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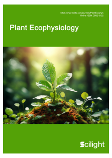

Review

Survival below Zero: Overlooked Aspects of Freezing-Tolerance in Photosynthetic Fern Tissues

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Abstract: The consequences of climate change on the ecophysiology of cryptogams, generally, and in ferns, particularly, are understudied. Phenomena induced by climate change, such as increased frequency of extreme weather events, shifts in precipitation patterns and temperature fluctuations, can significantly impact the physiology and distribution of ferns. The clade of ferns evolved about 400 million years ago and represents the sister group of seed plants. Given their long evolutionary history, ferns offer insights into the resilience and adaptability of plant lineages over geological time scales. Both from an evolutionary and functional perspective, ferns represent a crucial group with intermediate physiological properties between earlier-evolving bryophytes and spermatophytes. Additionally, their life cycle with single-celled reproductive spores and with two independent generations, gametophyte and sporophyte, which have strong anatomical and physiological differences and even different ecological requirements, make ferns a unique case study. While most ferns avoid freezing by living in the tropics or shedding their fronds, wintergreen species deal with subzero temperatures in temperate and cold ecosystems. Additionally, the chlorophyll-containing spores and/or gametophytes of many species also face subzero temperatures. Despite all this, our current knowledge of low temperature- and freezing-tolerance mechanisms in ferns is minimal. In this review we make a comprehensive compilation and re-evaluation of the available knowledge in this topic with a focus on photosynthetic cells/organs of ferns (class Polypodiopsida). We include some recent and relevant findings, identify major gaps and provide baseline for future lines of research.

Keywords: cold stress; frost; gametophyte; pteridophyte; chlorophyllous spore; sporophyte

1. Introduction: Background and Key Concepts on "Below-Zero Plant-Physiology"

Despite the ongoing warming of our planet, coping with freezing conditions is becoming more frequent for many plant species. Due to the presence of warmer temperatures, spring is arriving earlier across much of the globe, which causes plants to emerge from dormancy earlier and increases their exposure to late frosts (Augspurger, 2013). Extreme cold, including cold waves, has become less frequent and less severe at global scale (Lee et al., 2023). However, the frequency of severe winters and late frosts has increased in several regions of both hemispheres as a result of disruptions in atmospheric circulation patterns, which allow cold air from polar regions to reach lower latitudes unexpectedly (Cohen, Pfeiffer, & Francis, 2018; Crimp et al., 2016). Similarly, unseasonal warming episodes during winter or early spring can trigger premature loss of acclimation, leaving non-acclimated tissues susceptible to subsequent cold events. In addition, the reduction of snow cover resulting from warmer winters can impose a double risk on evergreen species, exposing them to both cold and excessive light stress. A comprehensive understanding of the cellular sites and mechanisms underlying injury related to freeze-thaw events in plants is essential to develop breeding programs or genetic modifications aimed at enhancing cold hardiness, as well as for devising effective frost-protection measures in the management of either crops or at natural environments. While processes and cellular effects of freezing (e.g., intratissular ice formation) on plants were initially studied as early as in the late 1800s (Muller-Thurgau, 1886; Sachs, 1873), many aspects of freeze-thaw effects on plants are still understudied (Arora, 2018). Moreover, while an extensive bibliography is now available concerning crops and conifers, very few works have comparatively focused on ferns (Sutinen et al., 2001; Bannister & Neuner, 2001).

Low temperatures impact plant performance through two main processes (1) a reduction in enzymatic activity and the disruption of membrane function, and (2) the formation of ice and mechanical injury within tissues (freezing stress). The former can induce the overexcitation of the photosynthetic apparatus (photooxidative stress) in chlorophyll-containing tissues, as a result of the unbalance between light absorption by chlorophylls and energy use by photosynthesis, and is regarded as one of the most challenging stress factors in plants and terrestrial algae (Míguez et al., 2017; Van Hasselt & Van Berlo, 1980). Thus, freeze-thaw events predominantly induce two main injuries: oxidative damage induced by reactive-oxygen-species and structural and/or functional perturbations in cell integrity. In vascular plants, freeze-thaw cycles can additionally induce cavitation within the xylem increasing the risk of embolism and producing a third type of damage: loss of hydraulic conductivity (Choat et al., 2011). To investigate this phenomenon, the centrifuge method, which is used to measure xylem resistance to drought-induced cavitation, has been modified to account for additional cavitation caused by freeze-thaw cycles (Davis, Sperry, & Hacke, 1999). The results of these studies indicate a strong correlation between freeze-induced cavitation and average conduit diameter. According to these findings, plants other than ferns with tracheids or smaller xylem conduits (mean diameter <30 µm) do not exhibit freeze-induced cavitation under moderate water stress (xylem pressure = −0.5 MPa). In contrast, species with larger conduits (mean diameter >40 µm) experience almost complete cavitation under the same conditions. Species with intermediate conduit diameters (30–40 µm) show partial freeze-induced cavitation. These results align with a critical conduit diameter of 44 µm, at which or above which cavitation occurs during freeze-thaw cycles at −0.5 MPa. As expected, vulnerability to freeze-induced cavitation is also correlated with hydraulic conductivity relative to the stem cross-sectional area. These findings confirm and expand previous studies, particularly regarding the greater resistance of small-diameter conduits to freeze-induced cavitation. Moreover, the modified centrifuge method, which incorporates freeze-thaw cycles, may be useful in distinguishing the interactive effects of xylem pressure and freezing on cavitation in plants in general (Davis, Sperry, & Hacke, 1999). The loss of hydraulic conductivity can threaten the water transport to photosynthetic cells and thus reduce the photosynthetic capacity of the plant (Choat et al., 2011). Very likely, all these effects, although mostly known from the study of spermatophytes (gymnosperms and angiosperms) also affect to ferns (Figure 1).

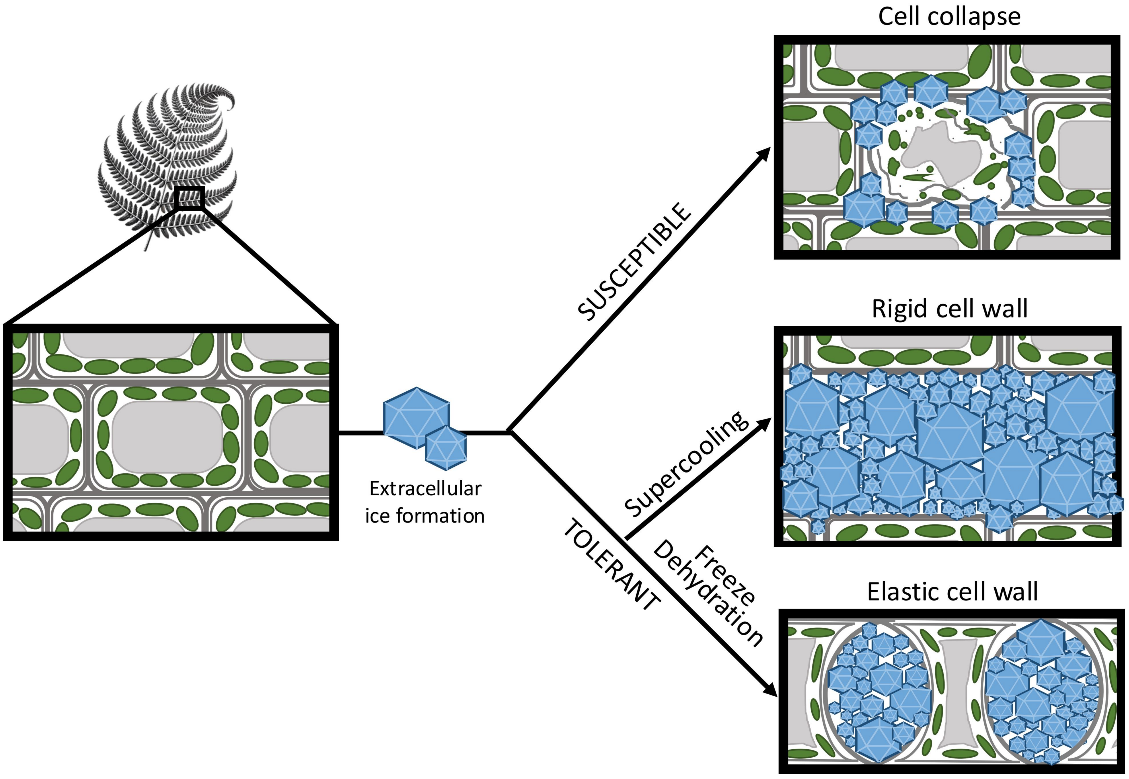

Figure 1. Potential main effects of intra-tissular ice formation on photosynthetic cells, as inferred from studies on seed plants. Essentially, either ice tolerant or ice intolerant cells can be found. Ice intolerant cells will suffer irreversible cell collapse induced by freeze-dehydration and membrane disruption during intratissular ice formation that is initiated in the apoplast. Ice tolerant cells, on the other hand, can show two different strategies. Cells with rigid cell wall will prevent freeze-dehydration and experience supercooling, while ice will be usually accommodated extracellularly in intercellular spaces. Cells with flexible cell wall will reversibly freeze-dehydrate maintaining membrane and organelle functionality upon thawing.

During a natural frost event, plant tissues will typically experience extracellular ice formation, since extracellular fluid has a higher freezing point than the intracellular content as a result of a higher solute concentration. Upon extracellular ice formation, plants can have either ice-tolerant or ice-intolerant cells. Once extracellular ice has been formed, ice-intolerant cells typically collapse (inwards) and suffer irreversible damage to the cell wall (Ashworth & Pearce, 2002), while ice-tolerant cells can either freeze-dehydrate or resist the dehydrating forces and supercool. Some ice-tolerant cells will then freeze-dehydrated and experience "freezing plasmolysis", which means shrinkage of protoplast together with the cell wall, and which will be reversible during thawing (Zhu & Beck, 1991). Other ice-tolerant cells can prevent the plasmolysis and resist the dehydrating forces thanks to relatively rigid cell walls. Finally, after thawing, provided no irreparable damage of the membrane has occurred, melted water will re-enter the cells (in freezing tolerant tissues), which will recover normal functioning. In nature, freezing-tolerant plants typically freeze at relatively slow cooling rates of up to 3 °C/h (Neuner et al., 2013) and show extracellular ice nucleation at relatively high temperatures of −0.5 to −3 °C (Fernández-Marín et al., 2018). During this cooling process, a sequence of processes/structures are affected. With few exceptions, most tissues will first experience a block on photosynthesis (carbon assimilation), while respiration will be maintained until much lower temperatures (Arora, 2018). At cellular level, trans-membrane transporters will be affected (due to alterations in transport proteins), while chloroplast and mitochondria will keep their ultrastructure (Palta & Li, 1978a, 1978b). This makes ultrastructural disorganization a typical symptom of irreversible (lethal) damage after freeze-thaw. Thus, chloroplast, mitochondrial and cell membrane function differ in their sensitivity to a realistic, freeze-thaw stress (Steffen, Arora, & Palta, 1989). While most of these evidences have been obtained in crops, model species, alpine angiosperms and/or conifers, very few studies have focused on fern responses to freezing/thawing events.

2. Biogeography and Phylogeny of Frost-Tolerance in Ferns

The clade of ferns evolved approximately 400 million years ago and is the sister group to seed plants (spermatophytes) (Pryer et al., 2001; PPG, 2016; Shen et al., 2018; Nitta et al., 2022). Specifically, ferns represent one (Polypodiopsida) of the two classes traditionally recognized within pteridophytes, being Lycopodiopsida (lycophytes) the other one. This review is focused on Polypodiopsida. Currently, ferns, with 11,916 species in 337 genera and 51 families, are the second most diverse group of vascular plants, after angiosperms. Ferns comprise four subclasses: Equisetidae (horsetails) with a single genus; Ophioglossidae with 12 genera; Marattiidae with 6 genera; and Polypodiidae (leptosporangiates) which contains the vast majority of extant fern species (PPG, 2016).

The life cycle of ferns is characterised by an alternation of generations: a diploid sporophyte, which produces spores by meiosis, and the haploid gametophyte, which forms the egg and sperm (Figure 2). Most ferns are homosporous, i.e., they produce only one type of spore that gives rise to potentially bisexual gametophytes. However, these gametophytes have several mechanisms that promote outcrossing, such as the asynchronous formation of male and female gametangia (Haufler et al., 2016). Fertilization results in a new sporophyte, which has traditionally been considered the 'dominant' generation based on its significantly larger individual size compared to the gametophyte. The fern gametophyte (prothallus) is generally a photosynthetic heart-shaped individual, although some species have other shapes or even lack chlorophyll (Raghavan, 1989). The gametophytes typically have a surface area of less than 1 cm^2, mostly consisting of a single cell layer, and may have a rudimentary cuticle or lack it entirely. Gametophytes can also be much larger and several cells thick under the notch meristem (Watkins & Cardelús, 2012; Dong et al., 2015) and is thus highly dependent on availability of liquid water for metabolic activity and for the movement of sperms during fertilization (see Section 4 of this review). The alternating generations gametophyte and sporophyte are able to live independently in ferns, in contrast with seed plants.

Fern sporophytes are typically larger and more conspicuous than fern gametophytes. Not to mention that several species (including *Vittaria appalachiana*) are only known to exist as a gametophyte. Like other vascular plants, the sporophytes of ferns consist of three types of vegetative organs: roots, stems and leaves. Fern root systems show high variation comparable to that observed in seed plants (Dong et al., 2015). Stems of ferns are traditionally called rhizomes, most of which are horizontal and located at or just below the soil surface. However, some ferns, particularly tree ferns, may grow their rhizomes vertically, eventually forming a "trunk.". The stems of some ferns with reduced leaves (*Equisetum*) or absent leaves (i.e., *Psilotum* and *Tmesipteris*) are photosynthetic, but this function is normally performed only by the leaves. Fern leaves, called fronds, do have stomata and a well-developed cuticle. Leaf size and shape also vary greatly, and the blade is usually divided into smaller leaflets called pinnae. Spore-producing organs (sporangia) are typically formed on the underside of the blade, and group together into clusters called sori. The spore is the first cell of the gametophytic generation and allows long-distance dispersal by wind. Spores of some species contain chlorophylls and functional chloroplasts (chlorophyllous spores) at maturity (Sundue, Vasco, & Moran, 2011) (see Section 5 of this review). Overall, this alternation of biologically and structurally different photosynthetic generations makes ferns a unique group to study ecophysiological aspects in vascular plants. Despite it, biogeographical and systematic aspects of ferns have been much more extensively addressed than their physiological ecology, as recently reviewed (Anderson, 2021).

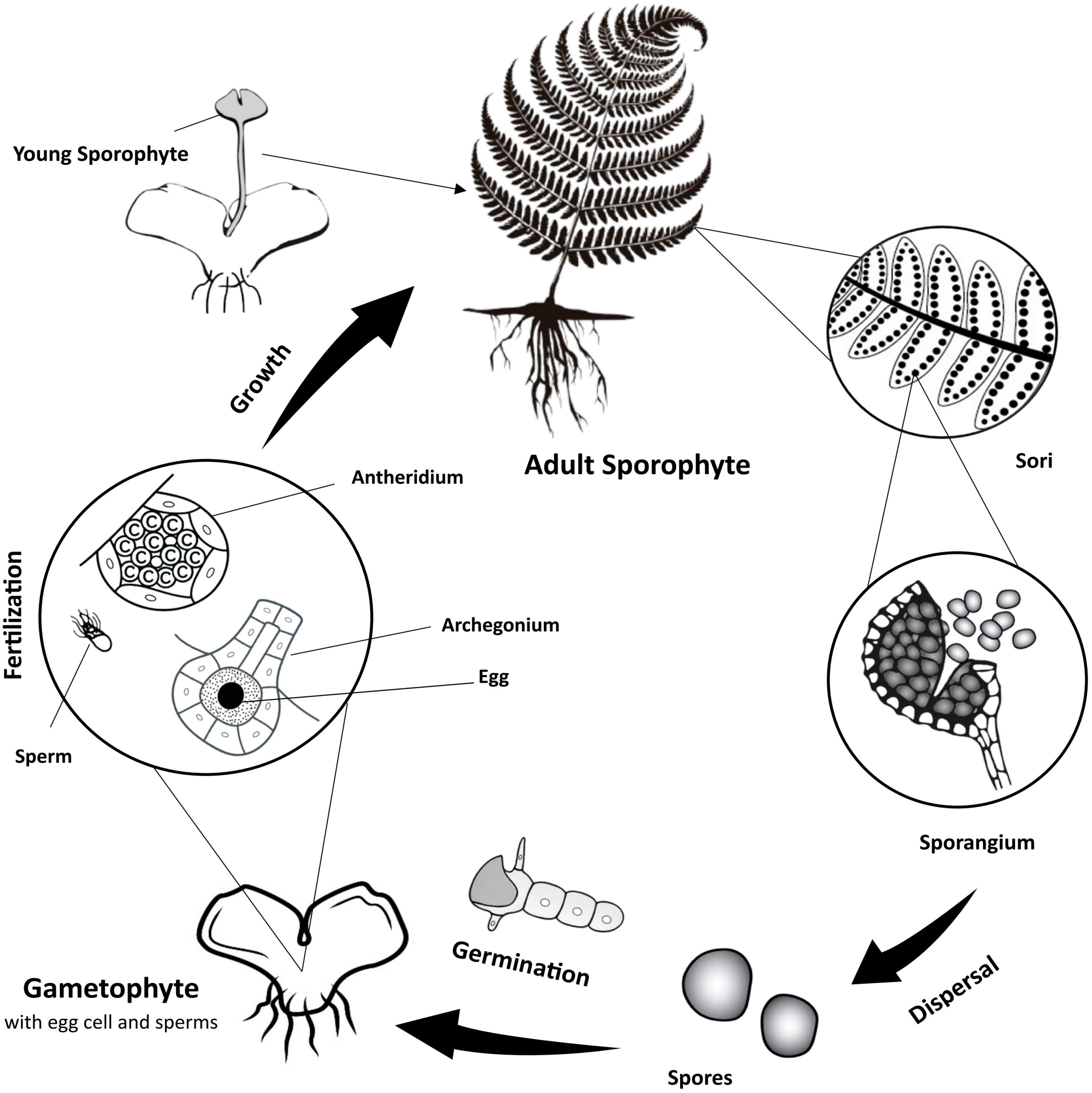

Figure 2. Simplified view of fern life cycle, illustrating the two generations (sporophyte and gametophyte) and the spores, which represent the main focus of this review. Adult sporophyte leaves produce sori with sporangia full of spores. At maturity, the sporangia shed the spores. Spores germinate and produce the prothalli of the gametophyte. Within the gametophyte, fertilization takes place and a new young sporophyte emerges.

In terms of evolutionary diversification, the clade of ferns experienced the most flourishing event during a relatively warm period on the history of the planet (Nitta et al., 2022). Currently, highest species richness is found in tropical moist forests, especially at high elevations (Weigand et al., 2020). Due to these two facts, ferns are often recognized as plants of warm, humid, and shady environments, but many species also thrive in sunny or dry conditions. However, ferns are present in all terrestrial biomes of the world, including the taiga and tundra, and grow well above the timberline in mountains. Therefore, many ferns inhabit areas that experience freezing events, whose intensity, frequency, and temporal distribution vary depending on latitude and altitude. For instance, arctic species are largely exposed to freezing temperatures (Gureyeva & Timoshok, 2016), ferns of temperate habitats (mid to moderately high latitudes) experience subzero temperatures seasonally (wintertime), and ferns at high elevations, even in tropical regions, may face subzero temperatures on a daily basis (Kessler & Kluge, 2022; Sato & Sakai, 1981a; Hill, 1976). Although the sporophyte individual is highly tolerant to overcome hard winters in many species, its annual fronds can be sensitive to freezing. As an example, the underground rhizome of the boreo-alpine fern *Botrychium lunaria* is freeze-tolerant, but its fronds are sensitive to spring frost (Watt, 1981). In this review we focused specially on the freezing tolerance of photosynthetic tissues, including leaves, chlorophyllous stems, as well as chlorophyllous gametophytes and spores. We standardized species names using the Taxonomic Name Resolution web application, version 5.1 (Boyle et al., 2013) and adopted the families recognized by PPG I (2016).

The highest latitudinal record for fern species has been documented in the Arctic, where a few species exceed 80° N in Canada and Greenland (GBIF Secretariat, 2022), while no pteridophytes are currently inhabiting Antarctica (Peat, Clarke, & Convey, 2007; Colesie et al., 2023). Early works on Russian Arctic flora enumerated up to 22 species of ferns (Gureyeva & Timoshok, 2016). Six species are included in the flora of Svalbard (latitude 74–81° N): *Botrychium lunaria*, *Cystopteris fragilis*, *Equisetum arvense*, *E. scirpoides*, *E. variegatum* and *Woodsia glabella* (Stavdal, 2020; Rønning, 1996). Nevertheless, most of these species lose their leaves or, in the case of *E. arvense*, their green shoots at the end of summer, and their sporophyte photosynthetic tissues do not necessarily face freezing temperatures. By contrast, *Equisetum scirpoides* and *E. variegatum* have evergreen shoots adapted to the extreme cold of the Artic winter. An early work compiling the temperature-resistance of Alaskan plants revealed interesting data about *E. scirpoides*. It has a very strong resistance to low temperature (−80 °C) plus the capacity to modulate it across the time, presenting a higher resistance in winter and even higher upon an unusual strong winter occurred in 1968/1969 (Riedmüller-Schölm, 1974).

Fern biodiversity across elevational gradients has been evaluated in the highest mountain ranges on Earth. For instance, Salazar et al. (2015) evaluated diversity patterns in Andean tropical forests up to 4000 m in elevation. None of the locations considered, however, presented average temperatures below zero (provided the data shown in the original articles). Also in America, another study conducted in the Trans-Mexican Volcanic Belt deeply evaluated fern biodiversity, and highlighted three species for the highest elevational upper limit: *Asplenium castaneum* (4,569 m), *Polystichum speciosissimum* (4490 m), and *Cystopteris fragilis* (4377 m) (Hernández-Cárdenas et al., 2019). More recently, in Asia, Umair (2023) and coworkers have evaluated climatic factors affecting fern species richness along an elevational gradient (100–5300 m a.s.l.) at the Tibetan Plateau. In this study, among the 441 fern species found, the family Dryopteridaceae was the richest, with 97 species, 32 of which exceeded 4000 m in elevation (Umair et al., 2023). Some ferns that grow even at an elevation of 5300 m represent a remarkable milestone in this work by Umair and co-workers. In Europe, Marini et al. (2011) studied fern richness along a 0–3000 m elevational gradient in the Alps and suggested that lethal effects of frost are among the factors explaining the decline in richness at high elevations. However, none of these studies addressed ecophysiological questions. Accordingly, Kessler and Kluge (2022) recently brought attention to the lack of ecophysiological knowledge on this aspect after a thorough review of available data. They also concluded that numerous physiological processes determine the elevational ranges of ferns, including not only cold and drought at high elevations but also drought, high temperatures and, importantly, limited frost tolerance at low elevations (Kessler & Kluge, 2022).

3. Freezing-Tolerance in Fern Sporophytes

Among the three types of photosynthetic cells considered in this review (sporophyte, gametophyte, chlorophyllous spores), sporophytes have been more widely studied regarding freezing-tolerance in terms of number of genera and families evaluated (Figure 3, see also Supplementary Table S1). Despite of it, only 14 families have been studied so far. Among them, Dryopteridaceae, Aspleniaceae, Blechnaceae and Polypodiaceae comprise most of the available data (Figure 3). Table 1 presents a summary of the findings on freezing-tolerance in ferns, categorized by tissue type (sporophyte, gametophyte, spore). In the next subsections, several aspects related to either the vascular system, the evolution of research on this topic, the ice formation and propagation and the photoprotection mechanism will be summarised.

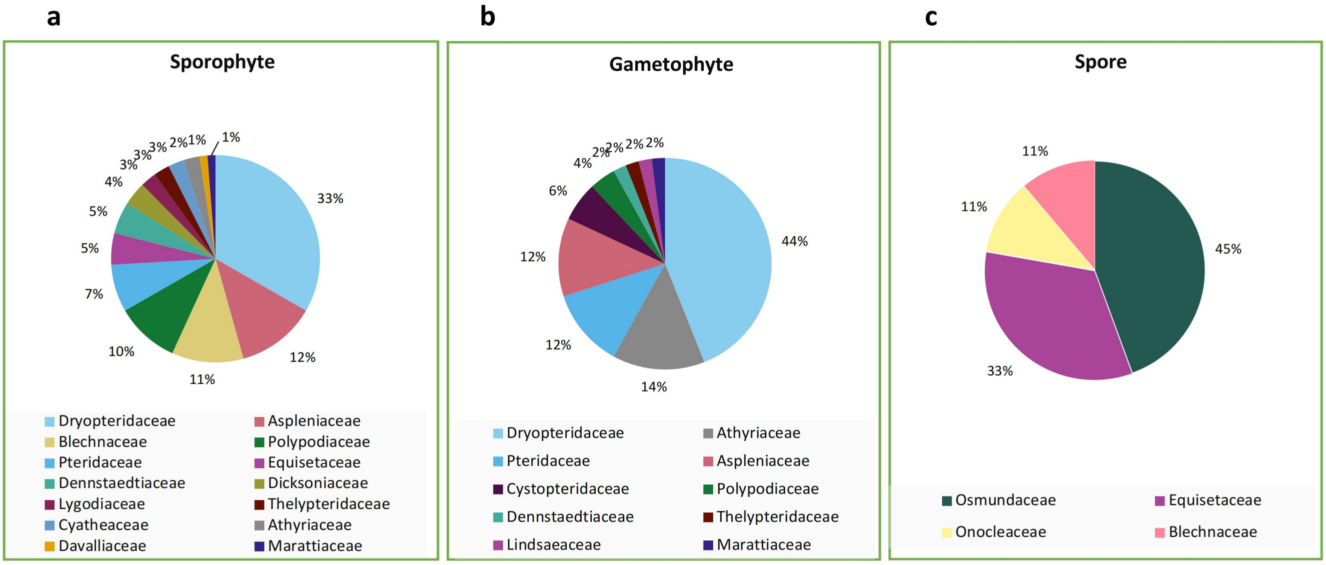

Figure 3. Data available per fern family in the bibliography regarding freezing tolerance of photosynthetic tissues: (a) sporophyte fronds; (b) gametophytes, and (c) photosynthetic spores. Charts are based on data compiled in Table 1 and in Supplementary Table S1.

Table 1. Compilation of already published works in which freezing tolerance was tested in ferns. Data are organized by tissue type. The temperature tested and the key physiological/biological parameters used to test for tolerance in the original references is also shown.

Tissue Type	Freezing tolerance range (°C)	Total number of species	Key Parameters	Reference
Sporophyte	(−2 to −47)	90	Ice nucleation, leaf damage, regreening, Survival, chlorophyll fluorescence (Fv/Fm), Plasma Resistance, Fatty acid composition of fronds	Sato & Sakai, 1981a; Kappen, 1964, 1966; Bannister, 1973, 1984, 2003; Sato, 1982; Bannister & Fagan, 1989; Noodén & Wagner, 1997; Tessier, 2018; Fernández-Marín et al., 2021a; Voronkov & Ivanova, 2022; Farrar, 1978; Warrington & Stanley, 1987; Bremer & Jongejans, 2010; Klinghardt & Zotz, 2021; Nokhsorov et al., 2021
Gametophyte	(−3 to −196)	57	Survival, regreening, habitat analysis	Sato & Sakai, 1981a; Kappen, 1965; Sato, 1982; Farrar, 1978; Pickett, 1914
Spore	(−18 to −196)	6	Germination rate, survival	Kato, 1976; Whittier, 1996; Ballesteros et al., 2011; Magrini & Scoppola, 2012; Li & Shi, 2014

3.1. Subzero temperatures and vascular system

The sporophyte is typically the vascular generation of ferns. Tracheids have been reported in fern gametophytes, albeit rarely (Whittier, 1976; Goswami & Sharma, 1996). As such, photosynthetic organs, i.e., leaves and, in some species stems, contain a specific tissue for water and nutrient transport, the vascular system, which consists of bundles of specialised cells including tracheids (Pittermann et al., 2011; McElwain, 2011). This is a relevant feature when facing freezing temperatures. Specifically, in addition to photoprotection and maintaining suitable osmolyte and antioxidant metabolism for preserving cell integrity, fern fronds and stems must also be prepared for rapid ice propagation through the xylem and the potential embolization of its conduits during freeze-thaw cycles (Figure 1). Drought-induced xylem embolism has already been quantified in many fern species, although the peculiarities of their stem anatomy result in frequent discrepancies when compared with seed-plants (Pittermann, Baer, & Sang, 2021; Prats & Brodersen, 2020). On the contrary, freeze-thaw induced embolism has not been evaluated on ferns so far.

3.2. Subzero temperatures tolerance in sporophytes: A chronological revision of early literature

Early works evidencing freezing tolerance in fern sporophytes were conducted by Kappen (1964, 1965, 1966) in the 60s (see also Supplementary Table S1). He observed that freezing-tolerance varies both within species along the year, being higher (fronds withstand lower temperatures) in winter than in spring, and among species. After testing 7 different species (*Cystopteris fragilis*, *Phegopteris connectilis*, *Gymnocarpium dryopteris*, *Dryopteris carthusiana*, *Athyrium filix-femina*, *Thelypteris limbosperma*, and *Pteridium aquilinum*), tolerance ranged between −2.5 to −12 °C (Kappen, 1964) (Supplementary Table S1). He also brought attention to the relevance of water content of the frond in the survival to low temperature. Specifically, he evidenced that hydrated leaves of *Polypodium vulgare* withstand −17 °C, and that the same leaves are able to survive even immersion in liquid nitrogen when in the dry state (Kappen, 1965, 1966; Sakai & Larcher, 1987).

In the 70s, a couple of studies complemented these results. Bannister (1973) reported the effects of an early frost event (of around −4 °C) at the end of summer over a couple of species. Among other interesting observations, he highlighted the vegetative fronds of *Blechnum spicant* and *Dryopteris filix-mas* as tolerant, while pinpointed some damage in the fertile fronds of *Blechnum spicant*. Few years later, Riedmüller-Schölm (1974) compiled an extensive set of data under natural conditions in Alaska for more than one year. He evidenced (i) very tough tolerance to low temperature in *Equisetum scirpoides* in winter (down to −80 °C, from November to January), and (ii) a strong seasonal variation (acclimation) in this tolerance, which was only around −10 °C in the warmer months (May to August) (Riedmüller-Schölm, 1974).

An increasing number of works was published along the 80s. Sato and co-workers did an extensive work testing freezing tolerance of a wide number of species (>60) in the north of Japan and comparing, in many cases, sporophytes with other tissues (Sato & Sakai, 1981a, 1981b; Sato, 1982). Frost resistance of the leaves was related to the phenology and ecology of the species, with the leaves of wintergreen and evergreen ferns generally being the most tolerant in the range of −20 to −40 °C (Supplementary Table S1). Almost in parallel, Bannister and co-workers evaluated tolerance to subzero temperatures in other species in New Zeland (Bannister & Fagan, 1989) They concluded that (i) for a similar latitude, fern species original from the Northern Hemisphere had higher frost tolerance than Southern Hemisphere species (Bannister, 1984), (ii) frost tolerance varies within a species temporally and spatially (with season and elevation) (Bannister & Fagan, 1989) and (iii) fertile fronds are less tolerant than vegetative fronds, i.e., in *Blechnum penna-marina* (Bannister, 1984). At the end of this decade, Niklas (1989) contributed to the understanding of possible tolerance mechanisms and highlighted that *Equisetum hyemale* tolerance to subzero temperatures was based on the extracellular freezing of water in the pith cavities of its aerial shoots. Later studies by Schott, Voigt, and Roth-Nebelsick, (2017) further confirmed this by demonstrating that, in addition to the pith cavity, ice formation also occurs in the vallecular canals, suggesting a crucial role of pre-existing lacunae in cold resistance. Thus, main findings until the 90s on fern sporophyte tolerance to freezing indicated that (1) relatively diverse wintergreen taxa can withstand subzero temperatures; (2) interspecific differences are remarkable; (3) most species show seasonal acclimation with stronger tolerance in winter; (4) when present, fertile fronds can show different tolerance than the vegetative fronds within the same species, (5) frond age can be a relevant factor for freezing tolerance and (6) water content of the frond greatly influence the tolerance extent at least in desiccation tolerant species. From the 90s onwards, different ecophysiological aspects have been addressed mostly related to (i) the role of snow protection, (ii) the physiology of ice formation and propagation and (iii) the relevance of photoprotection as is summarised in the following subsections.

3.3. Snow protection

The buffering effect of snow cover over extreme low temperature during wintertime as well as its shading effect (i.e., providing photoprotection) are well known for flowering plants (Briceno et al., 2014; Neuner, Ambach, & Aichner, 1999; Bannister et al., 2005; Wipf et al., 2015). Although largely understudied, similar effects have been observed in ferns. Nooden and Wagner (1997) already evidenced in the 90s that snow cover protection can be so relevant for wintergreen species that some have evolved a specific anatomical mechanism to facilitate snow covering. *Polystichum acrostichoides* and *Dryopteris intermedia* possess a controlled-senescence mechanism at the base of the stipe, which causes it to soften and bend, resulting in the prostration of the fronds. This adaptation helps maintain xylem flow and provides cold protection beneath the snow. Throughout winter, the fronds retain chlorophyll content and PSII photochemical efficiency, remaining capable of CO_2 assimilation at a rate of approximately 2 μmol m^{-2} s^{-1} (Noodén & Wagner, 1997). Additionally, decreasing temperatures lead to the formation of a necrotic zone at the stipe base, allowing fronds to lie flat on the ground and maintain optimal leaf temperatures without disrupting hydraulic function. This mechanism, along with the preservation of xylem conductivity, supports the survival of *P. acrostichoides* in northeastern forests, enabling it to sustain photosynthesis throughout winter (Prats & Brodersen, 2020). Deepening on the consequences of this ecophysiological strategy, the effect of unusual diminished snowfall in the overwintering survival of *Dryopteris intermedia*, *Dryopteris marginalis* and *Polystichum acrostichoides* was evaluated more recently (Tessier, 2014). The results added new evidences on the protective effect over overwintering leaves, so more damage was found on those not covered by snow during winter. The study also revealed higher damage on the leaves with lower Leaf Mass per Area (LMA) value, suggesting higher susceptibility to frost damage for thin leaves (Tessier, 2014). More recently, two manipulative experiments performed in the field with *Polystichum acrostichoides*, by artificially changing the prostrate into erect position of the frond during winter, shed more light into this aspect (Forget, Parker, & Hughes, 2018; Tessier, 2018). Prostration seems to additionally benefit the leaves by reducing leaf-to-air vapor pressure, enhancing stomatal conductance and photosynthesis during winter. Additionally, prostration enabled slight warming of the fronds on sunny days becoming leaf temperature closer to its optimal for photosynthesis (Forget, Parker, & Hughes, 2018).

3.4. Ice propagation and location within the photosynthetic tissue

While physical aspects of ice formation and propagation are crucial to understand species strategies to deal with freezing temperatures, these parameters have been rarely evaluated in fern sporophytes. Only a couple of works have evaluated this phenomenon in ferns. In *Equisetum hyemale*, extracellular ice formation occurs in the pith cavities and in the vallecular canals within the aerial shoots (hollow stems), inducing tissue dehydration (Niklas, 1989; Schott, Voigt, & Roth-Nebelsick, 2017; Konrad, Schott, & Roth-Nebelsick, 2019). In another recent study, ice propagation was evaluated in 5 temperate European species by using Infrared Differential Thermal Analysis (IDTA): *Adiantum capillus-veneris, Asplenium ceterach, A. trichomanes, A. scolopendrium, and Polypodium vulgare* (Fernández-Marín et al., 2021a). Ice propagation pattern within the frond was fast and disorganized in the freezing-sensitive species, compared to a slower and segmented pattern in the freezing-tolerant species. These observations suggest anatomical controls and barriers to ice propagation probably related to xylem anatomy. Ice nucleation temperature was also evaluated in this work by differential scanning calorimetry in well hydrated fronds. The ice nucleation temperature occurred around −3 °C in the evaluated species in the field and ranged between −3 and −9 °C in ex situ assays in the lab (Fernández-Marín et al., 2021a).

3.5. Cell wall properties and osmotic adjustment

Very few works have dealt with physiological aspects at cell level that may be involved on freezing-tolerance in fern fronds. By combining pressure-volume curves with gas-exchange measurements, Lösch and co-workers compared the physiological performance of three temperate wintergreen species belonging to the same genus (*Asplenium*) and co-habiting in a limestone rock: *A. trichomanes*, *A. ruta-muraria* and *A. scolopendrium* (Loesch et al., 2007). They found that *A. trichomanes* and *A. ruta-muraria* had fully poikilohydric fronds in winter (that is, tolerant to desiccation), whereas *A. scolopendrium* utilized osmotic adaptation as strategies to deal with winter stress (Loesch et al., 2007). This last species changed its leaf osmotic potential and increased the rigidity of its cell walls (higher modulus of elasticity) during autumn, so the leaves did not dehydrate during wintertime. Interestingly, *A. trichomanes* additionally shifted its optimal temperature for photosynthesis from 20–25 °C in summer to 8–10 °C in winter (Loesch et al., 2007).

3.6. Photoprotection

The deleterious effects of subzero temperatures plus high irradiance for fern leaves can be inferred from evidences obtained with evergreen angiosperms. Thus, most of the studies considering snow-cover demonstrated exacerbated damage to the exposed leaves (uncovered by snow) when additionally exposed to high light for flowering plants (Briceno et al., 2014; Bannister et al., 2005). Although not much research has been conducted with ferns in these directions, at least two studies evidence the need for photoprotection in wintergreen fern fronds (Fernández-Marín et al., 2021a; Putzier, Polich, & Verhoeven, 2022). Both works demonstrate the activation of the photoprotective xanthophyll cycle induced by freezing even in the absence of light, in five different species: *A. trichomanes*, *A. scolopendrium*, *Polypodium vulgare*, *P. virginianum* and *Adiantum capillus-veneris*. Interestingly, this enzymatic response in darkness was first described during the dehydration of the desiccation tolerant fern *Asplenium ceterach* (Fernández-Marín et al., 2009). The dark-induction of zeaxanthin formation has also been observed in vascular plants tolerant to low temperatures in response to freezing (Fernández-Marín et al., 2018). Apparently, this carotenoid can help in the maintenance of integrity of the chloroplast photosynthetic membranes in addition to its direct implication in the dissipation as heat of excessive light energy (Fernández-Marín et al., 2021b). In other species, such is the case of *Dryopteris filix-mas*, the plant optimizes its photosynthetic period through phenological changes, such as differences in the timing of frond development and longevity between sterile and fertile sporophytes. While the fertile sporophytes are summer green, the sterile sporophytes, produce new fronds in mid-summer and remain green throughout winter, extending their photosynthetic period and increase their productivity. This suggests that plants use various mechanisms, including structural and physiological adjustments such as changes in timing of leaf growth or protection of photosynthetic structures, to maintain their photosynthetic efficiency under varying environmental conditions (Bauer, Gallmetzer, & Sato, 1991).

4. Ecophysiological Aspects of Fern Gametophytes and Below-Zero Temperatures

Since gametophytes lack specialized epidermis, stomata, and vascular tissue, they are poikilohydric (López-Pozo et al., 2018), meaning they do not have mechanisms to maintain their water content. As a consequence, gametophytes are exposed to different selective pressures than their conspecific sporophytes, resulting in niche specialization that ultimately determines the establishment of fern populations (Krieg & Chambers, 2022). One of the first studies directly addressing freezing tolerance in ferns was conducted in gametophytes of *Phlebodium aureum* in the 30s (Stuckey & Curtis, 1938). The authors found cellular sensitivity to freezing, and described the freezing sequence of cellular compartments: cytosol, followed by mitochondria and chloroplast (Stuckey & Curtis, 1938).

Given that ferns are present in climates with cold winters, and that the lifespan of fern gametophytes can be longer than one year, as has been recently documented by photographic analysis in Switzerland (Farrar et al., 2008), it is obvious that they have to face recurrent episodes of freezing temperatures. In fact, gametophytes have been found in extreme environments such as the periglacial areas of the Altai mountains (Gureyeva & Timoshok, 2016) or the mountains of Hokkaido (Sato, 1982). Gametophytes are considered to be more stress tolerant than sporophytes (Kessler & Kluge, 2022; Gureyeva & Timoshok, 2016; Farrar et al., 2008). This is, for example, the case of the greater desiccation tolerance of gametophytes in some tropical ferns (Watkins et al., 2007). Furthermore, gametophytes are well adapted to surviving winter in temperate/cold climates. This was described in a study performed in Hokkaido (Japan) showing that gametophytes are in general more tolerant to freezing than their respective sporophytes (Sato, 1982; Sato & Sakai, 1980) and in fact most species were able to survive to experimental treatments at −40 °C (Sato & Sakai, 1981a). Furthermore, in another study, these authors described that the marginal cells of *Polystichum retroso-paleaceum* were able to recover even after exposure to −196 °C (Sato & Sakai, 1981b). Survival to −23 °C under field conditions in Switzerland has been documented by photographic analysis in gametophytes of *Athyrium filix-femina* and *Dryopteris dilatata* (Schneller & Farrar, 2022). In contrast, in a field study in Florida, gametophytes of subtropical *Lygodium microphyllum* and the invasive *L. japonicum* were not able to survive to 6-h exposure to −2.2 °C (Hutchinson & Langeland, 2014), limiting the expansion of the latter northwards. These results indicate that freezing tolerance is not a universal trait among fern gametophytes.

The so-called ‘independent gametophytes’ are a well-known example of increased abiotic stress tolerance in the gametophytic generation. These gametophytes are capable of reproducing and dispersing through asexual propagules, which allow them to form populations without the involvement of sporophytes (Figure 2). Independent gametophytes have been found in several epiphytic and epilithic species of five fern families (Yoneoka et al., 2024). Interestingly, in some species, the sporophytes have an exclusively tropical distribution, but the gametophytes reach temperate latitudes due to a greater tolerance to low temperatures and desiccation (Farrar, 1990). Independent gametophytes of exclusively temperate species also withstand harsher conditions at higher latitudes compared to their conspecific sporophytes (e.g., Ben-Menni et al., 2022). The different ecophysiological tolerances of gametophytes and sporophytes can partially explain the observed niche segregation between them, but other factors, such as dispersal and competition may also be relevant. Asexual propagules are larger and, as a result, have less effective wind dispersal compared to spores (Farrar, 1990). Thus, it has been hypothesized that the current populations of independent gametophytes in temperate latitudes could have originated from nearby sporophyte populations that were eliminated by glaciations due to their lower tolerance to extreme cold (Farrar, 1990; PPG, 2016).

The physiological responses of gametophytes to low and freezing temperatures have been rarely studied. It is known that the response of carbon assimilation to low temperature is similar to that of sporophytes, but their respiration is less sensitive to temperature (Johnson et al., 2000). One of the consequences of low temperatures is the metabolic impairment between light absorption and energy use, which is compensated by the so-called photoprotection mechanisms. In this sense it has been described that low temperatures induce a light avoidance chloroplast relocation mechanism in gametophytes of *Adiantum capillus-veneris* (Kodama et al., 2008). This mechanism, which is activated by blue light, also contributes to photoprotection by reducing the absorption of photosynthetic radiation.

A high proportion of fern gametophytes can be considered as desiccation tolerant (López-Pozo et al., 2018; Blake-Mahmud et al., 2024). Since desiccation tolerance and freezing tolerance share many common mechanisms (Verhoeven, García-Plazaola, & Fernández-Marín, 2018), and in fact one of the main effects of freezing is cellular dehydration (Figure 1), it could be expected a constitutive protection against freezing. Moreover, studies of cryopreservation of gametophytes show that a pre-treatment with abscisic acid (ABA) is required to enhance survival (Mikuła, Jata, & Rybczyński, 2009), suggesting a role for ABA in the activation of freezing tolerance mechanisms.

5. Ecophysiological Aspects of Chlorophyllous Fern Spores and Below-Zero Temperatures

Chlorophyllous fern spores occur in around 14% of fern species, mainly included in the families Equisetaceae, Hymenophyllaceae, Onocleaceae, Osmundaceae, and Polypodiaceae (Mellado-Mansilla et al., 2021, 2022). The presence or absence of chlorophyll in spores reflects the diverse strategies that ferns have evolved to optimize their chances of survival and reproduction in varying ecological niches (Mellado-Mansilla et al., 2021; López-Pozo et al., 2019). In particular, the fern species with chlorophyllous spores belong to two ecologically distinct functional groups: epiphytes abundant in humid tropical forests at middle or high elevation; and hydrophytes inhabiting waterlogged soils, particularly in temperate regions, belonging to Onocleaceae (e.g., *Onoclea struthiopteris*), Osmundaceae (e.g., *Osmunda regalis*), and Equisetaceae (*Equisetum* spp.). The presence of chlorophyll on spores has been associated with a lack of dormancy, shorter longevity, and rapid germination (Lloyd & Klekowski, 1970). Moreover, chlorophyllous spores have also been associated with a lower tolerance to stressful environmental conditions, including low or below-zero temperatures, compared to achlorophyllous spores (Lloyd & Klekowski, 1970; Ballesteros, Hill, & Walters, 2017).

Most of the studies regarding chlorophyllous fern spores and below-zero temperatures are focused on effective preservation methods for germplasm banks research or educational purposes (Kato, 1976; Whittier, 1996; Ballesteros et al., 2011). Overall, preservation studies performed in achlorophyllous spores under below zero-temperatures (−10 to −30 °C) indicate a negative effect on their viability (Lindsay, Williams, & Dyer, 1992; Quintanilla et al., 2002; Aragon & Pangua, 2004). In the case of chlorophyllous spores, negative effects of freezing temperatures have also been observed in *Osmunda japonica* (Li & Shi, 2014). However, some chlorophyllous spores present less sensitivity to low temperatures, as observed in *Equisetum ramosissimum* and *Osmunda regalis* (Ballesteros et al., 2011; Magrini & Scoppola, 2012), which show a decrease in spore viability but do not loss it completely at −20 °C for a period of at least 28 months. As for low non-freezing temperatures, green spores of *Osmunda regalis*, *O. claytoniana*, and *Osmundastrum cinnamomeum* also retained viability after long periods of dry storage at fridge temperatures between +2 and +6 °C (Stokey, 1951). Beyond natural freezing temperatures, studies focusing on preservation or storage have also proved the capacity of chlorophyllous spores to survive very low freezing temperatures. This is the case of the green spores of *Equisetum hyemale*, which are able to maintain their viability after been frozen at −70 °C for over a year (Whittier, 1996), and the particular the case of the green spores of *Osmunda regalis*, which can survive up to 18 months stored in liquid nitrogen (Pence, 2000).

The microscopic size of spores (a few tens of micrometres in diameter) is a major challenge for field studies. Although the phenology of spore release has been studied in many species, especially in temperate regions of the Northern Hemisphere (Lee, Huang, & Chiou, 2018), the tolerance of spores to low temperatures after dispersal has not been studied under natural conditions. Spore of many species of northern temperate regions are dispersed from June to September and germinate before winter begins, giving rise to gametophytes that survive the winter (Sato, 1982). However, there is also indirect evidence of the presence and survival of spores during winter. Specifically, in *Onoclea sensibilis*, a species that reaches high latitudes in North America, the sporangia retain mature green spores during the winter and release them in early spring (DeMaggio & Stetler, 1980; Suissa, 2022). Moreover, in some *Equisetum* species, such as *E. arvense*, spore formation and dispersal take place during early spring, a time in which the spores are exposed to cold temperatures below 5 °C in many areas of the species' range (Cody & Wagner, 1981; Zhao et al., 2015).

The capacity to withstand freezing is closely related to desiccation tolerance. Indeed, studies on spore conservation in germplasm banks highlight the drought tolerance of chlorophyll-containing spores, which is crucial for their survival at subzero temperatures (Ballesteros et al., 2011; Magrini & Scoppola, 2012; Li & Shi, 2014). This is the case for green spores of the fern *Onoclea struthiopteris*, which exhibit significant physiological adaptations that allow them to survive below-zero temperatures during winter, when spore dispersal occurs (López-Pozo et al., 2019). Mature spores of *O. struthiopteris* demonstrated good physiological recovery after desiccation and freezing, showing resilience under harsh conditions. This tolerance is associated with the antioxidant capacity of the spores, as seen in the high levels of α-tocopherol and proline that protect them from oxidative stress. Another example are the green spores of *Osmunda regalis*, which exhibit desiccation tolerance (López-Pozo et al., 2019) and are able to maintain their viability after stored at −20 °C without any pre-treatment (freshly collected with a water content of approximately 17%) (Magrini & Scoppola, 2012). Nonetheless, very few studies have focused on the effects of freezing on green spores and more data are required to understand this interaction between freezing and desiccation tolerance on chlorophyllous spores.

6. Conclusions

Ferns are able to live in cold ecosystems, but very few is known on the physiological mechanisms that ferns have developed to face winter stress. Most of the scarce knowledge currently available on freezing tolerance of photosynthetic tissues in ferns has been obtained either under natural, garden or laboratory conditions mainly in temperate regions with cold winters (Supplementary Table S1). Thus, studies on species covering a much wider biogeographical range would be needed. There are also significant taxonomic biases and gaps that need to be addressed (Figure 3). Overall, although interspecific differences are evident, the gametophyte generally shows higher tolerance than the sporophyte within a single species (Sato, 1982). Fertile vs. vegetative leaves may have different frost-tolerance (being vegetative frond more tolerant), although very scarce literature is available in that sense. There are also interspecific differences in the lethal temperature, being some species able to tolerate temperatures ≤−40 °C in their photosynthetic tissues (Riedmüller-Schölm, 1974; Sato, 1982). Another overall observation is the importance of acclimation and hardening, so a same population can show enhanced tolerance during winter time when compared to summer or after a frost event (Riedmüller-Schölm, 1974; Bannister, 1984; Voronkov & Ivanova, 2022). Water content of the tissue determines the tolerance to subzero temperatures, with higher tolerance reached at lower water contents (Kappen, 1966). In fact, as in the case of flowering plants, a certain relationship between tolerance to desiccation and freezing has also been observed in spores, gametophytes and leaves of ferns. In agreement with this, and although the biochemical/anatomical mechanism behind needs deeper study, ABA could have a role on the enhancement of freezing-tolerance mechanisms in ferns. Also, the preservation of chloroplast functionality by enhancing antioxidant, excess energy dissipation and ultrastructural stabilization, seems to be relevant for freezing tolerance in photosynthetic tissues of ferns. Freeze-thaw induced embolism has not been directly evaluated in ferns but can be inferred from data obtained in *Polystichum acrostichoides* under subzero temperatures. Prats and Brodersen (2020) measured a 25% loss of conductivity in the stipes suggesting the occurrence of embolism. Almost no information is available at cellular level regarding freezing-tolerance mechanisms.

Supplementary Materials

The additional data and information can be downloaded at: https://media.sciltp.com/articles/others/2505201437432693/plantecophys-577-Supplementary-final.pdf. Supplementary Table S1. Compilation of fern species, from which the tolerance to subzero temperatures has already been tested in the literature in green tissues (either sporophyte, gametophyte or chlorophyllous spore). The parameter/approach used to estimate tolerance to subzero temperatures, as well as the country and the original reference are specified.

Author Contributions

BF-M conceived the idea and drafted the work. JIG-P and MIA led the gametophyte- and spore-related information, respectively. LGQ led the taxonomy- and ecology-related information. SF and MIA prepared the figures. SF and BF-M wrote the main body of the manuscript and built the supplementary table. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

Original data are obtained from the literature. Literature sources are detailed in Supplementary Table S1.

Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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