonemata of *Physcomitrella patens* to -4°C under normal growth conditions kills more than 90% of the cells. Application of ABA for 24 hours causes a marked increase in the freezing tolerance (see also Nagao et al. 2001, 2005, 2006). Cold treatment only slightly increases the freezing tolerance within the same period. Treatment with ABA causes a marked increase in expression of all the PPAR genes within 24 hours. Several of these genes also respond to cold, but much more slowly than they respond to ABA. Treatment with hyper-osmotic concentrations of NaCl and mannitol also increases the expression levels of eleven PPAR genes and the freezing tolerance of the protonemata. Minami and coworkers (2003) suggest that these relationships indicate that stresses increase the expression of genes that result in protection of the protonemata, but the nature of that relationship is unclear.

Nevertheless, in *Physcomitrella patens* (Figure 87) protonemata, as in tracheophytes, freezing tolerance increases following incubation at low temperatures in the range of 0-10°C, indicating the importance of acclimation (Minami et al. 2005). This tolerance is accompanied by an accumulation of several transcripts for late-embryogenesis-abundant (LEA) proteins and boiling-soluble proteins. De-acclimation causes reduction in expression of these proteins and loss of freezing tolerance. But surprisingly, unlike events in tracheophytes, in *P. patens* low-temperature-induced freezing tolerance does not coincide with an increase in endogenous ABA, despite increases in expression of stress-related genes. In short, the acclimation is somewhat different from that of tracheophytes.

These observations are further confounded by the experiments of Minami et al. (2003) on *Physcomitrella patens* (Figure 87). They found that treatment with ABA for 24 hours greatly increases the freezing tolerance of the protonemata; cold treatment alone has only a slight effect on freezing tolerance. Even slow freezing to -4°C kills more than 90% of the cells. On the other hand, hyperosmotic concentrations of NaCl and mannitol increase freezing tolerance of protonemata.
At the same time, research by Takezawa and Minami (2004) identified genes coding for membrane transporter-like proteins. These newly identified proteins increase considerably following treatment with low temperatures, hyperosmotic solutes, or ABA. These genes are regulated by calmodulin.

**Arachidonic Acid**

Prins (1982) suggested that one reason small mammals eat mosses in winter is the content of arachidonic acids. These fatty acids make membranes more pliable and may make it easier for these rodents to run around on frozen ground and snow. But what do these do for bryophytes in winter? Does this extra flexibility also make it easier for them to survive? One protection against freezing is the ability to lose water, avoiding crystal formation that could damage membranes and organelles. With flexible membranes and withdrawal of water, the cells could shrink within the walls during the cold (and dry) period.

In Physcomitrella patens (Figure 87), production of arachidonic acid increases with higher concentrations of sugar (Chodok et al. 2010). Al-Hasan (1989) found that in Bryum bicolor (Figure 88) more arachidonic acid is produced at 5°C than at 25°C. Both of these studies support the production of arachidonic acid as winter approaches.

![Figure 88. Bryum bicolor, a species that produces more arachidonic acid at low temperatures than in warm ones. Photo by Michael Lüth, with permission.](image)

**Polyribosomes**

Polyribosomes (cluster of ribosomes connected by a strand of messenger RNA and active in protein synthesis) respond to cooling temperatures. In the xerophytic moss Syntrichia ruralis (Figure 61-Figure 62), when temperatures descend to 2°C an accumulation of polyribosomes occurs while the single ribosomes decrease (Malek & Bewley 1978). This change in numbers reflects rearrangement, but does not involve a change in the number of ribosomal units. Slowly dried S. ruralis does not contain any polyribosomes when rehydrated, but these reform at 2, 8, and 20°C. Leucine rapidly incorporates into the protein when the plants are rehydrated at 20°C, but its incorporation is less dramatic at 2°C. Cold-hardened S. ruralis has no changes in the rate of protein synthesis at low temperatures (2°C). In fact, even in summer this species can carry out protein synthesis at low temperatures.

**Summary**

One of the dangers of frost damage is desiccation. Ice crystals on the inside of cells damage membranes and those on the outside pull water from the cells. Some bryophytes are protected by being encased in ice, preventing the formation of crystals and insulating against severe cold. Absence of vacuoles or having only small vacuoles can help to protect the interior of cells.

Many species survive winter and are ready for photosynthesis when the snow disappears, using the snowmelt water to rehydrate their tissues. Slow cooling, like slow drying may be important in survival. Extracellular freezing can protect against intracellular freezing. Some macromolecular substances can modify the shape of ice crystals in ways that do not damage the cells. Some ice-nucleating structures, made by the plants or available from the atmosphere, including proteins, create a small crystalline structure likewise protecting against damage from larger crystals. On the other hand, some ice crystals on the outsides of the cells can sequester water that is available at suitable temperatures. Desiccation can protect the cells by preventing crystal formation. Cell shrinkage helps to prevent crystal formation. Frequent freeze-thaw cycles, like dehydration-rehydration cycles, can damage the cells if the hydration and photosynthetic period is insufficient to repair membranes and accomplish a carbon gain.

Polyribosomes are active immediately following the freeze-thaw cycle. Lipids, starch bodies, sucrose, ABA, bibenzyls, bis(bibenzyls), and phenylpropanoids help to increase freezing and desiccation tolerance. Arachidonic acid helps to make membranes more pliable. These compounds permit some bryophytes to have seasonal tolerance. In desert habitats winter is often the best growing season because mosses remain hydrated for several days following rainfall events. Some species become bleached from frost damage, but shoot tips and other parts may remain healthy and provide new growth in spring.

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