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Freezing induces an increase in leaf spectral transmittance of forest understorey and alpine forbs

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1 Title: Freezing induces an increase in leaf spectral transmittance of forest-understorey and alpine forbs

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13

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15

16 Highlights/Running Headline:

- 17
- Transmittance increased in frozen vs fresh leaves
 - Freezing leaves shifted green reflectance to longer λ
 - Freezing caused chlorophyll degradation in understorey leaves
- 18
- 19

20

21 1 Abstract

22 Evergreen plants growing at high latitudes or high elevations may experience freezing events in their
23 photosynthetic tissues. Freezing events can have physical and physiological effects on the leaves which alter leaf
24 optical properties affecting remote and proximal sensing parameters. We froze leaves of six alpine forbs
25 (*Soldanella alpina*, *Ranunculus kuepferi*, *Luzula nutans*, *Gentiana acaulis*, *Geum montanum*, and *Centaurea*
26 *uniflora*) and three evergreen forest understorey species (*Hepatica nobilis*, *Fragaria vesca* and *Oxalis acetosella*),
27 and assessed their spectral transmittance and optically measured pigments, as well as photochemical efficiency of
28 photosystem II (PSII) as an indicator of freezing damage. Upon freezing, leaves of all the species transmitted
29 more photosynthetically active radiation (PAR) and some species had increased ultraviolet-A (UV-A)
30 transmittance. These differences were less pronounced in alpine than in understorey species, which may be related
31 to higher chlorophyll degradation, visible as reduced leaf chlorophyll content upon freezing in the latter species.
32 Among these understorey forbs, the thin leaves of *O. acetosella* displayed the largest reduction in chlorophyll (-
33 79%). This study provides insights into understanding the changes in leaf optical properties upon freezing of wild
34 forbs which could be used to set a baseline for upscaling optical reflectance data from remote sensing. Changes
35 in leaf transmittance may also serve as an indicator of changes in photosynthetic sufficiency and physiological
36 tolerance of freezing events, but this still requires experimental research to establish their functional association.

37

38

39 2 Introduction

40 Perennial plants in alpine environments and in temperate understory forests are at natural risk of freezing damage.
41 This risk may be highest in early spring, when photosynthetic tissues emerge from under insulating snow cover,
42 before attaining full frost hardiness [1, 2]. Exposure to such freezing events can damage buds and leaves, leading
43 to reduced flowering or fruiting and ultimately mortality in both alpine plants [3-6] and forest understorey species
44 [4, 7, 8]. Plants have evolved various adaptations against freezing but nevertheless freezing resistance varies
45 greatly across species, ranging from those that are very temperature sensitive to some alpine and boreal species
46 that even survive extreme freezing temperatures [2, 9, 10]. Canopy cover partially buffers understorey plants from
47 the sharp drops in temperature experienced in open environments in boreal and alpine regions [11], but alternately
48 canopy interception and heterogeneity can reduce snow cover leaving plants exposed to freezing temperatures.
49 Alpine plants are typically better adapted to freezing conditions than forest understorey species, however even
50 they can also suffer significant loss of biomass, fecundity and ultimately competitive ability following freezing
51 events [12]. Freezing resistance can also vary through the seasons, among populations within a single species, and
52 even among plant organs and developmental stages [2, 13-15]. Freezing definitely triggers physiological,
53 biochemical and physical alterations in the leaves, regardless the capability of tissues to tolerate sub-zero
54 temperatures.

55 Sharp drops in temperature can occur during spring in alpine zones and forest understories at high
56 elevation or latitude [11]. These sudden fluctuations in temperature during periods when full physiological
57 function is still returning to overwintering leaves often causes chlorophyll degradation [16] and stimulates
58 accumulation of antioxidant pigments, such as anthocyanins, which ameliorate stress at low temperatures [17, 18],
59 and other flavonoids which have additional roles including UV screening, frost protection and herbivory defence
60 [17, 19-21]. Freezing, also generates changes in the pigment composition of leaves: for instance, in frost-tolerant
61 leaves freezing induces the de-epoxidation of xanthophyll cycle carotenoids involved in photoprotection [22],
62 even in darkness [23-25].

63 In addition to the changes in pigments, freezing temperatures may cause structural changes to the leaf
64 and leaf surface affecting their optical properties. On the one hand, physiological and anatomical responses to
65 avoid leaf damage at cold temperatures, including acclimation of the cell membrane composition and cell
66 structure, may modify leaf optical properties at time-scales of days to seasons [26]. On the other hand, ice
67 accumulation in apoplastic spaces, cellular cytorrhysis, or changes to the cuticle have associated effects on the
68 scattering of radiation in the leaf [26-29]. In fact, ice optical properties greatly differ to those from liquid water,

69 and its birefringence has recently been used to locate ice crystals within leaves and buds through reflected light
70 microscopy [30].

71 Very few approaches are available to detect exotherms, to visualize ice formation and/ propagation
72 thermally, or to evaluate ice allocation within plant tissues in order to address the mechanisms and consequences
73 of plant freezing [27, 31-35]. However, by examining the transmittance of radiation in frozen leaves and
74 comparing it with that of fresh leaves we can infer its functional significance, as well as providing information on
75 reflectance that can be scaled-up to interpret remote sensing data [36] with broad applications in crop and
76 ecosystem science.

77 All previous points considered, we aimed to determine the changes induced by freezing in the spectral
78 reflectance and transmittance of leaves from alpine and understorey plants, spanning the ultraviolet (UV; 280-
79 400 nm), PAR (400-700 nm) and far-red (700-900 nm wavelengths) regions of the spectrum. In order to provide
80 insights into physiological processes potentially associated with changes in the spectra, we assessed the
81 photosynthetic yield of photosystem II (F_v/F_m) and optically measured leaf pigments, before and upon freezing.
82 To examine the consistency of these responses between alpine meadow species and boreal-forest understorey
83 forbs, we tested several plant species from each environment in spring soon after snowmelt. We expected alpine
84 plant species, from a habitat where fluctuating temperatures and high irradiances are common, to be better
85 acclimated to freezing, including having more photoprotective pigments. Because of this, we also expected alpine
86 species to display a smaller increase in leaf spectral transmittance and smaller drop in photosynthetic yield
87 following freezing compared to the forest understorey species.

88 3 Materials and Methods

89 3.1 Collection sites, plant material and experimental design

90 Leaves of six of the most prominent springtime alpine species [37] were sampled from an alpine meadow in the
91 sub-nival zone of the western French Alps adjacent to the *Station Alpin Joseph Fourier* (SAJF) botanical garden
92 at the Col du Lautaret (45.0359 °N, 6.4052 °E) at 2150 m a.s.l, on 28th May 2019. These species were: *Soldanella*
93 *alpina* (alpine snowbell), *Ranunculus kuepferi* (Pyrenean buttercup), *Luzula nutans* (wood rush), *Gentiana acaulis*
94 (trumpet gentian), *Geum montanum* (alpine avens) and *Centaurea uniflora* (alpine knapweed). Likewise, leaves
95 of three common forb species were sampled from the understorey of a mixed boreal forest [38], dominated by
96 *Betula pendula*, *Betula pubescens* with some *Picea abies* trees, at Lammi Biological Station LBS, central southern
97 Finland (61.05 °N, E 25.04 °E) at 130-135 m a.s.l on 15th June 2020. These understorey forbs were: *Hepatica*

98 *nobilis* (liverwort), *Fragaria vesca* (wild strawberry) and *Oxalis acetosella* (wood sorrel). In the alpine meadow,
99 only leaves from previous year that had survived under snow-cover through the winter were sampled while in the
100 understorey new leaves were also sampled. All leaves were collected close to solar noon between 12:00 and 14:00
101 to control for diurnal variation in leaf traits. A parallel set of leaves were sampled concurrently from which leaf
102 area, fresh weight and dry mass (after drying to a constant weight at 60°C) were recorded. For alpine species 6
103 groups of 5 leaf disks were weighed, whereas among understorey species 20-25 entire leaves were used, to
104 calculate Specific Leaf Area (SLA: leaf or disk area divided by dry mass) and Leaf Water Content (LWC: one
105 minus dry mass over fresh weight) [39] (Table 1). Mean daily temperature, precipitation and relative humidity in
106 the alpine meadow during the experiment were 5.0° C, 0 mm and 73 % (SAJF weather station, source:
107 <https://www.davis-meteo.com/Vantage-Pro2.php>) and in the understorey they were 20.6° C, 0 mm and 80 %
108 (LBS, source: <https://en.ilmatieteenlaitos.fi/>) (Fig. 1).

109 3.2 Freezing treatments and chlorophyll fluorescence measurements

110 One mature leaf was harvested from three-to-four different plants of each species (and leaf age class, where
111 applicable) growing naturally in the alpine meadow and the forest understorey, and immediately after sampling
112 were taken to the lab for optical measurements. During the transfer period from field to lab of about 10-15 mins,
113 the fresh leaves were kept in sealed plastic bags in a cool box. All three sets of measurements: optical properties,
114 chlorophyll fluorescence and leaf pigments were made on every leaf. To freeze the leaves, they were placed
115 horizontally flat between two sheets of paper towel to ensure that they didn't crinkle on freezing, returned to
116 sealed plastic bags and transferred to the freezer at -18° C for 24 hours in darkness, directly following room-
117 temperature measurements on fresh leaves. This same set of leaves were remeasured when frozen, keeping the
118 leaves *in situ* in the freezer to ensure that they remained frozen and at the same temperature throughout all the
119 different measurements.

120 Indices of leaf chlorophyll content, and epidermal flavonoid glycosides (flavanols in dicots., flavones in
121 monocots.) and epidermal anthocyanins were measured once from the adaxial side of each leaf with a Dualex
122 Scientific+ (Force-A, Paris Sud, France). Maximum quantum yield of PSII (F_v/F_m), as indicator of photosynthetic
123 capacity, was measured with a FluorPen (PSI, Drásov, Czechia) following 30 min of dark acclimation using leaf
124 clips. We made a set of measurements of leaf spectral transmittance and reflectance of radiation (Jaz Spectro-
125 Clip) for each of the three leaves per alpine species or four leaves per understorey species and leaf-age class.
126 These measurements were repeated on the same leaves once frozen, except for reflectance of alpine species which

127 was not logistically possible. Following these measurements and 30-min dark-adaptation, F_v/F_m (mini-PAM,
128 Heinz-Walz, Effeltrich, Germany) was recorded as a means of indirectly assessing true freezing within the tissues
129 of the same set of frozen leaves. The frozen leaves of alpine species were too fragile to allow Dualex and
130 reflectance measurements before leaves defrosted.

131 3.3 Measuring and processing of leaf optical properties

132 Leaf spectral transmittance and reflectance were measured across the spectral region (250-892 nm),
133 encompassing all the wavelengths of ambient UV-B and UV-A radiation, and visible and far-red light that plants
134 naturally receive in sunlight. Reflectance of radiation from the adaxial (upper) side and transmittance to the abaxial
135 (lower) side of one leaf from three-to-four replicate plants per species and age class were measured with a dual
136 integrating-sphere system, Jaz Spectro-Clip (Ocean Optics, Dundane, FL, USA). These measurements were all
137 taken from the same part of the fresh and frozen leaves selected for all other optical measurements, avoiding the
138 midrib where possible. The Jaz Spectro-clip comprises several modules including a dual spectrometer, a pulse
139 xenon light source, data processing unit and battery. The two integrating spheres in the system collect light
140 transmitted though the leaf as well as reflected light. The Jaz modular spectrometer was used as a standalone for
141 optical measurements, the trigger rate was set to 10 ms with a hold-off time and trigger delay of 1 ms, and the
142 spectrometer integration time was set to 1000 ms. Each leaf's transmittance/reflectance spectrum was an average
143 of six consecutive individual recordings of spectra without both boxcar-smoothing and non-linearity detection.
144 Measurements of each leaf took c 4 min., during which time there was no noticeable trend to suggest any short-
145 term time-dependent variation in optical properties. The flash rate was set at 200 Hz (or one flash every 5 ms)
146 with an intensity of 400 Volts. Each leaf transmittance and reflectance spectra measured on a sample (S), were
147 matched with a dark (D) and reference (R) measurement using black and white Spectralon diffuse-reflectance
148 reference targets (WS-1-SL, Ocean Optics). The instrument calculates the spectral transmittance ($T\lambda$) and
149 reflectance($R\lambda$) according to Eq.1.

$$T\lambda/R\lambda = \frac{S - D}{R - D} * 100 \quad (\text{Eq.1})$$

150

151 Post-processing of the raw spectra was done using the Photobiology suite of packages in R [40], to detect
152 out-of-range values. A "lowess" function was selected to smooth the spectrum, after testing and comparing those
153 smooth functions available, reducing the effect of bad pixels without overfitting the data.

154 3.4 Statistical analyses

155 Differences in the transmittance, reflectance and absorptance of leaves were compared in two ways, treated either
156 as continuous spectra or as discrete spectral regions calculated from these spectra. The mean of spectra (from 3-4
157 leaves) for a species, or freezing treatment, were considered to differ significantly when their 95% confidence
158 bands were non-overlapping, plotted in the same figure. Whereas, species or freezing-related differences for
159 discrete spectral regions were tested through ANOVA.

160 Two-way ANOVA was used to test whether the spectral transmittance in blue, green, red and far-red
161 (and likewise F_v/F_m) differed among species, before vs upon freezing. Differences in leaf epidermal pigments
162 (flavonols, anthocyanin and chlorophyll content) due to leaf age, among understory species, and before vs upon
163 freezing, were tested by a three-way ANOVA, and among alpine species leaf epidermal pigments were test using
164 a single factor ANOVA. Function `glht` from ‘multcomp’ package was used to estimate within-species pair-wise
165 comparisons and multiple comparisons between fresh and frozen leaves.

166 4 Results & Discussion

167 Freezing caused changes in the optical properties (reflectance and transmittance across the UV, PAR and far-red
168 spectrum), as well as pigments (optically measured flavonols, anthocyanins and chlorophylls) of frozen leaves
169 compared with fresh leaves from species growing in alpine and forest understory environments. While a few
170 studies have addressed spectral characterization of leaf-injury induced after freezing in crop plants [36, 41] and
171 in trees [42], to the best of our knowledge, this is the first study examining the spectral transmittance and
172 reflectance of frozen leaves in wild species. The results of this series of measurements of leaf optical properties
173 and associated leaf pigments in the same leaves, could provide insights into variation in the amount of light
174 reaching the mesophyll upon freezing and set a baseline for remote sensing where such data on leaf transmittance
175 and reflectance of frozen leaves in winter are required as inputs for modelling radiative transfer.

176 4.1 Freezing increased transmittance of leaves of both alpine and understory species.

177 The transmittance spectra of fresh leaves from our studied species followed characteristic patterns similar to those
178 previously reported for leaves from a variety of species [43, 44] (Fig. 2). A typical leaf appears green to the human
179 eye, due to the strong absorption of light by photosynthetic pigments mainly in the visible spectrum
180 (Photosynthetically Active Radiation - PAR, 400-700nm). At longer wavelengths, in the near and short-wave
181 infrared (700-2500nm) green leaves typically have prominently higher reflectance and transmittance, with very
182 little absorptance; properties linked to their water content and structural features [45-49]. In our experiment, leaf

183 spectral transmittance increased in response to freezing in the leaves but the size of this effect differed across the
184 spectrum. Overall, the increase in the percentage of radiation transmitted was largest in the far-red (increasing by
185 4.1% in alpine sp & 7.6% in understorey sp) and in the PAR (1.4% alpinesp & 4.5% understorey sp); where
186 specifically the difference in transmittance between fresh and frozen leaves was greater in the green (500-600 nm;
187 1.84% alpine sp & 7.74% understorey sp) than the red (600-700 nm; 0.14% alpine sp & 5.45% understorey sp)
188 and blue (400-500 nm; 0.40% alpine sp & 1.85% understorey sp) spectral regions (Fig. 2 & 4, Table S5). Overall,
189 frozen leaves also transmitted 0.60% (alpine sp) and 0.24% (understorey sp) more UV-A radiation (315-400 nm)
190 than fresh leaves of both the understorey and alpine species, but this effect was minimal in the UV-B (280-315 nm)
191 because all leaves transmitted negligible radiation (<0.1%) in this spectral region, decreasing at shorter
192 wavelengths (Fig. 2, Table S5).

193 The enhancement of transmittance induced by freezing was generally greater in the leaves of understorey
194 than alpine species (Fig. 2a&b). However, in both environments there were some species which deviated from
195 this general pattern of response. Even once frozen, the leaves of most alpine species (five of the six species) were
196 opaque to UV-A radiation and blue light (Fig. 2a, & 4), and *L. nutans* only transmitted less than 0.02% UV-A
197 radiation (Fig. 2a & 4). *Luzula nutans* was the only monocot among the species that we tested, and had high SLA
198 and LWC compared with the other alpine species (Table 1), as well as a very different leaf structure; which may
199 explain this difference in transmittance [50, 51]. A typical monocot leaf has a compact palisade mesophyll often
200 referred to as chlorenchyma and lacks spongy mesophyll; this causes light interception, scattering and
201 transmittance to be very different from species with both spongy and palisade mesophyll cells [52]. Among alpine
202 species, only leaves of *G. montanum* and *R. keupferi* transmitted significantly more green light (increasing by
203 2.7% & 4.8%) and far-red light (increasing by 6.5% & 11.3%) when frozen than fresh (Fig. 2a, Table S4),
204 indicating that freezing had a greater impact on leaf optical properties in these species than in the other alpine
205 forbs. Among the understorey species, fresh leaves of *O. acetosella* had the highest spectral transmittance among
206 the species we measured; and likewise, once frozen, its leaves transmitted the most spectral irradiance (Figs. 2b,
207 4 & Table S4). Irrespective of damage, the fact that *O. acetosella* has very thin leaves [53-56] likely explains why
208 their transmittance was highest, this contrasts with *H. nobilis* which has thick leaves through which very little
209 radiation is transmitted (Figs 2b & 4, Table 1) [54]. As well as leaf structure, specific leaf area, and leaf anatomy,
210 difference in transmittance among species may change due to acclimation to the physical environment, such as
211 temperature fluctuations and high incident irradiance, e.g., inducing phototropin-mediated chloroplast stacking,
212 whereby chloroplasts align against the anticlinal cell walls increasing light transmittance through the leaf [55, 57].

213 The processes leading to apoplastic ice allocation, organelle reorganization and other ultrastructural changes upon
214 freezing are as yet poorly understood in plant leaves. However, recent methodological advances have enabled ice
215 accommodation within leaf cross-sections to be visualised [30], and for ultrastructural evaluation of frozen leaves
216 by transmission electron microscopy [58]. These techniques appear to be promising new tools to complement the
217 interpretation of changes in spectral signatures of frozen leaves in the near future.

218 Notably, the reflectance of *O. acetosella* leaves in the PAR increased when frozen, with the green peak
219 seemingly shifting towards longer wavelengths (Fig. 2b). Leaf absorbance in this spectral region is mainly
220 attributable to chlorophyll, hence this change may be the result of cell lysis affecting the pH, degrading chlorophyll
221 and producing pheophytin [42]. It is also possible that disruption of the membrane and cell structure of the leaf
222 during freezing contributed to the changes recorded in transmittance and reflectance, reducing the scattering of
223 radiation by internal structures, which would also result in reduced back-scattered radiation escaping the leaf. In
224 all three understorey species, freezing reduced leaf reflectance in the far-red region. The amount of radiation
225 reflected in the far-red is thought to be controlled by surface properties, air spaces and the internal structure of the
226 leaf [52]. Additionally, the phase transition from water to ice has been found to displace reflectance spectra to
227 longer wavelengths in the green and far-red regions, during freezing of oil-seed rape (*Brassica napus*) leaves [36].

228 When exposed to freezing temperatures and high irradiance, leaves can suffer severe photo-inhibitory stress
229 and damage to the photosynthetic apparatus. In freezing-tolerant species, this risk is effectively counteracted with
230 photoprotective mechanisms [59-61]. Our experiment, however, was conducted in darkness to avoid
231 photoinhibition. The fresh leaves from the understorey species showed high F_v/F_m values of 0.82, and similarly,
232 fresh leaves of the alpine species had high F_v/F_m at 0.78 on average. Values of F_v/F_m slightly below 0.8, such as
233 these, are common under optimal conditions for alpine species generally [24, 25, 62]. Acclimation of
234 photosynthetic apparatus from freezing-tolerant species to low temperatures is usually accompanied by a
235 downregulation of predawn F_v/F_m values [23, 24]. Interestingly, in our study, the F_v/F_m depression due to freezing
236 was higher in the understorey than the alpine species. The greater susceptibility of understorey species to freezing
237 damage may have been exacerbated because they were sampled later into the growing season than the alpine
238 species, meaning that they are likely to have completely dehardened prior to freezing [63, 64]. Amongst the
239 understorey species, F_v/F_m was lowered most by freezing in *O. acetosella* (Fig. 3) which was consistent with the
240 largest increase in transmittance occurring in this species, along with the greatest loss of chlorophyll and lowest
241 flavonoid indices (Fig. 2b & 5). Amongst alpine species, *S. alpina* had both the lowest F_v/F_m in fresh leaves (0.72
242 ± 0.01), and the biggest decrease in frozen leaves (to 0.37 ± 0.04), compared with F_v/F_m in other alpine species

243 which only declined to about 0.60 (Fig. 3). While we didn't find a difference in the extent to which F_v/F_m in
244 mature leaves, that had overwintered, was depressed by freezing compared with current year's leaves, previous
245 studies [65, 66] have reported young leaves of alpine species to be more freezing sensitive, since ice formation
246 can more easily damage their photosynthetic apparatus than in mature leaves.

247 4.2 Changes upon freezing in optically measured leaf pigments.

248 Leaf chlorophyll, epidermal flavonol and anthocyanin contents were measured optically on the leaves of
249 understory species before and upon freezing, whereas these optical traits were only measured in fresh leaves of
250 alpine species. The fresh leaves of alpine species contained higher epidermal flavonoids as well as higher
251 chlorophyll content than the fresh leaves of understory species (Fig. 5a & b). This presumably is a consequence
252 of their accumulation in response to the higher solar radiation, in particular UV-B radiation, received in the alpine
253 environment than in the boreal understory, and its colder springtime temperatures with large diurnal fluctuations
254 [38, 67], which stimulate increased photoprotection and chlorophyll accumulation [60, 67]. It is conceivable that
255 excision of the fresh leaves prior to measurement could affect their physiology and pigment values prior to
256 freezing, but comparison with other attached leaves of these species did not provide evidence for such an effect
257 (unpublished data).

258 For all the understory species, the index of epidermal flavonols was higher than that of anthocyanins,
259 but these indices differed in response to freezing (Fig. 5b & c). In understory species, the epidermal anthocyanin
260 index significantly increased upon freezing in darkness ($p < 0.001$) (Fig. 5c). However, there was no parallel
261 increase in the optical index of UV-screening epidermal flavonols upon freezing in *O. acetosella* or *F. vesca*, it
262 only increased in *H. nobilis* (Fig. 5b). Without a quantitative comparison of the metabolites of fresh and frozen
263 leaves, we are not able to attribute these increases in absorbance to changes in the concentration or location of
264 these classes of flavonoid. Even well-acclimated alpine species such as *Ramonda myconi* cease enzymatic activity
265 at -15°C [24], so at most these leaves would have been metabolically active only as their temperature dropped
266 prior to freezing, meaning there would have been little opportunity for anthocyanin synthesis.

267 In contrast to flavonols, the drop in optically measured leaf chlorophyll content upon freezing differed
268 significantly among species. Chlorophyll content was substantially reduced in frozen leaves of *H. nobilis* (-39 %)
269 and *O. acetosella* (-79 %) compared with fresh leaves, but only reduced to a lesser extent in leaves of *F. vesca* (-
270 11 %) (Fig. 5a, Table S2). These results suggest that there was degradation of leaf chlorophyll during freezing
271 [68], which is consistent with the larger increase in PAR transmittance in *H. nobilis* and *O. acetosella* than in

272 *F. vesca* upon freezing (4.1). Transmittance of frozen leaves also increased at longer wavelengths, but a study of
273 the leaf ultrastructure would be required to distinguish the relative contributions to this effect from leaf structural
274 changes and pigment degradation.. The two obligate understorey species were presumably less-well adapted to
275 tolerate sudden freezing to -18°C or less-well acclimated to cold conditions at the time of sampling than *F. vesca*
276 which grows across a broader environmental niche [69, 70].

277 Interpretation of the changes in optically measured leaf pigments and leaf optical properties that we
278 report could be improved by identifying whether these differences are underpinned by biochemical changes in the
279 leaf or structural changes and cellular damage during freezing. These effects can be related to the freezing
280 resistance strategy and acclimation of the different species that we studied. Upscaling the changes in leaf optical
281 properties, measured for the alpine and understorey species in our study, provides possibilities to interpret
282 remotely sensed canopy-level changes in absorbed PAR on freezing [36, 71]. Leaf reflectance spectra provide
283 reflectance indices useful in understanding the physiological status of plants, e.g., chlorophyll indices and the
284 photochemical reflectance index (PRI), which allow scaling from the leaf to the canopy. The species-level
285 information we provide could be incorporated in ecosystem-scale models to improve estimates of their
286 contribution to photosynthesis [48, 72, 73].

287 4.3 Conclusions

288 We found that across alpine and understorey species, freezing increases the spectral transmittance of mature leaves
289 in spring. The largest reductions in transmittance were in the PAR, particularly the red and green, but there was
290 also a small species-specific increase in transmittance of UV-A radiation. Transmittance of far-red increased and
291 its reflectance decreased upon freezing. In understorey species, the spectral peak characteristic of *in vivo* leaf
292 absorbance by chlorophyll shifted towards longer wavelengths on freezing, possibly as a symptom of membrane
293 damage and a change in its molecular configuration or pH, though this suggestions require specific examination.
294 Among obligate species understorey, optically assessed leaf chlorophyll content was particularly decreased by
295 freezing.

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303 6 References

- 304 1. Körner, C. (2012). Treelines will be understood once the functional difference between a tree and a shrub is.
305 *Ambio*, 41(3), 197-206. <https://doi.org/110.1007/s13280-13012-10313-13282>.
306 <https://doi.org/10.1007/s13280-012-0313-2>.
- 307 2. Neuner, G. (2014). Frost resistance in alpine woody plants. *Frontiers in Plant Science*, 5(654.), 00.
308 <https://doi.org/10.3389/fpls.2014.00654>.
- 309 3. Bianchi, E., Bugmann, H., & Bigler, C. (2019). Early emergence increases survival of tree seedlings in
310 Central European temperate forests despite severe late frost. *Ecology and Evolution*, 9(14), 8238-8252.
311 <https://doi.org/8210.1002/ece8233.5399>.
- 312 4. Gerdol, R., Siffi, C., Iacumin, P., Gualmini, M., & Tomaselli, M. (2013). Advanced snowmelt affects
313 vegetative growth and sexual reproduction of *Vaccinium myrtillus* in a sub-alpine heath. *Journal of*
314 *Vegetation Science*, 24(3), 569-579. <https://doi.org/510.1111/j.1654-1103.2012.01472.x>.
- 315 5. Inouye, D. W. (2008). Effects of climate change on phenology, frost damage and floral abundance of
316 montane wildflowers. *Ecology*, 89(2), 353-362. <https://doi.org/310.1890/1806-2128.1891>.
- 317 6. Shen, W., Zhang, L., Liu, X., & Luo, T. (2014). Seed-based treeline seedlings are vulnerable to freezing
318 events in the early growing season under a warmer climate: Evidence from a reciprocal transplant
319 experiment in the Sergyemla Mountains, southeast Tibet. *Agricultural and Forest Meteorology*, 187, 83-92.
320 <https://doi.org/10.1016/j.agrformet.2013.1012.1004>.
- 321 7. Bokhorst, S., Bjerke, J. W., Bowles, F. W., Melillo, J., Callaghan, T. V., & Phoenix, G. K. (2008). Impacts
322 of extreme winter warming in the sub-Arctic: growing season responses of dwarf shrub heathland. *Global*
323 *Change Biology*, 14(11), 2603-2612. <https://doi.org/2610.1111/j.1365-2486.2008.01689.x>.
- 324 8. Rixen, C., Dawes, M. A., Wipf, S., & Hagedorn, F. (2012). Evidence of enhanced freezing damage in
325 treeline plants during six years of CO2 enrichment and soil warming. *Oikos*, 121(10), 1532-1543.
326 <https://doi.org/https://doi.org/10.1111/j.1600-0706.2011.20031.x>.
- 327 9. Sklenář, P. (2017). Seasonal variation of freezing resistance mechanisms in north-temperate alpine plants.
328 *Alpine Botany*, 127(1), 31-39. <https://doi.org/10.1007/s00035-016-0174-6>.
- 329 10. Wheeler, J. A., Hoch, G., Cortés, A. J., Sedlacek, J., Wipf, S., & Rixen, C. (2014). Increased spring freezing
330 vulnerability for alpine shrubs under early snowmelt. *Oecologia*, 175(1), 219-229.
331 <https://doi.org/10.1007/s00442-013-2872-8>.
- 332 11. Lamichhane, J. R. (2021). Rising risks of late-spring frosts in a changing climate. *Nature Climate Change*,
333 11(7), 554-555. <https://doi.org/10.1038/s41558-021-01090-x>.
- 334 12. Bokhorst, S. F., Bjerke, J. W., Tømmervik, H., Callaghan, T. V., & Phoenix, G. K. (2009). Winter warming
335 events damage sub-Arctic vegetation: consistent evidence from an experimental manipulation and a natural
336 event. *Journal of Ecology*, 97(6), 1408-1415. <https://doi.org/https://doi.org/10.1111/j.1365-2745.2009.01554.x>.
- 337 13. González-Rodríguez, Á. M., Pérez-Martín, E. M., Brito, P., & Fernández-Marín, B. (2021). Unexpected
338 vulnerability to High Temperature in the Mediterranean Alpine Shrub *Erysimum scoparium* (Brouss. ex
339 Willd.) Wettst. *Plants*, 10(2), 379. <https://doi.org/10.3390/plants10020379>.
- 340 14. Larcher, W., Kainmüller, C., & Wagner, J. (2010). Survival types of high mountain plants under extreme
341 temperatures. *Flora - Morphology, Distribution, Functional Ecology of Plants*, 205(1), 3-18.
342 <https://doi.org/https://doi.org/10.1016/j.flora.2008.12.005>.
- 343 15. Wisniewski, M., Gusta, L., & Neuner, G. (2014). Adaptive mechanisms of freeze avoidance in plants: a brief
344 update. *Environmental and Experimental Botany*, 99, 133-140.
345 <https://doi.org/https://doi.org/10.1016/j.envexpbot.2013.11.011>.
- 346 16. Ward, K., Scarth, R., & Daun, J. K. (1992). The effect of freezing on the analysis of chlorophyll content of
347 canola seed (*Brassica napus* L.). *Journal of the American Oil Chemists' Society*, 69(10), 1039-1040.
348 <https://doi.org/10.1007/BF02541074>.
- 349 17. Chalker-Scott, L., & Scott, J. D. (2004). Elevated Ultraviolet-B Radiation Induces Cross-protection to Cold
350 in Leaves of Rhododendron Under Field Conditions¶. *Photochemistry and Photobiology*, 79(2), 199-204.
351 <https://doi.org/https://doi.org/10.1111/j.1751-1097.2004.tb00010.x>.
- 352 18. Oberbauer, S. F., & Starr, G. (2002). The role of anthocyanins for photosynthesis of Alaskan arctic
353 evergreens during snowmelt. In *Advances in Botanical Research* (Vol. 37, pp. 129-145). Academic Press.
354 [https://doi.org/https://doi.org/10.1016/S0065-2296\(02\)37047-2](https://doi.org/https://doi.org/10.1016/S0065-2296(02)37047-2).
- 355 19. Jordan, B. R. (2017). *UV-B radiation and plant life: molecular biology to ecology*. CABI publishing,
356 Oxford.
- 357 20. Nybakken, L., Aubert, S., & Bilger, W. (2004). Epidermal UV-screening of arctic and alpine plants along a
358 latitudinal gradient in Europe. *Polar Biology*, 27(7), 391-398. <https://doi.org/10.1007/s00300-004-0601-9>.
- 359 21. Schulz, E., Tohge, T., Zuther, E., Fernie, A. R., & Hincha, D. K. (2016). Flavonoids are determinants of
360 freezing tolerance and cold acclimation in *Arabidopsis thaliana*. *Scientific Reports*, 6(1), 34027.
361

- 362 <https://doi.org/10.1038/srep34027>.
- 363 22. Peguero-Pina, J. J., Gil-Pelegrín, E., & Morales, F. (2013). Three pools of zeaxanthin in *Quercus coccifera*
- 364 leaves during light transitions with different roles in rapidly reversible photoprotective energy dissipation
- 365 and photoprotection. *J Exp Bot*, 64(6), 1649-1661. <https://doi.org/10.1093/jxb/ert024>
- 366 23. Fernández-Marín, B., Arzac, M. I., López-Pozo, M., Laza, J. M., Roach, T., Stegner, M., Neuner, G., &
- 367 García-Plazaola, J. I. (2021). Frozen in the dark: interplay of night-time activity of xanthophyll cycle, xylem
- 368 attributes, and desiccation tolerance in fern resistance to winter. *Journal of Experimental Botany*, 72(8),
- 369 3168-3184. <https://doi.org/10.1093/jxb/erab071>.
- 370 24. Fernández-Marín, B., Neuner, G., Kuprian, E., Laza, J. M., García-Plazaola, J. I., & Verhoeven, A. (2018).
- 371 First evidence of freezing tolerance in a resurrection plant: insights into molecular mobility and zeaxanthin
- 372 synthesis in the dark. *Physiologia Plantarum*, 163(4), 472-489.
- 373 <https://doi.org/https://doi.org/10.1111/ppl.12694>.
- 374 25. Fernández-Marín, B., Roach, T., Verhoeven, A., & García-Plazaola, J. I. (2021). Shedding light on the dark
- 375 side of xanthophyll cycles. *New Phytologist*, 230(4), 1336-1344.
- 376 <https://doi.org/https://doi.org/10.1111/nph.17191>.
- 377 26. Pearce, R. S. (2001). Plant Freezing and Damage. *Annals of Botany*, 87(4), 417-424.
- 378 <https://doi.org/https://doi.org/10.1006/anbo.2000.1352>.
- 379 27. Stegner, M., Lackner, B., Schäfermolte, T., Buchner, O., Xiao, N., Gierlinger, N., Holzinger, A., & Neuner,
- 380 G. (2020). Winter nights during summer-time: stress physiological response to ice and the facilitation of
- 381 freezing cytorrhysis by elastic cell wall components in the leaves of a nival species. *International Journal of*
- 382 *Molecular Sciences*, 21(19), 7042. <https://doi.org/10.3390/ijms21197042>.
- 383 28. Wisniewski, M.E., Gusta, L.V., Fuller, M.P., Karlson, D. (2009). Ice Nucleation, Propagation, and Deep
- 384 Supercooling: The Lost Tribes of Freezing Studies. pp 1-11. *Plant Cold Hardiness: From the Laboratory to*
- 385 *the Field*. eds. Gusta, L., Wisniewski, M., and Tanino, K. CABI, Oxfordshire, UK 317pp.
- 386 <https://doi.org/10.1079/9781845935139.0001>.
- 387 29. Xin, Z., & Browse, J. (2000). Cold comfort farm: the acclimation of plants to freezing temperatures. *Plant,*
- 388 *Cell & Environment*, 23(9), 893-902. <https://doi.org/https://doi.org/10.1046/j.1365-3040.2000.00611.x>.
- 389 30. Stegner, M., Wagner, J., & Neuner, G. (2020). Ice accommodation in plant tissues pinpointed by cryo-
- 390 microscopy in reflected-polarised-light. *Plant Methods*, 16(1), 73. [https://doi.org/10.1186/s13007-020-](https://doi.org/10.1186/s13007-020-00617-1)
- 391 [00617-1](https://doi.org/10.1186/s13007-020-00617-1).
- 392 31. Kovaleski, A. P., Londo, J. P., & Finkelstein, K. D. (2019). X-ray phase contrast imaging of *Vitis* spp. buds
- 393 shows freezing pattern and correlation between volume and cold hardiness. *Scientific Reports*, 9(1), 14949.
- 394 <https://doi.org/10.1038/s41598-019-51415-2>.
- 395 32. Livingston, D. P. (2018). Investigating Freezing patterns in plants using infrared thermography. In M.
- 396 Iwaya-Inoue, M. Sakurai, & M. Uemura (Eds.), *Survival strategies in extreme cold and desiccation:*
- 397 *adaptation mechanisms and their applications* (pp. 117-127). Springer Singapore.
- 398 https://doi.org/10.1007/978-981-13-1244-1_7.
- 399 33. Neuner, G., Monitzer, K., Kaplenig, D., & Ingruber, J. (2019). Frost survival mechanism of vegetative buds
- 400 in temperate trees: deep supercooling and extraorgan freezing vs. ice tolerance. *Frontiers in Plant Science*,
- 401 10(537). <https://doi.org/10.3389/fpls.2019.00537>.
- 402 34. Quamme, H. A. (1995). Deep supercooling in buds of woody plants. Biological ice nucleation and its
- 403 applications. Edited by R.E. Lee, P.183-199.. American Phytopathological Society Press, St. Paul, MN,
- 404 USA. ISBN: 0890541728 9780890541722
- 405 35. Wisniewski, M., Lindow, S. E., & Ashworth, E. N. (1997). Observations of ice nucleation and propagation
- 406 in plants using infrared video thermography. *Plant Physiology*, 113(2), 327-334.
- 407 <https://doi.org/10.1104/pp.113.2.327>.
- 408 36. Wei, C., Huang, J., Wang, X., Blackburn, G. A., Zhang, Y., Wang, S., & Mansaray, L. R. (2017).
- 409 Hyperspectral characterization of freezing injury and its biochemical impacts in oilseed rape leaves. *Remote*
- 410 *Sensing of Environment*, 195, 56-66. <https://doi.org/https://doi.org/10.1016/j.rse.2017.03.042>.
- 411 37. Aubert, S., Bec, S., Choler, Ph., Douzet, R., Michalet, R., Thuiller W. (2011). Découverte botanique de la
- 412 région du Lautaret et du Briançonnais. Partie 1. Eléments d'écologie alpine. Les cahiers illustrés du Lautaret.
- 413 No2(1). Ed. SAJF, 76.
- 414 38. Hartikainen, S. M., Pieristè, M., Lassila, J., & Robson, T. M. (2020). seasonal patterns in spectral irradiance
- 415 and leaf UV-A absorbance under forest canopies. *Frontiers in Plant Science*, 10(1762).
- 416 <https://doi.org/10.3389/fpls.2019.01762>.
- 417 39. Pérez-Harguindeguy, N., Díaz, S., Garnier, E., Lavorel, S., Poorter, H., Jaureguiberry, P., Bret-Harte, M. S.,
- 418 Cornwell, W. K., Craine, J. M., Gurvich, D. E., Urcelay, C., Veneklaas, E. J., Reich, P. B., Poorter, L.,
- 419 Wright, I. J., Ray, P., Enrico, L., Pausas, J. G., de Vos, A. C., Buchmann, N., Funes, G., Quétier, F.,
- 420 Hodgson, J. G., Thompson, K., Morgan, H. D., ter Steege, H., Sack, L., Blonder, B., Poschlod, P., Vaieretti,
- 421 M. V., Conti, G., Staver, A. C., Aquino, S., & Cornelissen, J. H. C. (2013). New handbook for standardised

- 422 measurement of plant functional traits worldwide. *Australian Journal of Botany*, 61(3), 167-234.
423 <https://doi.org/https://doi.org/10.1071/BT12225>.
- 424 40. Aphalo, P. J. (2015). The r4photobiology suite: spectral irradiance. *UV4Plants Bulletin*, 2015(1), 21-29.
425 <https://doi.org/10.19232/uv4pb.2015.1.14>.
- 426 41. Duddu, H. S. N., Pajic, V., Noble, S. D., Tanino, K. K., & Shirliffe, S. J. (2018). Image-Based Rapid
427 Estimation of Frost Damage in Canola (*Brassica napus* L.). *Canadian Journal of Remote Sensing*, 44(2),
428 169-175. <https://doi.org/10.1080/07038992.2018.1462660>.
- 429 42. Nicotra, A. B., Hofmann, M., Siebke, K., & Ball, M. C. (2003). Spatial patterning of pigmentation in
430 evergreen leaves in response to freezing stress. *Plant, Cell & Environment*, 26(11), 1893-1904.
431 <https://doi.org/https://doi.org/10.1046/j.1365-3040.2003.01106.x>.
- 432 43. Gorton, H. L., Brodersen, C. R., Williams, W. E., & Vogelmann, T. C. (2010). Measurement of the optical
433 properties of leaves under diffuse light. *Photochem Photobiol*, 86(5), 1076-1083.
434 <https://doi.org/10.1111/j.1751-1097.2010.00761.x>.
- 435 44. Vogelmann, T. C., & Evans, J. R. (2002). Profiles of light absorption and chlorophyll within spinach leaves
436 from chlorophyll fluorescence. *Plant, Cell & Environment*, 25(10), 1313-1323.
437 <https://doi.org/https://doi.org/10.1046/j.1365-3040.2002.00910.x>.
- 438 45. Carter, G. A. (1991). primary and secondary effects of water content on the spectral reflectance of leaves.
439 *American Journal of Botany*, 78(7), 916-924. <https://doi.org/10.2307/2445170>.
- 440 46. Carter, G. A., & Knapp, A. K. (2001). Leaf optical properties in higher plants: linking spectral characteristics
441 to stress and chlorophyll concentration. *American Journal of Botany*, 88(4), 677-684.
442 <https://doi.org/https://doi.org/10.2307/2657068>.
- 443 47. Gates, D. M., Keegan, H. J., Schleter, J. C., & Weidner, V. R. (1965). Spectral properties of plants. *Applied*
444 *Optics*, 4(1), 11-20. <https://doi.org/10.1364/AO.4.000011>.
- 445 48. Jacquemoud, S., & Baret, F. (1990). PROSPECT: A model of leaf optical properties spectra. *Remote Sensing*
446 *of Environment*, 34(2), 75-91. [https://doi.org/https://doi.org/10.1016/0034-4257\(90\)90100-Z](https://doi.org/https://doi.org/10.1016/0034-4257(90)90100-Z).
- 447 49. Merzlyak, M. N., Melø, T. B., & Razi Naqvi, K. (2004). Estimation of leaf transmittance in the near infrared
448 region through reflectance measurements. *Journal of Photochemistry and Photobiology B: Biology*, 74(2),
449 145-150. <https://doi.org/https://doi.org/10.1016/j.jphotobiol.2004.03.003>.
- 450 50. Day, T. A. (1993). Relating UV-B radiation screening effectiveness of foliage to absorbing-compound
451 concentration and anatomical characteristics in a diverse group of plants. *Oecologia*, 95(4), 542-550.
452 <https://doi.org/10.1007/BF00317439>.
- 453 51. Deng, Y., & Lu, S. (2017). Biosynthesis and regulation of phenylpropanoids in plants. *Critical Reviews in*
454 *Plant Sciences*, 36(4), 257-290. <https://doi.org/10.1080/07352689.2017.1402852>.
- 455 52. Ustin, S. L., & Jacquemoud, S. (2020). How the optical properties of leaves modify the absorption and
456 scattering of energy and enhance leaf functionality. In J. Cavender-Bares, J. A. Gamon, & P. A. Townsend
457 (Eds.), *Remote Sensing of Plant Biodiversity* (pp. 349-384). Springer, Cham. [https://doi.org/10.1007/978-3-](https://doi.org/10.1007/978-3-030-33157-3_14)
458 [030-33157-3_14](https://doi.org/10.1007/978-3-030-33157-3_14).
- 459 53. Packham, J. R. (1978). *Oxalis acetosella* L. *Journal of Ecology*, 66(2), 669-693.
460 <https://doi.org/10.2307/2259158>.
- 461 54. Urbas, P., & Zobel, K. (2000). Adaptive and inevitable morphological plasticity of three herbaceous species
462 in a multi-species community: field experiment with manipulated nutrients and light. *Acta Oecologica*,
463 21(2), 139-147. [https://doi.org/https://doi.org/10.1016/S1146-609X\(00\)00115-6](https://doi.org/https://doi.org/10.1016/S1146-609X(00)00115-6).
- 464 55. Haupt, W., & Scheuerlein, R. (1990). Chloroplast movement. *Plant, Cell & Environment*, 13(7), 595-614.
465 <https://doi.org/https://doi.org/10.1111/j.1365-3040.1990.tb01078.x>.
- 466 56. Myers, D.A., Vogelmann, T.C. and Bornman, J.F. (1994), Epidermal focussing and effects on light
467 utilization in *Oxalis acetosella*. *Physiologia Plantarum*, 91: 651-656. [https://doi.org/10.1111/j.1399-](https://doi.org/10.1111/j.1399-3054.1994.tb03001.x)
468 [3054.1994.tb03001.x](https://doi.org/10.1111/j.1399-3054.1994.tb03001.x).
- 469 57. Wada, M., Kagawa, T., & Sato, Y. (2003). Chloroplast Movement. *Annual Review of Plant Biology*, 54(1),
470 455-468. <https://doi.org/10.1146/annurev.arplant.54.031902.135023>.
- 471 58. Buchner, O., Steiner, P., Andosch, A., Holzinger, A., Stegner, M., Neuner, G., & Lütz-Meindl, U. (2020). A
472 new technical approach for preparing frozen biological samples for electron microscopy. *Plant Methods*, 16,
473 48-48. <https://doi.org/10.1186/s13007-020-00586-5>.
- 474 59. Körner, C., Riedl, S., Keplinger, T., Richter, A., Wiesenbauer, J., Schweingruber, F., & Hiltbrunner, E.
475 (2019). Life at 0 °C: the biology of the alpine snowbed plant *Soldanella pusilla*. *Alpine Botany*, 129(2), 63-
476 80. <https://doi.org/10.1007/s00035-019-00220-8>.
- 477 60. Solanki, T., Aphalo, P. J., Neimane, S., Hartikainen, S. M., Pieristè, M., Shapiguzov, A., Porcar-Castell, A.,
478 Atherton, J., Heikkilä, A., & Robson, T. M. (2019). UV-screening and springtime recovery of photosynthetic
479 capacity in leaves of *Vaccinium vitis-idaea* above and below the snow pack. *Plant Physiology and*
480 *Biochemistry*, 134, 40-52. <https://doi.org/https://doi.org/10.1016/j.plaphy.2018.09.003>.
- 481 61. Starr, G., & Oberbauer, S. F. (2003). Photosynthesis of arctic evergreens under snow: implications for tundra

- 482 ecosystem carbon balance. *Ecology*, 84(6), 1415-1420. <https://doi.org/https://doi.org/10.1890/02-3154>.
- 483 62. Míguez, F., Fernández-Marín, B., Becerril, J. M., & García-Plazaola, J. I. (2015). Activation of
484 photoprotective winter photoinhibition in plants from different environments: a literature compilation and
485 meta-analysis. *Physiologia Plantarum*, 155(4), 414-423. <https://doi.org/https://doi.org/10.1111/ppl.12329>.
- 486 63. Larcher, W., Kainmüller, C., & Wagner, J. (2010). Survival types of high mountain plants under extreme
487 temperatures. *Flora - Morphology, Distribution, Functional Ecology of Plants*, 205(1), 3-18.
488 <https://doi.org/https://doi.org/10.1016/j.flora.2008.12.005>.
- 489 64. Bokhorst, S., Bjerke, J. W., Davey, M. P., Taulavuori, K., Taulavuori, E., Laine, K., Callaghan, T. V., &
490 Phoenix, G. K. (2010). Impacts of extreme winter warming events on plant physiology in a sub-Arctic heath
491 community. *Physiologia Plantarum*, 140(2), 128-140. [https://doi.org/https://doi.org/10.1111/j.1399-
492 3054.2010.01386.x](https://doi.org/https://doi.org/10.1111/j.1399-3054.2010.01386.x).
- 493 65. Ladinig, U., Hacker, J., Neuner, G., & Wagner, J. (2013). How endangered is sexual reproduction of high-
494 mountain plants by summer frosts? Frost resistance, frequency of frost events and risk assessment.
495 *Oecologia*, 171(3), 743-760. <https://doi.org/10.1007/s00442-012-2581-8>.
- 496 66. Taschler, B., Beikircher, B., & Neuner, G. (2004). Frost resistance and ice nucleation in leaves of five
497 woody timberline species measured in situ during shoot expansion. *Tree Physiology*, 24(3), 331-337.
498 <https://doi.org/10.1093/treephys/24.3.331>.
- 499 67. Fernández-Marín, B., Sáenz-Ceniceros, A., Solanki, T., Robson, T. M., & García-Plazaola, J. I. (2021).
500 Alpine forbs rely on different photoprotective strategies during spring snowmelt. *Physiologia Plantarum*,
501 172(3), 1506-1517. <https://doi.org/https://doi.org/10.1111/ppl.13342>.
- 502 68. Liu, B., Wang, X.-Y., Cao, Y., Arora, R., Zhou, H., & Xia, Y.-P. (2020). Factors affecting freezing
503 tolerance: a comparative transcriptomics study between field and artificial cold acclimations in
504 overwintering evergreens. *The Plant Journal*, 103(6), 2279-2300.
505 <https://doi.org/https://doi.org/10.1111/tpj.14899>.
- 506 69. Chabot, B. F. (1978). Environmental influences on photosynthesis and growth in *Fragaria vesca*. *The New*
507 *Phytologist*, 80(1), 87-98. <https://doi.org/10.1111/j.1469-8137.1978.tb02267.x>.
- 508 70. Jurik, T. W., & Chabot, B. F. (1986). Leaf dynamics and profitability in wild strawberries. *Oecologia*, 69(2),
509 296-304. <https://doi.org/10.1007/bf00377637>.
- 510 71. Atherton, J., Olascoaga, B., Alonso, L., & Porcar-Castell, A. (2017). Spatial variation of leaf optical
511 properties in a boreal forest is influenced by species and light environment. *Frontiers in Plant Science*,
512 8(309). <https://doi.org/10.3389/fpls.2017.00309>.
- 513 72. Hu, J., Liu, X., Liu, L., & Guan, L. (2018). Evaluating the performance of the SCOPE model in simulating
514 canopy solar-induced chlorophyll fluorescence. *Remote Sensing*, 10(2), 250.
515 <https://doi.org/10.3390/rs10020250>.
- 516 73. van der Tol, C., Verhoef, W., Timmermans, J., Verhoef, A., & Su, Z. (2009). An integrated model of soil-
517 canopy spectral radiances, photosynthesis, fluorescence, temperature and energy balance. *Biogeosciences*,
518 6(12), 3109-3129. <https://doi.org/10.5194/bg-6-3109-2009>.

519 7 Tables

520 Table 1: Leaf morphological traits from the alpine and understory species used in the experiment. Leaves of plants growing in the experimental
 521 sites at the time of measurement were used to calculate Specific Leaf Area (SLA), Leaf Area (LA) and Leaf Water Content (LWC), as the mean (± 1 standard
 522 deviation) of 20-25 leaves of understory plants, and of 6 groups of 5 leaf disks from alpine species.

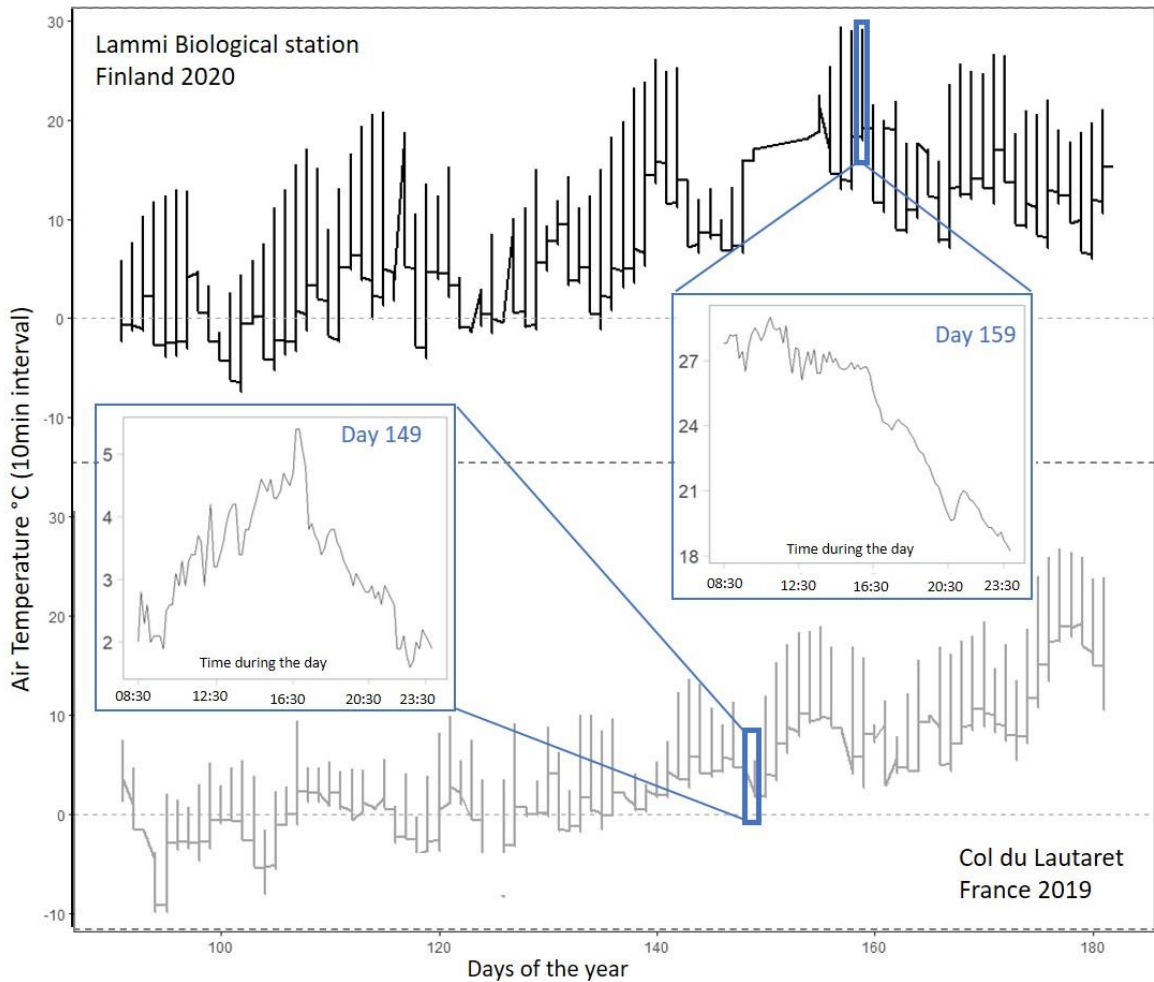
523

	Leaf Production	Leaf Anatomy	Leaf Shape	Trichomes	Specific Leaf Area (mm ² mg ⁻¹) [‡]	Leaf Area (cm ²) [‡]	Leaf Water Content (%)
Alpine Species							
<i>Centaurea uniflora</i>	Perennial	Dorsiventral	Narrow lanceolate	Dense short rough hair	15.647 \pm 1.532	2.69 \pm 0.75	76.78 \pm 1.01
<i>Gentiana acualis</i>	Annual/ perennial	Dorsiventral	Elliptical to lanceolate	Hairless	11.914 \pm 1.405	10.16 \pm 3.52	70.05 \pm 2.10
<i>Geum montanum</i>	Perennial	Dorsiventral	Pinnate	Densely Hairy	11.305 \pm 0.805	5.87 \pm 0.56	64.54 \pm 0.64
<i>Luzula nutans</i>	Perennial	Isobilateral	Narrow lanceolate	Hairy	18.752 \pm 1.375	1.76 \pm 0.17	81.07 \pm 1.36
<i>Ranunculus kuepferi</i>	Perennial	Dorsiventral	Narrow lanceolate		13.291 \pm 1.372	3.82 \pm 0.29	79.32 \pm 0.06
<i>Soldanella alpina</i>	Perennial	Dorsiventral	Kidney	Hairless	12.407 \pm 0.77	3.59 \pm 1.01	70.57 \pm 1.14
Understorey Species							
<i>Hepatica nobilis</i>	Once annually	Dorsiventral	Lobate 3 leaflets	Few hairs	25.64 \pm 4.33	9.28 \pm 3.23	71.49 \pm 0.25
<i>Oxalis acetosella</i>	Sequentially growing season	Isobilateral	Palmate trifoliate	Few hairs	81.97 \pm 13.16	5.53 \pm 1.44	82.86 \pm 2.59
<i>Fragaria vesca</i>	Dimorphic spring/autumn	Dorsiventral	Palmate 3 leaflets	Hairy	48.09 \pm 4.54	14.82 \pm 2.16	67.08 \pm 2.52

524

525 8 Figures

526 **Figure 1:** Time series of mean air temperature measured at 10 mins interval at both sampling locations; Lammi
527 Biological Station, Finland (understorey species) and the Col du Lautaret, France (alpine species). Inset plots in
528 blue boxes zoom in on the temperature on the day when plant leaves were sampled from both the locations.

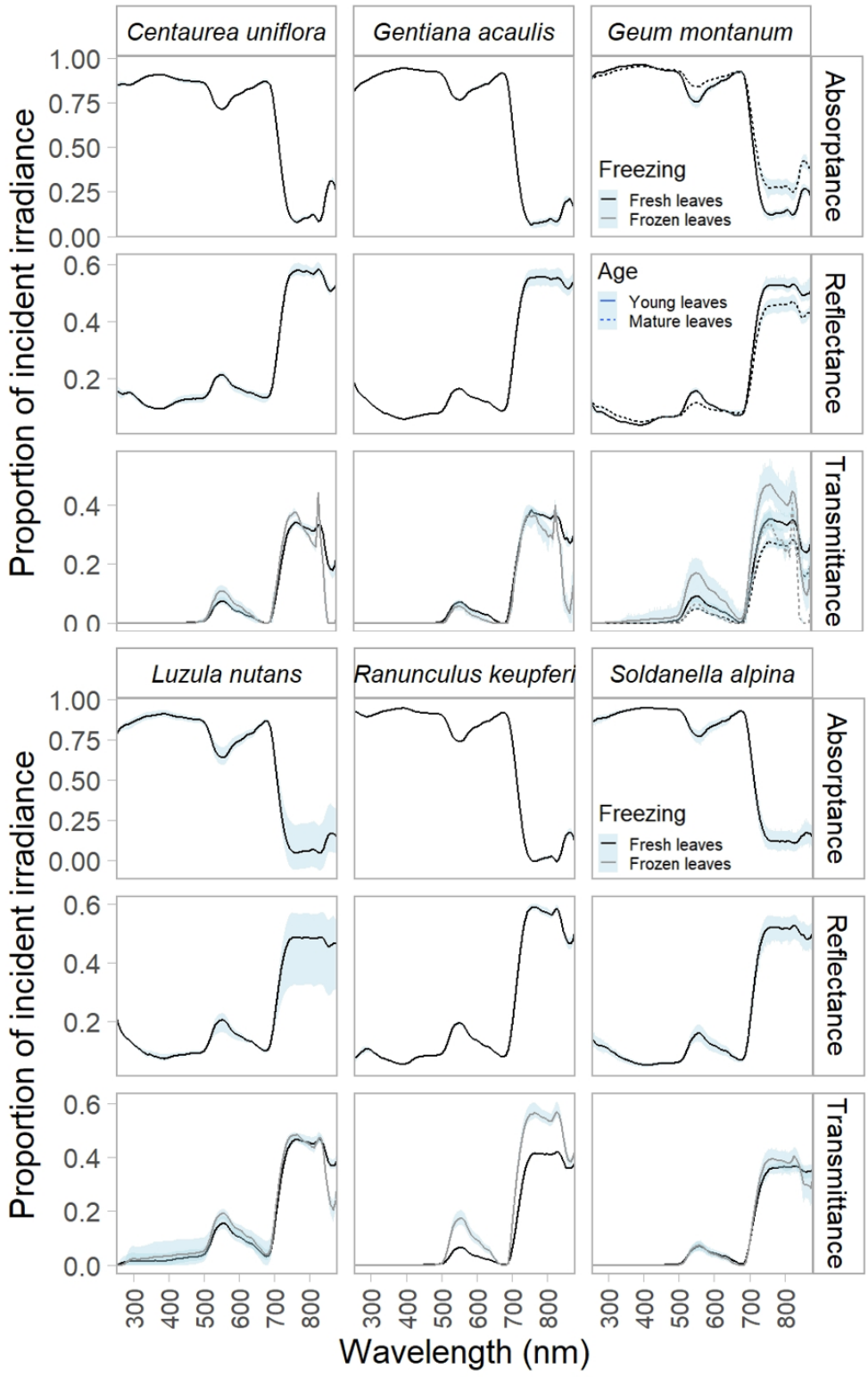


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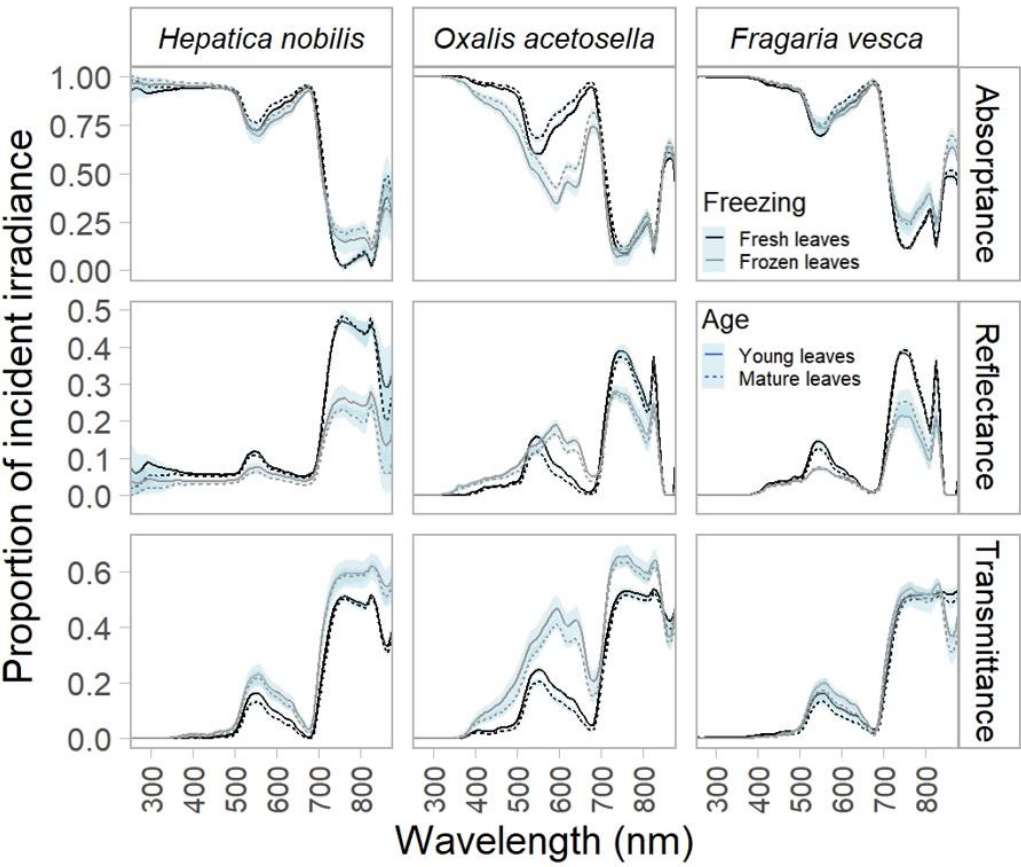
531 **Figure 2:** Measured spectral reflectance and transmittance and calculated absorptance (280-892 nm) for fresh leaves prior to freezing
532 (black line) and for frozen leaves (grey line). The blue band indicates the 95% confidence interval around each spectrum, for mature
533 leaves (solid line) and young leaves (dashed line). Where the band around spectra are non-overlapping the difference between them
534 is considered statistically significant.

535 **Figure 2a.** Alpine species: Each spectrum is the mean of three leaves, with each leaf's spectrum composed of six consecutive spectra
 536 from a single position on the leaf. Note that reflectance spectra were not recorded from frozen leaves except for *G. montanum*.



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538 **Figure 2b** Understorey species: Each spectrum is the mean spectra from four leaves collected from different locations in the
 539 understorey of the stand. Each leaf's spectrum is composed of six consecutive spectra from a single position on the leaf.



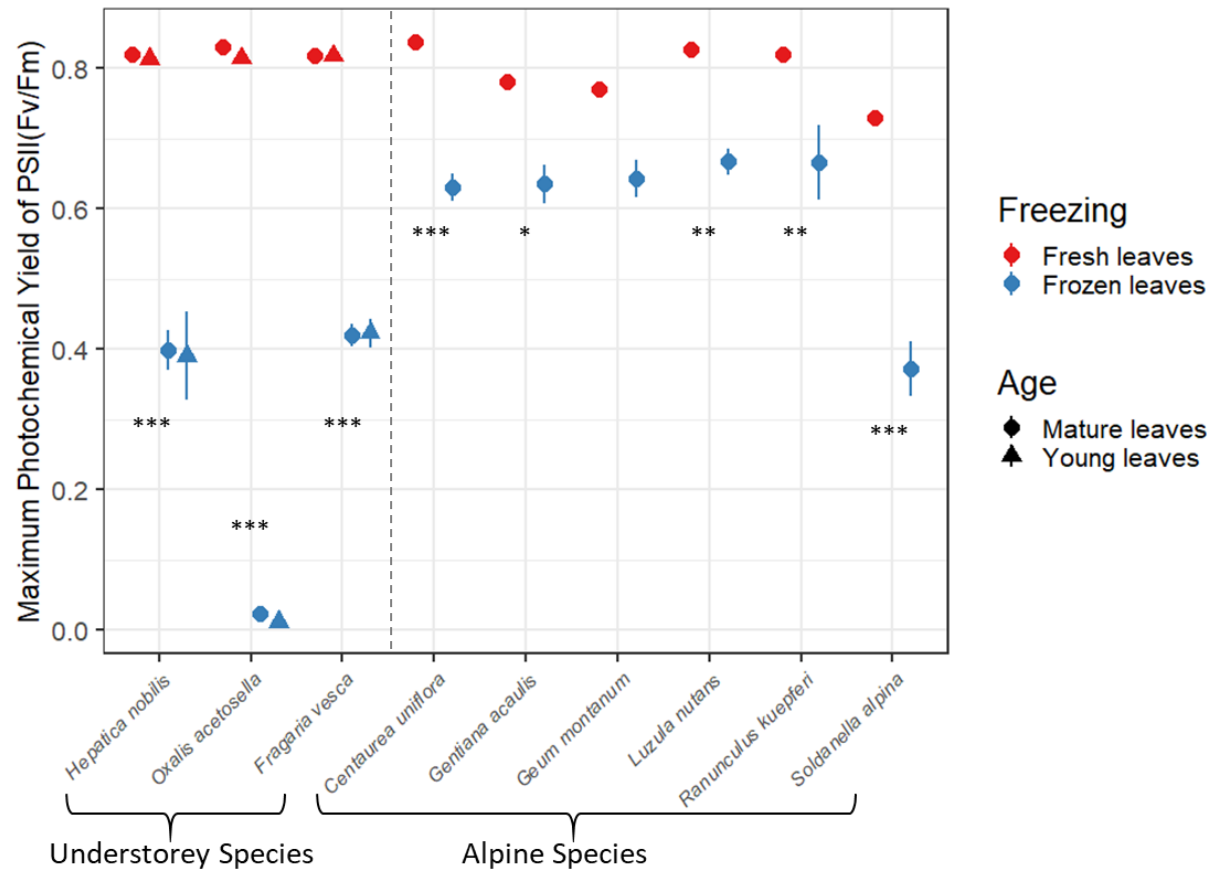
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541 **Figure 3:** Maximum quantum yield of photosystem II (PSII) photochemistry (F_v/F_m) of six alpine species for fresh leaves prior to
 542 freezing and for frozen leaves. Each point is the mean \pm 1 SE of four leaves. Significant differences between fresh and frozen
 543 leaves for each species are indicated as follow: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. *S. alpina* among alpine species, and *O.*
 544 *acetosella* among all understorey species, had significantly lower value of F_v/F_m than the rest ($p < 0.001$).

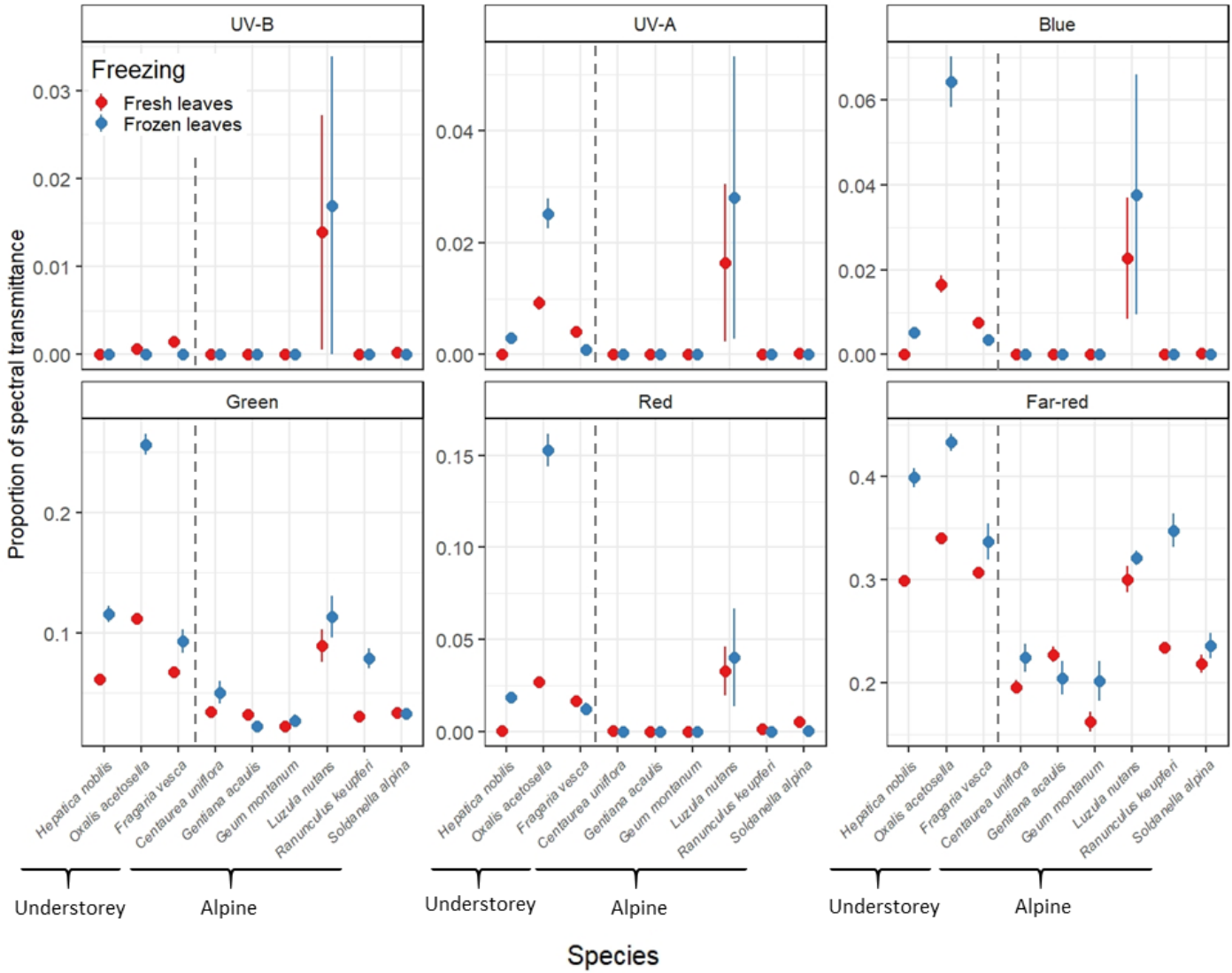
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548 **Figure 4:** Spectral reflectance, absorptance and transmittance plotted for different spectral regions: UV-B, UV-A, blue, green, red
 549 and far-red for all understorey and alpine plant species, comparing fresh leaves prior to freezing against frozen leaves. Spectral
 550 regions were calculated from the spectra plotted in Fig. 2A, B. Only mature leaves were used in this plot (Fig. 2A, B).



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553 **Figure 5:** Leaf chlorophyll (a), and epidermal
 554 flavonol (b) and anthocyanin (c) contents of fresh
 555 leaves of six alpine species (Lautaret) before
 556 freezing, and three understory forbs (Lammi) for
 557 fresh leaves prior to freezing (red points) and
 558 frozen leaves (blue points). Significant differences
 559 between fresh and frozen leaves for each species
 560 are indicated as black asterisks, and differences
 561 between mature and young leaves as blue and red
 562 asterisks, follow: * $p < 0.05$, ** $p < 0.01$, *** $p <$
 563 0.001 . Mean values with different letters are
 564 significant differences among understory species
 565 (upper case) and alpine species (lower case) Each
 566 point is the mean \pm 1 SE of 3 and 4 leaves for alpine
 567 and understory species, respectively. Where
 568 Dualex data are absent, leaves were too delicate for
 569 this measurement when frozen.

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