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ORIGINAL ARTICLE

Life at 0 °C: the biology of the alpine snowbed plant *Soldanella pusilla*

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Abstract

All plant species reach a low temperature range limit when either low temperature extremes exceed their freezing tolerance or when their metabolism becomes too restricted. In this study, we explore the ultimate thermal limit of plant tissue formation exemplified by a plant species that seemingly grows through snow. By a combination of studies in alpine snowbeds and under controlled environmental conditions, we demonstrate and quantify that the clonal herb *Soldanella pusilla* (Primulaceae) does indeed grow its entire flowering shoot at 0 °C. We show that plants resume growth under 2–3 m of snow in mid-winter, following an internal clock, with the remaining period under snow until snow melt (mostly in July) sufficient to produce a flowering shoot that is ready for pollination. When snow pack gets thin, the flowering shoot intercepts and re-radiates longwave solar radiation, so that snow and ice gently melt around the fragile shoot and the flowers emerge without any mechanical interaction. We evidence bud preformation in the previous season and enormous non-structural carbohydrate reserves in tissues (mainly below ground) in the form of soluble sugars (largely stachyose) that would support basic metabolism for more than 2 entire years under snow. However, cell-wall formation at 0 °C appears to lack unknown strengthening factors, including lignification (assessed by confocal Raman spectroscopy imaging) that require between a few hours or a day of warmth after snow melt to complete tissue strengthening. Complemented with a suite of anatomical data, the work opens a window towards understanding low temperature limits of plant growth in general, with potential relevance for winter crops and trees at the natural climatic treeline.

Keywords Anatomy · Development · Growth · Low temperature · Non-structural carbohydrates · Phenology · Tissue formation

Introduction

Plant life in cold environments must cope with both, low temperature extremes and the gradual effect of low temperature on metabolism. While extremes may prevent taxa from occurring in certain cold areas (due to insufficient frost tolerance), slow metabolism constrains growth, development and reproduction and thus diminishes long-term fitness. Here, we explore such long-term, gradual effects of low temperature on a very special alpine plant species.

Soldanella pusilla belongs to the 'classical' herbaceous snowbed flora over acidic siliceous bedrock (Salicion herbaceae) of the Alps, but it also occurs in the Carpathians, Apennines, and Balkan mountains (Zhang and Kadereit 2003). Pink flowers are emerging at snow edges and adjacent, recently snow-free ground in thousands (Fig. 1). This plant species seems to perform the unthinkable for an angiosperm: it appears to be capable of growing at 0 °C, the

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Fig. 1 *Soldanella* inflorescences emerging from late laying snow in snowbeds in midsummer

temperature of melting snow. Does it just expand existing cells as many spring geophytes do? When exactly are the flower and its stalk formed? How does *Soldanella* 'know' the right time? How can such a fragile flower bell make it through often compact snow physically? How does this plant manage to complete the seasonal life cycle, with only a few weeks left before snowed in again? What if snow does not disappear in a year?

The current theory of plant life in seasonally cold environments rests on the 'small by design' concept (Körner 2003). Alpine plants 'engineer' their microclimate by adopting short stature, and thus allow heat accumulation near the ground during the short growing season. The evidence for a favourable microenvironment in the vicinity of shoot meristems during the snow-free period is overwhelming (Scherrer and Körner 2009, 2011), with weather station data not reflecting the real life conditions of such low stature plants (Körner and Hiltbrunner 2018). While benefiting from snow cover during the coldest part of the year (insulation), a second prerequisite for life in a snow dominated world is the capability to develop extremely fast and complete tissue maturation, seed set, and reserve formation in a short period of time. The so far observed minimum duration for seasonal growth and survival in an angiosperm (*Saxifraga oppositifolia*) is in the order of 5–6 weeks if one counts days with at least 1 h above 0 °C to belong to the growing season of such cold adapted taxa (Körner 2011). However, *S. oppositifolia* is living at the outposts of higher plant life, on rocks of the nival belt up to 4500 m elevation (where reproduction is unlikely or very rare), while *Soldanella* grows and reproduces amid the lushest part of the alpine belt, yet at locations with late laying snow, leading to a short, but otherwise comparatively warm growing season.

Because of the short growing season, many alpine species preform the flower buds in the previous season (Körner 2003), and so does *Soldanella*. Yet, there is no rule for seasonal reproductive strategies, with similarly successful early as well as late flowering taxa, with a tendency for the early flowering ones to perform outbreeding and the late flowering ones allowing for a high selfing rate (Molau 1993). However, failure of both seed production and seedling establishment is very common, so that persistence and spreading has to be ensured by a clonal life strategy (Hartmann 1957; De Witte et al. 2012). Relative to the time of snow melt, *Soldanella* represents the earliest flowering species within the snowbed plant community (and we will show here, how this is achieved), and it forms dense clonal networks (Fig. 2).

Freezing damage is a summer, rather than a winter issue in alpine plants outside the tropics, because snow protects against low temperature extremes, and soils under a continuous snow cover commonly remain unfrozen in the Alps. Accordingly, snowbed taxa are the least frost tolerant alpine plant taxa, and reproductive parts are more sensitive than vegetative parts (Körner 2003; Larcher et al. 2010; Ladinig et al. 2013). In general, carbon metabolism of alpine plants shows very high rates of CO_2 uptake (Körner and Diemer 1987) and respiration is tuned in such a way that high rates of ATP production are possible at very low temperature, with Q_{10} values up to 4 (quadrupling dark respiration for a rise in temperature by 10 K, Larigauderie and Körner 1995). As a net outcome, there is commonly no carbon limitation of growth, as evidenced by unresponsiveness of seasonal above-ground biomass to experimental CO_2 enrichment (Schäppi and Körner 1996;

Fig. 2 Clonal network of *Soldanella* excavated and washed free from the top 4 cm of soil. Note the short (thick) rhizomes often carrying flower stalks and the long (thinner) stolons (note the nodes with scale leaves) contributing to spatial expansion. Roots emerge from both rhizomes and stolons

Inauen et al. 2012). A common statement that growth and productivity in cold climate plants are nutrient limited is a truism that holds for almost any type of ecosystem globally, and is certainly not specific to alpine plants including snowbed communities. Within snowbeds, there is often a sharp zonation with lower soil nutrients, lower abundance of vascular plants, lower annual productivity, but higher occurrence of mosses in the centre of the snowbed than at the edges with earlier snow release (Green and Pickering 2009).

Rated by the actual duration of the growing season, productivity in alpine vegetation is, in fact, not particularly low, but falls within the daily rates observed in other ecosystems (2.2 ± 0.9 g m^{-2} day^{-1} of above-ground net biomass accumulation until peak season for different alpine vegetation types; see Tab. 15.3 in Körner 2003). Hence, snowbed communities are clearly less productive per year, but not necessarily per day of growing season, and they represent the low end of the alpine productivity spectrum in temperate mountains (100–300 g m^{-2} a^{-1}; Billings and Bliss 1959; Körner 2003).

Soldanella pusilla and the congeneric *Soldanella alpina* belong to the Primulaceae family and evolved independently into snowbed specialist (Steffen and Kadereit 2014). The genome is packed in 36–40 (2n), relatively small chromosomes (Zhang and Kadereit 2003), and does not meet the large genome size proposed by Grime and Mowforth (1982) for cold adapted taxa. Both species occur in our test region, and are separated by bedrock chemistry. While *S. alpina* with its larger leaves and flowers is found in late snow release, but otherwise lush herb fields at slightly lower elevation on carbonate bedrock, *S. pusilla* with smaller flowers and very tiny, kidney-shaped leaves, dominates snowbed habitats at elevations between 2300 and 2650 m a.s.l. on siliceous bedrock. Surprisingly, this species also occurs outside snowbeds in late successional alpine grassland, but rarely flowers at such locations.

This paper explores the various facets of the seasonal developmental cycle of this perennial plant, its microclimate, its developmental controls (including the internal clock), its carbon relations, biomass production, dry matter allocation, tissue quality, and reproduction. The work includes field data and controlled environment data. The *Soldanella* "case" will be used to discuss the very basis of our understanding of growth and metabolism at the thermal edge of life (Chap. 14 in Körner 2003, 2006; Nagelmüller et al. 2017). Specifically, we will focus on three questions: (1) When does *Soldanella pusilla* initiate its growth? (2) How do the fragile stalks and flower bells protrude through the snow without any mechanical damage? (3) What are the specific anatomical, developmental, and physiological adjustments for long life under snow.

Materials and methods

Site conditions

The field work and plant sampling was conducted in the Swiss Central Alps at the Furka Pass at 2440–2460 m a.s.l. The snowbed sites are next to the alpine research station ALPFOR (http://www.alpfor.ch/general.shtml). An automatic weather station operates year round in the centre of the sampling area (46°34′40″N, 8°25′15″E). Snowbeds accumulate ca. 2–4 m snow during winter, and they melt free largely during July, but the first *Soldanellas* may already appear by mid June near the shallower edges of snowbeds, and the last ones as late as mid August in the centre of very deep snowbeds. Therefore, emergence may vary by 2 months. The growing season ends roughly by mid September, but late August snow falls are frequent and ground frost is regular in the later part of September. Snowing-in dates are irregular, and may be as early as beginning of October and as late as end of November. Snow may fall, and air temperatures below zero may occur on any day during the growing season. The area receives ca. 1500 mm of annual precipitation and the mean air temperatures for June, July, and August are between 5 and 11 °C, depending on year (2012–2017). During 7–9 months, *Soldanella* is under snow.

Soils in the test area are derived from silica mica-schist, granite, and gneiss, are sandy silts with soil organic carbon content declining from the edge to the centre of snowbeds, the latter often covered by a dark cryptogram crust. The upper 5 cm are organic (peat-like) and host most (>80%) of the below-ground biomass, with pH commonly between 3.5 and 3.9 (in 0.01 M $CaCl_2$). Soils pass through water saturation after snow melt, and usually remain moist for the rest of the season.

Soldanella pusilla, in the following named *Soldanella*, forms large clones, with all vegetative shoots below ground (mostly in the uppermost 10 mm), buds at the soil surface and winter-green leaves of mostly 2–5 mm diameter very close to the ground on short petioles (Fig. 2). Rated by the size of an isolated, sterile, colour mutant in the test area (ESM Fig. 1), the size of such clones may be several m^2 (patch diameter 3–4 m); hence, clones may be more than 1000 years old (given the very low, perhaps a few mm, annual length growth of stolons and irregular growth direction). Flowering stalks are between 5 and 8 cm long at flowering. Each flower produces c. 60 seeds in a ca. 1 cm long capsule, and seeds mature between end of August and end of September. *Soldanella* seeds germinate well, and seedlings can be seen regularly. Most frequent companion plant species are *Luzula alpinopilosa*, *Gnaphalium supinum, Alchemilla pentaphyllea, Sibbaldia procumbens, Salix herbacea,* and *Polytrichum sexangulare* (moss), all with foliage confined to a narrow layer of no more than 2 cm above-ground surface. Tussocks of *Carex foetida, Carex curvula,* and *Nardus stricta* can occur interspersed.

Microclimate

We documented the temperatures at two locations in three different snowbeds at the soil–snow interface in the uppermost 2 cm soil layer, where all meristems are, and where the flower buds enter winter (one-channel data loggers, Tidbit, Onset Corp., USA; logger exposed October 2015 to early July 2016). IR thermometry was employed to visualize temperatures of flowering shoots emerging through snow (IR camera, VarioCAM Infratec, Dresden, Germany). Air temperature data were taken from the automatic weather station at the test site, and an additional temperature logger (Tidbit) was buried close to the weather station at 3–4 cm soil depth (long-term records).

Plant biomass and tissue quality

Above- and below-ground biomass and tissue samples for chemical analysis were obtained at late season (early September) from four 10 × 10 cm monolith blocks of 4 cm depth from different snowbeds. All below-ground structures were washed free and sorted into thick rhizomes (>2 mm diameter) and thin rhizomes addressed as 'stolons' (1–2 mm diameter) plus fine rots (<1 mm diameter), and all above-ground parts were obtained from the same blocks. Biomass was dried at 80 °C and subsamples ground for the analysis of non-structural carbohydrates (NSC, including fructans) using the enzymatic digestion method, by Hoch et al. (2002), with additional analytical steps for fructan quantification. Specific enzymes hydrolyse each of the different NSC compounds to glucose (or other monosaccharides were converted to glucose by invertase), the concentration of which was assessed photometrically (at 340 nm, HR 7000 Hamilton, Reno, USA). All enzymes were from Sigma-Aldrich (St. Louis, USA) or from Megaenzyme International (Bray; IRE, fructanase mixture consisting of exo- and endo-fructanase). The same plant material was also used for a gas chromatography–mass-spectrometry analysis of individual low molecular weight carbohydrates that were not covered by the above NSC analysis. Briefly, plant material was extracted with hot water, de-ionized by ion exchange and the resulting neutral fraction converted to trimethylsilyl derivatives and separated and quantified by mass spectrometry (Merchant et al. 2006). An enzymatic assay and gas chromatography–mass-spectrometry analysis of oligosaccharides may not arrive at the same concentrations of individual sugars, because enzymes may digest some additional carbohydrates, which were not soluble in the water extract analysed by the GC–MS method (Quentin et al. 2015). We, therefore, kept the results of the different analyses separately.

Furthermore, ground plant material was weighed into tin capsules and analysed for C and N concentrations, as well as $^{13}C/^{12}C$ isotope ratio by a CN element analyser connected to a mass spectrometer (Delta S Finnian Mat, Bremen, Germany) at the Paul Scherrer Institute (PSI, Villigen, Switzerland).

Shoot anatomy and lignification

A central task was the monitoring of shoot development, including stem histology (lignification in particular). We dug ca. 2 m deep snow pits in locations marked by long poles in autumn to assess the flowering shoot status at least a month before local snow melt, in June 2015. These and concurrently sampled flowering shoots taken from plants that were released from snow earlier and experienced between 1 and 10 days of mid summer conditions, were stored in 70% ethanol for later light microscopy. In the light microscope, we measured cell size in longitudinal thin cuttings of flowering stalks of different developmental stages (to differentiate shoot length increments due to increased cell number or increased cell length) and stained stem cross sections with phloroglucinol dihydrate (4% w/v), and HCl (30% v/v) to assess the presence of lignin (or its precursors, both staining red by phloroglucinol). Additional ethanol samples collected in early July 2018 where examined for lignification in the lab of F. Schweingruber (WSL, Birmensdorf, Switzerland) using combined safranin–astrablue and phloroglucinol/HCl dyes, as well as polarized light.

Samples for confocal Raman spectroscopy imaging

Once initial light microscopy revealed a weak phloroglucinol response in novel stem tissue that never experienced

temperatures above 0 °C, we sampled (with the same procedure) flowering stalks for Raman spectroscopy. Raman imaging permits to scan cell walls for their chemical composition in microtome cuts of any tissue with a laser beam at <0.5 µm spatial resolution and without destroying the sample. We describe two preparation methods that were employed, because the first, with frozen samples, failed in stalks collected from under snow, which in itself is an important result.

We transferred samples directly from their ca. 1 m deep snowbed on 25 June 2016 to liquid nitrogen, without ever experiencing temperatures >0 °C, plus controls that had already experienced 1–2 days warm summer weather. These samples were than stored at −80 °C for later Raman spectroscopy. Because the deep-frozen 2016 samples collected from the snow pit fully disintegrated after placing PEG 2000 (polyethylene glycol) microtome cuts in water (unlike the 'warm' controls that did not disintegrate), we collected new samples from a snow pit and control samples in early July 2018 and stored them in 70% ethanol (thus, unfrozen), and used these to repeat the Raman spectroscopy procedure described below, except for the PEG embedding. These slightly thicker microtome cuts from snow-pit samples did not disintegrate.

Standard Raman spectroscopy sample preparation: deeply frozen stem pieces of 3–4 mm length were first embedded in Cryo-M-Bed (a PEG-2000 based embedding compound; Bright Instruments, Luton, UK) and then cut into 10 µm slices in a cryo-microtome (Cryo Star NX70, Thermo Fisher, Waltham, USA; working temperature −20 °C). Immediately before the Raman laser scans, the frozen cuts were washed free of PEG with distilled water, placed on a microscope slide in water and covered with a cover slip (Gierlinger et al. 2012). Because of the problem with the 'cold' samples mentioned above, the alternative set of samples stored in 70% ethanol right in the field, with cuts prepared on a normal microtome with no embedding, were directly placed on a microscope slide for Raman spectroscopy. The Raman signals were not affected by this alternative procedure. These ethanol samples remained at room temperature throughout the entire procedure and the samples from under snow did not disintegrate.

Raman spectroscopy images were recorded with a Raman microscope (inVia, Renishaw, Wotton under Edge, UK), equipped with a 532 nm laser, an oil immersion objective (Nikon, 100×, NA = 1.4) and a 1800 mm^{-1} grating. A step size of 300 nm was chosen in the Streamline HR mode. After measurements, the software Wire (release 4.1; Renishaw, UK) was used to remove cosmic ray signals and to correct the spectra for a common baseline. The mean spectra were calculated in Cytospec (release 2.00.01; Cytospec, Berlin, Germany), a Matlab based software, and plotted in Origin Pro 8.1 (OrginiLab, Northampton, USA).

Shoot development

To test, whether clones under snow are influenced by traces of light penetrating snow, we covered three patches of *Soldanella* in two different snowbeds with thick, non-transparent plastic tablets (six tablets of 30×50 cm) just before snowing-in in October 2015 (pairwise controls without tablets, which makes 12 test plots in total). Immediately at release from snow, plots were uncovered and developmental stage was assessed. We recorded temperatures under the tablets and on uncovered ground over the entire snow covered period using Tidbit loggers (see microclimate), but we found no difference between treatments (hence, these temperature data are not shown).

Once field observations in snow pits made it obvious that shoots do grow under deep snow (see results), we collected 24 mono-specific monoliths with *Soldanella* of 10×10×4 cm size, in the first week of October 2015, and transferred them in cooled transport boxes into 0 °C phytotron chambers within 15 h. Monoliths were placed on trays to allow watering from underneath, half of them were covered with a 2 cm-thick piece of soft foam plastic with edges loaded with light weights to seal to the irregular soil surface (gently forcing any newly developing flowering stalks to horizontal growth and ensuring complete darkness as is the case under deep under snow). The other half of the monoliths experienced very dim white LED light (<50 µmol m^{-2} s^{-1}, but such readings of LED light are not corresponding to sunlight readings). The only purpose was to provide some dim light, actually more than would ever penetrate a snow pack of >30 cm in nature (Körner 2003). These two treatments should yield a yes or no influence of traces of light on shoot development beneath snow layers. The photoperiod was 8 h in November–February, 10 h from February until March, 12 h March–April, and 14 h April–May. Temperature was monitored between adjacent monoliths by Tidbit temperature loggers, set at 1 h reading interval (see above). Thermostating was perfectly achieved at the desired 0 ± 0.5 °C, confirmed by persistent, marginal ice formation on the trays. All monolith sides were lined with a strip of plastic tape to prevent water loss from the edges. On 30 May, monoliths were transferred to 15 °C daylight growth cabinets.

Monoliths were visited about once a week between December and 20 March and thereafter, on 15 April and 30 May. The inspection in dim light took 1–2 min per monolith and week, that is the maximum length that the 'dark' monoliths had to be uncovered, hence were not under complete darkness. The assessment started by looking for swelling of flowering buds (size <2 mm at the beginning of the census) and once they started to grow and emerged from the thin debris layer, total shoot length was measured with a pointed ruler. These measurements were always conducted at the end of the daily phytotron light period.

Pollination and fruiting

Twelve clusters of emerging flowering shoots spread over two snow fields were cached under mesh just before snow release, to test the ability to self-pollinate (pollinator exclusion). Fruits were inspected later in the season for pollination success. In addition, smart-phone movies of pollinator visits were recorded in flower populations 2–3 days after snow melt. The length of the fruiting shoots was measured and compared to those of just emerging flowering shoots (potential additional length growth during the fruiting period).

Results

Microclimate

Our 3–4 cm soil temperature data from six snowbeds confirmed the long-term records of the nearby weather station that soils never freeze under >1 m deep snow at that elevation (Fig. 3). Yet, 2015 was an exception, because snow depth at the weather station (on a small ridge) was less and snowing-in occurred very late, so that the reference soil temperature collected at the weather station was colder than usual and showed some soil freezing that was not seen in other years and in the concurrent readings in the nearby snowbeds. Once covered by snow, *Soldanella* was perfectly thermostated at 0 °C exactly at the interface between snow and soil, with the warmth of deep soil feeding a continuous, very slow melting process that physically prevents the shoot temperature of *Soldanella* to depart from zero (Fig. 3). Therefore, whenever shoot development might occur, it occurs in a 7–9 month zero-degree world. Manual temperature readings in 2 cm soil depth within 50 cm of melting snow edges revealed that temperatures can rise to 28 °C on bright days (ESM Fig. 2). Hence, the rhizome system passes from 0 °C to warm, almost tropical summer conditions within a few hours. Temperature data for the entire year evidence that once snow has melted, there is no difference in temperature between snowbed and reference grassland sites. So, snowbed plants have a shorter season, but compared for the same period of time after snow melt,

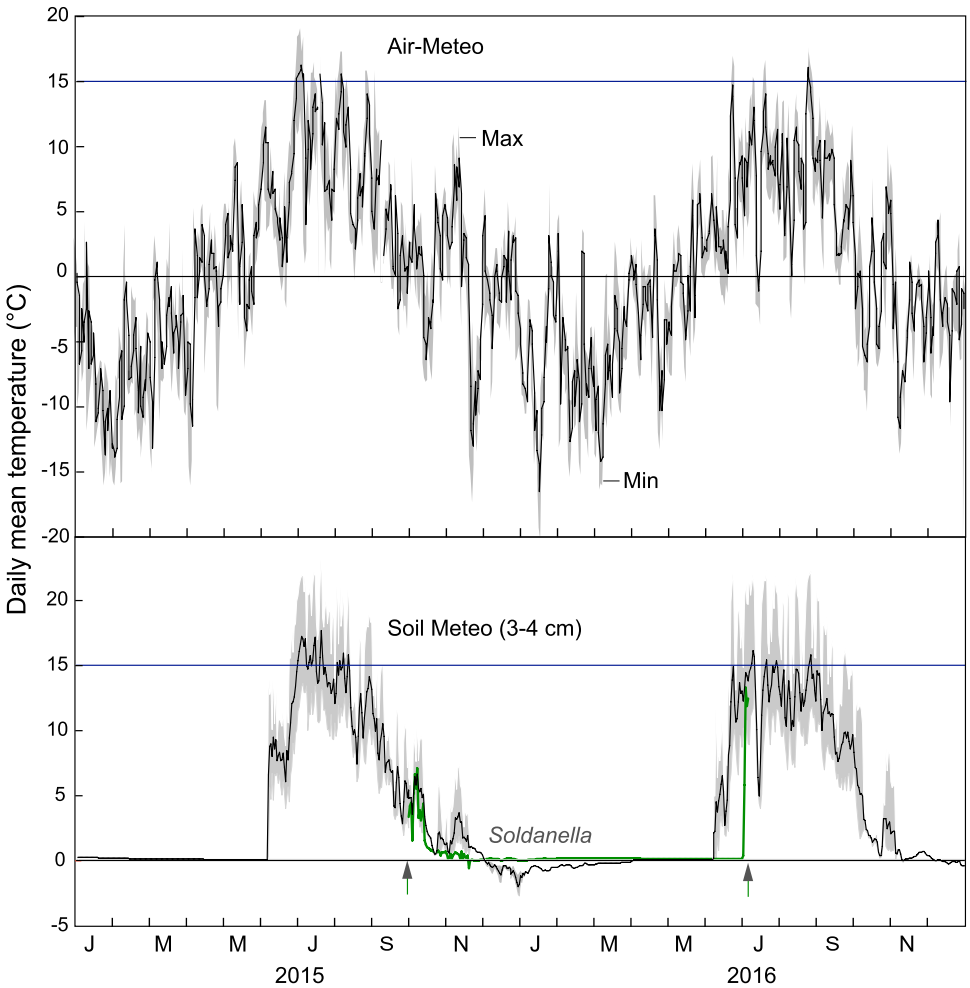

Fig. 3 Temperature regime in the *Soldanella* environment at 2440 m a.s.l. Air temperature from the nearby ALPFOR meteorological station which is located at a ridge, where snow depth is often diminished by strong wind (daily means, minima and maxima for 2015–2016). Concurrent soil temperature at this station at 3–4 cm depth indicates periodic top soil freezing during the 2015/2016 winter. In contrast, no ground freezing was observed in the nearby *Soldanella* snowbeds except before snow arrived (very late in 2015; green line)

their thermal life conditions are similar to nearby non-snowbed plants of similar exposure.

Development of flowering stalks in the field

Soldanella enters winter with no visible above-ground shoots. However, it is possible to detect ca. 1 mm size flower buds on a 1–2 mm stem 'socket' under a binocular (Fig. 4) in carefully washed clonal systems late in the season. In fact, we found the first buds for the next year's season already by 20 July. On the other hand, stalk and flower development in the field is clearly close to completion by the time snow melts in July of the subsequent year. We discovered fully developed flowering stalks, with fully developed flower bells flattened under a thin sheet of ground ice, 2 m under snow by end of June (Fig. 5). There is no doubt that these shoots have been formed before any measurable traces of light could penetrate the thinning, but still substantial snow pack (35 cm of snow produce full darkness, Fig. 5.1 in Körner 2003).

Shortly before release from snow in mid July, flowering stalks had reached a length of 40 mm, expanded to 50 mm immediately after snow melt, and added another 10 mm to a mean length of 60 mm length during the late flowering to fruiting stage, a size indifferent from that of the previous season (dead) stalks (Fig. 6). Since biomass changed in proportion with length, the mass/length ratio (ca. 1.30 mg d.m. cm^{-1}) hardly differed among these three developmental stages, confirming that the expansion of the flowering stalks was not just cell size expansion (water uptake), but dry matter increment. Stalks grown for several months under snow had actually the highest mass/length ratio (1.55 mg cm^{-1}), further underlining the occurrence of a net dry matter investment (cell production) during that zero-degree period rather than just expanding preformed tissues, as is often the case in geophytes in spring (cell expansion by water). Stalks fully darkened in situ by non-transparent plastic tablets over the entire winter arrived at the same stalk size at snow melt.

How can these flattened stalks become upright as the melting front approaches them? From in situ observations, it seems the mechanism includes four steps: (1) as the stalks become immersed in down-seeping melt water, their immediately surrounding snow softens. (2) The reddish horizontal stalk (see the dark trichomes/glands in Fig. 4) and the also dark inflorescence intercept transmitted solar radiation, once snow depth is <15 cm, and the re-radiated long-wave radiation creates a melting zone around the stalk and the flower, yielding space for the next step. (3) The stalk has two regions that are spring-loaded, one at the stalk's base producing a ca. 45° angle upward after being released, and the second one, just below the flower bell, adding another 45° angle, once released, again causing the shoot to bend upward (Fig. 5). At that point, (4) the dark and pointed top of the bell receives even more radiation and melts a tunnel through the snow (Fig. 1). Therefore, flowers emerge through snow without any mechanical force, purely driven

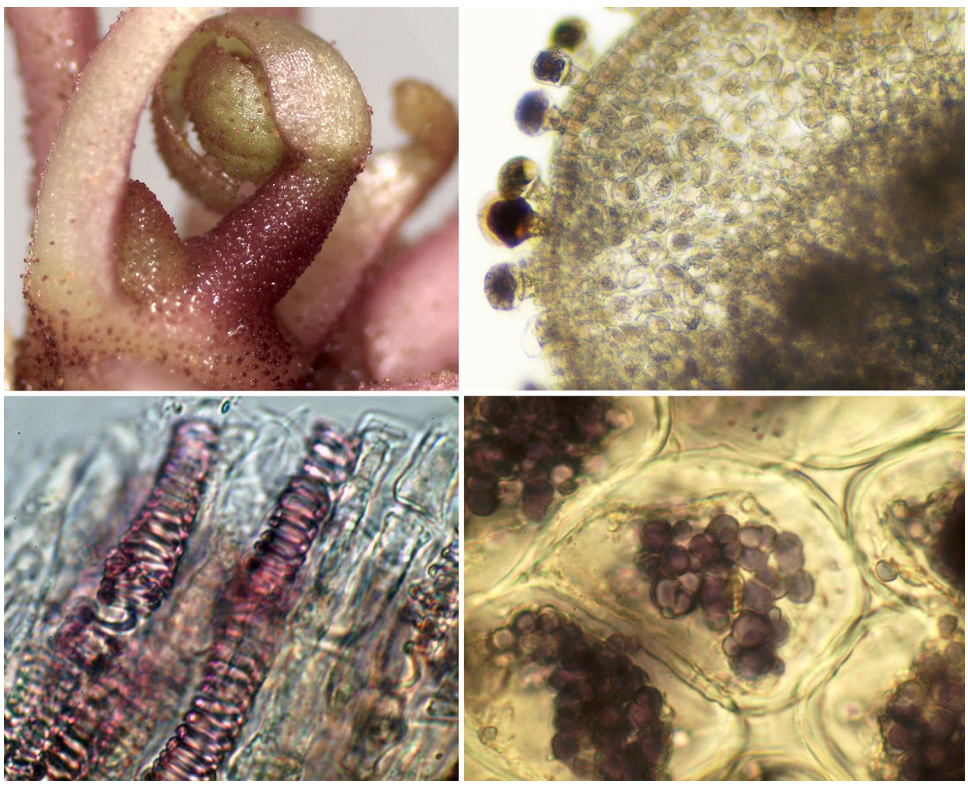

Fig. 4 Primordial flowering bud underneath the protective bract, dark glands at the surface of the bud's socket, fully lignified spiral vessels (phloroglucinol staining) and storage parenchyma packed with starch grains (potassium–iodine staining) in the cortex late in the growing season (early September)

Fig. 5 Flowering shoots of *Soldanella* underneath a thin ice crust at the bottom of a 2 m snow pack, excavated close to the centre of a snow bed on 19 June, 2015. Note the upward bending immediately after melting, and compare this with the melting slots seen in the bottom image of Fig. 1

by absorbing and releasing long-wave radiation (Fig. 7). As a consequence, as the melting snow surface is lowering, stalks are positioned in a little tunnel and emerge to sunlight in a vertical position (Fig. 1).

Development of flowering stalks in the phytotron

Sods with *Soldanella* plants maintained at 0 °C in the phytotron permitted tracking the shoot development under simulated winter conditions. Flower buds remain inactive until end of December with no visible traces of swelling. In the second half of January buds rapidly swelled to twice their autumn size became easier to spot among the moss and debris ground layer, and stalks started to lengthen. Similar to the zero-effect of in situ darkening, there was no difference between entirely darkened plants and plants that experienced 8 h of very dim LED light. Therefore, dim light at <50 µmol m^{-2} s^{-1} had no effect, and will be disregarded in the following (results from all sods pooled). Therefore, the onset of shoot growth under snow is clearly not light driven.

It took about 6 weeks for the entire cohort of a year's flowering shoots to emerge (became visible by eye, that is, became longer than 2 mm; Fig. 8). This partially delayed stalk development scales to stalk and inflorescence size variation by the time of snow melt. The stalk length variation shown in Fig. 8 resulted largely from the variation in the onset of stalk growth. The rate of length growth was 0.1 mm day^{-1} in January, ca. 0.2 mm day^{-1} in late February, 0.3 mm day^{-1} by end of March and arriving at ca. 0.4 mm day^{-1} between April and May (a mean of 0.33 mm day^{-1} over the entire ca. 150 day period between first signs of growth and release from the 0 °C environment on 30 May (Fig. 8). By that time, shoots reached 44 mm length and flowers arrived at near to final size. Abruptly exposed to 15 °C air temperature, as occurs at snow melt, stalks reached 64 mm after 10 days (the expansion may have occurred immediately after transfer to warm conditions). The flowers developed more or less proportionally to the stalks, so that a 10 mm stalk exhibited a 2–3 mm flower bud, a 40 mm stalk a flower of 8–12 mm length. By the time, the stalk reaches 12–15 mm (mid March), the calyx slightly opens, and first parts of the pink corolla appear (Fig. 9). Until that stage, the linear bract (or phaeophyll) exhibits a sort of 'helmet' function that protects the bud, while the stalk keeps lengthening. The data obtained during the phytotron experiment thus match the field observations, namely, that shoots grow for several months in full darkness under snow and reach almost full size at snow melt.

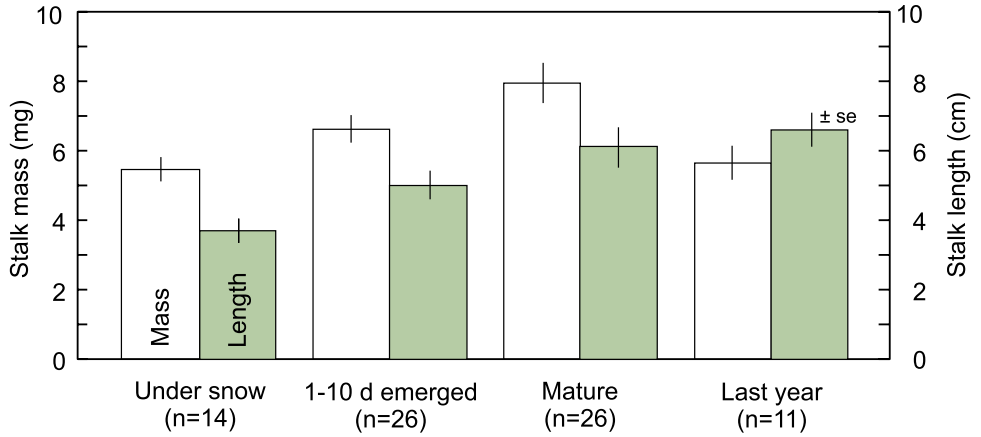

Fig. 6 Length and biomass of flowering stalks of *Soldanella* harvested mid July, 2013, across a developmental chronosequence from under the last snow pack, during the first 10 days after release from snow, and at fruiting stage several weeks after emergence. Dead, still standing stalks of last year are also included. Live samples were collected in groups of 3–6 stalks across 15 locations of increasing distance to the snow edge, lumped into 3 categories only, because there were no consistent differences among the cohorts released from snow by days 1–10

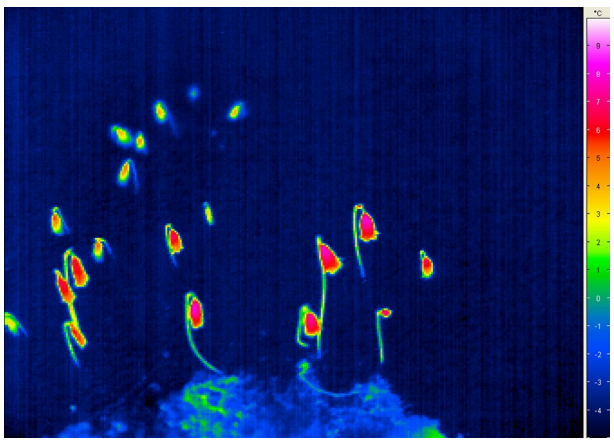

Fig. 7 Thermal image of emerging *Soldanella* shoots during the final phase of snow melt at the edge of a late snow-field in midsummer. Note the heat collecting bells and compare with Fig. 1

Stalk anatomy

As flowering stalks grow under snow, all types of stalk tissues are present from very early stages on. Fully lignified xylem and a lignified sclerenchyma ring were observed in the 1 mm long socket of preformed buds by the beginning of September (Fig. 4). When this socket had grown into a 15 mm long flowering stalk by mid March, vessels measured 8–12 µm in diameter and the spiral wall thickening stained for lignin with phloroglucinol/HCl (Fig. 4; ESM Fig. 3). In the epidermis of the stalk, cell size (length) at the stalk base increased a lot while being still under snow, but hardly after snow melt (Table 1). In addition, most of the cell enlargement in the middle part of the stalk occurred before release from snow, but another, smaller increment by ca. 14% occurred immediately after snow melt, with no further increase later on. Cell enlargement in the upper part of stalks is small under snow, shows no response to snow release, but these cells stretch in the later flowering phase after successful pollination.

As the entire stalk length triples, cell length rises by only ca. 40% (base) or ca. 60% (middle part) with most of this enlargement occurring still under snow). Final cell length is reached earliest (long before snow melt) at the base of stalks, shortly after snow melt in the middle part, and latest in the small fraction of uppermost stalk tissue (with generally smaller cells). This means that cell enlargement alone is insufficient to explain the stalk size increment both, under snow, as well as after release from snow. After release from snow, most of the cell expansion during the first day occurs in the middle part of the stalk. Cells are always largest in the middle part, shorter at the base and shortest at the top. Given that stalk diameter did not significantly differ across these developmental stages (Table 1), we assume that cell diameter also did not change.

Lignification

As mentioned above, lignin or lignin precursors were identified in the xylem by phloroglucinol/HCl staining, from the earliest stage of stalk development on (Fig. 4; ESM Fig. 3). Given the later length increment of cells in the epidermal tissue, this raises the question if and how lignified xylem conduits could have co-expanded. Presumably, this phenomenon is not restricted to *Soldanella,* but possibly has been solved in expanding shoots of many taxa. A preliminary count of the number of individual spiral loops in vessels per unit of

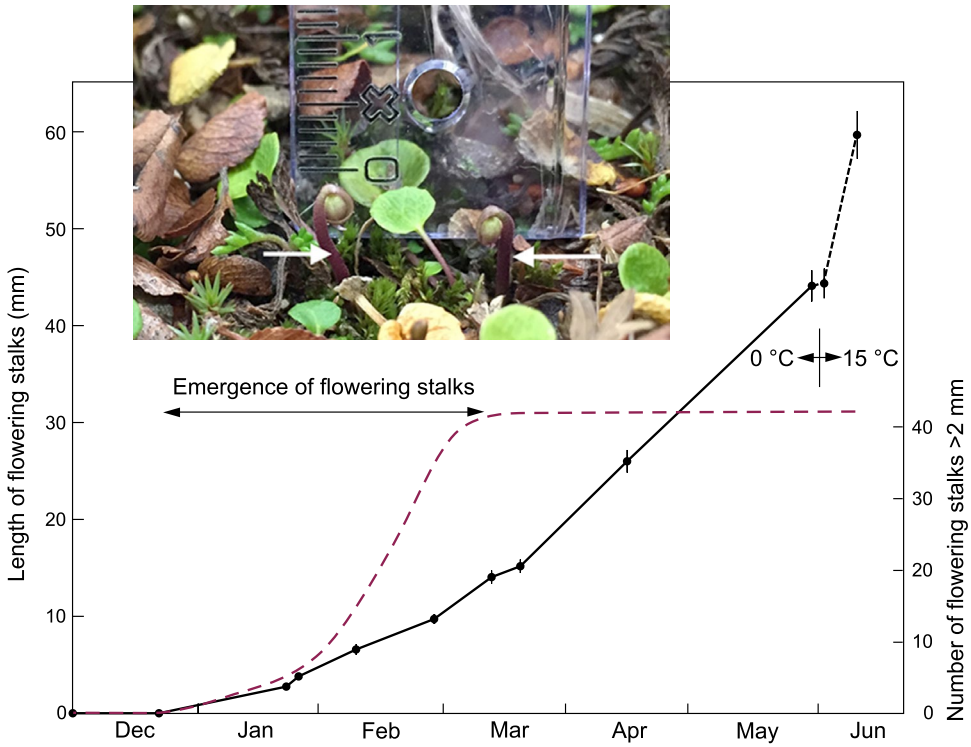

Fig. 8 Dynamics of flowering stalk emergence (the number of buds that exceeded 2 mm size, that is, became visible) and the mean length of all stalks visible at census time for what ended up as a total of 42 flowering shoots that developed under 0 °C in a phytotron. The inset shows two emerging inflorescences on 13 March

Fig. 9 Buds develop as the stalks lengthen, while still deep under snow. Left 13 March, right 17 March (not the same bud). Photographs taken during the 0 °C phytotron experiment

vessel length (ESM Fig. 3) indicated 34 spirals per 100 µm in the middle part of the stalks in both, 20 mm long stalks collected under snow and >60 mm long stalks that experienced several days of warm summer weather (3 stalks each). This suggests that no spiral stretching had occurred, an issue deserving further exploration with more replicates.

We compared the intensity of xylem staining by safranin and phloroglucinol in longitudinal hand cuttings of stalks by a visual 0–3 scoring (0 no staining and 3 most intense) in three types of stalk tissue: (1) small, clearly premature shoots dug out from deep under snow and (2) stalks from their first day after emergence from snow, and type (3), 3–5 days after snow melt in warm summer weather. Type 3 stalks revealed staining intensity 2–3 in xylem with both dyes, with safranin always a bit weaker (consistently 2) than

Table 1 Stalk size and cell size (microscopy) in four developmental stages of *Soldanella*

Developmental stage	Stalk size	Stalk size	Cell size[a]		
			Base	Middle	Top
	Length (mm)	Diameter (mm)	Length (µm)	Length (µm)	Length (µm)
Under snow, stage short	20 ± 4 (3)	1.2 ± 0.3 (3)	65 ± 16 (3)	90 ± 26 (3)	30 ± 9 (3)
Under snow, stage long	56 ± 14 (5)	1.3 ± 0.1 (5)	113 ± 16 (3)	133 ± 1 (2)	35 ± 4 (2)
Emerged <24 h	69 ± 10 (5)	1.2 ± 0.2 (5)	118 ± 37 (3)	158 ± 17 (2)	37 ± 9 (3)
Mature >3 days	63 ± 14 (10)	1.2 ± 0.1 (10)	100 ± 34 (4)	149 ± 42 (4)	61 ± 9 (5)

Two stages under deep snow (temperature never >0 °C), immediately after snow melt, and several days after snow melt (mean ± SE; n = number of stalks). Samples collected 5 July, 2013

[a]Cell size—base: the lowest 3 mm of the stem, top: the last 3–5 mm before the bract, middle: the "bulk" of the stalk between these extremes. 'Short' and 'long' under snow refers to stalks that arrived at different sizes by the time of excavation (see length data)

phloroglucinol (2–3, mostly 3) and type 2 stalks did not differ from type 3 stalks. However, type 1 stalks showed less pronounced xylem staining, with safranin revealing mostly 1, and phloroglucinol mostly 1–2. Within a given type 1 stalk, maximum staining occurred in cells at the stalk base with both dyes, and clearly less in the middle part of the stem. Therefore, based on these estimates, 0 °C stalks exhibited less lignified xylem and there was an abrupt change on day one after snow melt. An important caveat is that these classical histological dyes may also stain phenolic precursors of fully polymerization 'polyphenolic' lignin; hence, the weaker lignification we found in the xylem of samples excavated from deep snow might in fact have been even weaker if these dyes would stain fully polymerized forms of lignin only. The PEG embedded cross sections did not show a phloroglucinol response for lignin ('warm' stalks), which is due to PEG (Ferreira et al. 2017).

To explore this semi-quantitatively, we examined the same type of flowering stalks (type 1 versus type 3) by confocal Raman microscopy. As mentioned in methods, flowering stalks sampled under deep snow and freeze-stored in PEG-2000 disintegrated as soon as PEG was washed away from samples (ESM Fig. 4). In contrast, slices from shoots that experienced warm weather remained intact and could be analysed. The second cohort of samples collected in 2018 and stored in ethanol until cutting with a microtome at room temperature (without PEG embedding), permitted Raman scans also from 'cold' samples that never experienced a temperature >0 °C before sampling. These samples did not disintegrate. Raman images of the xylem vessel walls revealed perfect lignification as know from wood, irrespective of whether stems came from under deep snow or had experienced a few warm days (ESM Fig. 5). In contrast to the xylem (in essence the few vessels), the sclerenchyma ring embedded in the inner cortex did not stain for lignin with any dyes, nor did the Raman scans reveal cell-wall lignification when stems came from under snow, except for the cell corners (Fig. 10). The sclerenchyma walls in 'warm' stems were fully lignified. Therefore, this most prominent and mechanically most significant structure emerges from snow largely non-lignified, but becomes lignified rapidly once out of snow and experiencing warm summer weather (ESM Fig. 5). While the walls of vessels from 'cold' stems appeared less lignified based on dyes, the Raman signal did not reflect such a difference. It was very difficult to cut the fragile 'cold' stalks without embedding. Viewed in polarized light, the microtome cuttings of such unembedded cross sections (ethanol samples) clearly underpin the absence of lignin in the sclerenchyma ring of 'cold' stalks (Fig. 11).

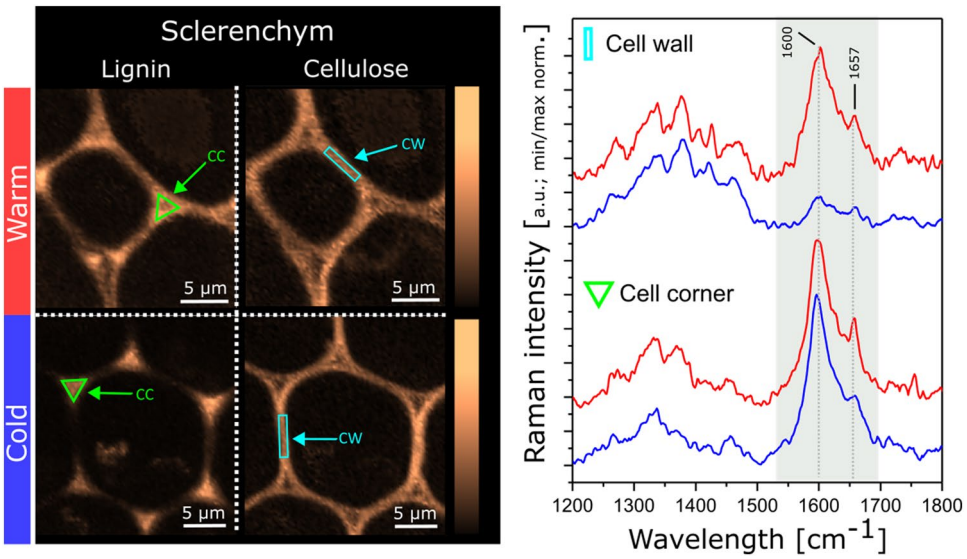

Fig. 10 Confocal Raman microscopy images of cross sections of the sclerenchyma ring in flowering stalks of *Soldanella* based on the two lignin marker bands at 1600 cm^{-1} and 1657 cm^{-1}, which are attributed to aromatic C=C vibrations in lignin and C=O vibrations of coniferylaldehyde and C=C coniferylalcohol, respectively. Samples were either collected from deep under snow in late spring ('cold' = blue line) or after 1–2 days of warm summer weather ('warm' = red line). Note the lack of lignification of the cell wall in the 'cold' samples except for the cell corners. The mean Raman spectra are shown in relative units. There were no differences in the cellulose specific bands (not shown), but clear differences in the lignin specific bands. For the sake of clearness, the curves for cell corners and cell walls were stacked, thus avoiding overlap

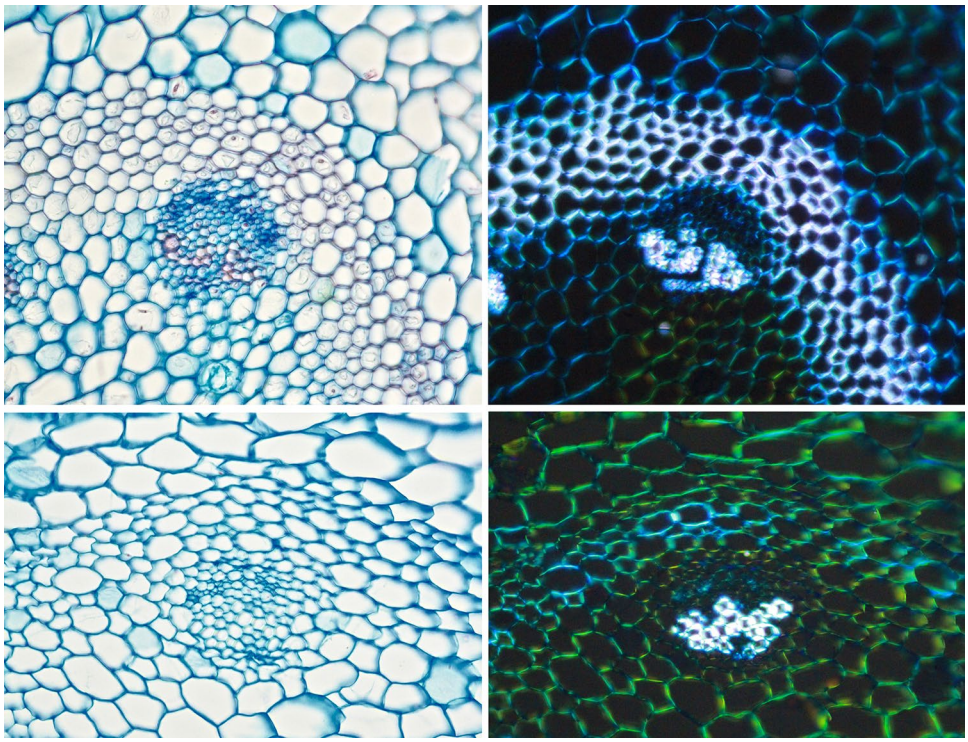

Fig. 11 Microscopic cross sections of flowering stalks of *Soldanella* either excavated from snow in late spring ('cold'; bottom) or exposed to 1–2 days summer weather immediately after release from snow ('warm'; top). Left stained with safranin–astrablue, right in polarized light to visualize lignin (×400). Note the near to complete lack of lignin in the 'cold' sclerenchyma, while the 'cold' vessels show lignification in polarized light, not obvious in the dye treatment

Table 2 Biomass of *Soldanella* clones harvested in nearly mono-specific stands in the centre of snowbeds at peak season, 30 July, 2015; all mean ± SE in g m^{-2} d.m., $n = 4$ stands

Flowers and stalks	Leaves and petioles	Above-ground biomass	Below-ground biomass (4 cm depth)	Total biomass *Soldanella*	Total phytomass *Polytrichum*
24 ± 4	92 ± 13	116 ± 14 31%	269 ± 79 69%	385 ± 68 100%	522 ± 155

Polytrichum sexangulare moss is the only other photosynthetically active component, but live and dead parts cannot be separated; hence, the term phytomass includes necromass

Biomass per unit land area and biomass allocation

When *Soldanella* clones dominate ground cover, the mono-specific above-ground biomass (live tissue) reaches ca. 120 g m^{-2}, with more than twice as much, that is ca. 270 g m^{-2} that accumulated below ground in the top 4 cm of the soil profile (Table 2). There is very little biomass deeper in the soil. While the above-ground part reflects biomass built during ca. 1.5 seasons (the fraction of overwintering leaves may be 30–50%, not quantified), the below-ground fraction is of unknown age, but short, thick rhizomes (Fig. 2) represent one-third to one half of the below-ground fraction and may be several years old. Stolons, and maybe also roots, may become >2 years as well. If we assume a 1:1 proportionality of new above- and new below-ground production per season (ca. 100 g m^{-2} in both compartments), the annual total net primary production may be in the order of 200 g m^{-2} or 2 t ha^{-1}, half of which above- and below ground. The fraction of *Polytrichum sexangulare* mosses that fill all niches in such pure *Soldanella* stands arrives at 500 g m^{-2} (or 5 t ha^{-1}). However, this moss biomass includes dead attached scale leaves of 2–3 previous seasons and the vertical stem, connected to a clonal system, embedded in raw humus, may also represent several-year-old tissue, so that the annual new growth may at most match that of *Soldanella* (thus, doubling total NPP).

Carbohydrate storage

Soldanella is a 'sugar' plant, with quite extraordinary concentrations, presumably co-related to the fact that tissues have very low structural dry matter density (not measured). Dry matter of leaves contains around 19% of soluble sugars in mid season, and leaves enter winter with around 40% sugar (Fig. 12). Rhizomes and roots contain ca. 26% sugars in summer and enter winter with ca. 41%, similar to leaves. After 5 months at 0 °C, leaves came down to 28% and rhizomes and roots to 36%.

The contributions of higher molecular weight carbohydrates to the total non-structural carbohydrate (NSC) concentration, that is, largely by starch and fructan is comparatively small, around 4% and 2%, respectively, in mid summer foliage, and 6% starch and still ca. 2% fructan at the beginning of winter (Fig. 12). Rhizomes and roots store 8% starch

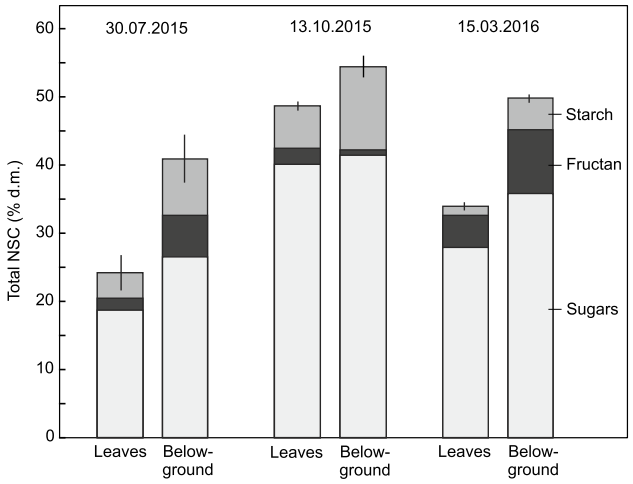

Fig. 12 Non-structural carbohydrate concentrations (% d.m; mean ± SE) in *Soldanella* at peak season (30 July), end of season/onset of winter (13 October) and in mid winter (15 March) after 5 months simulation of snowbed conditions with monoliths in a 0 °C phytotron chamber. In the first two cases, rhizomes and roots were pooled, for the last date, rhizomes and roots were analysed separately and values averaged for the sake of comparison (assuming a 1:1 contribution of roots and rhizomes to below-ground biomass)

and 6% fructan in summer, and 12% starch and 1% fructan at the onset of winter. Five months of life at 0 °C brings these osmotically inactive (or less active, in the case of small fructans) forms of NSC to 1% (starch) and 5% (fructan) in leaves, and to 5% starch and 9% fructan in rhizomes and roots. Hence, during the long 0 °C life (no freezing), there is a small relative shift to slightly higher fructan concentrations, but lower starch concentration.

In leaves, total NSC varies from 24% in summer to 49% at the beginning of winter, with the corresponding concentrations in rhizomes and roots of 41% and 54%. After 5 months at 0 °C, foliage contains 33% NSC, and rhizomes and roots 45%, in other words, metabolism and growth during that long cold period consumed about one-third of the reserves of leaves and a smaller fraction (a drop by 9%) of below-ground stores. Given the leaf-to-below-ground tissue ratio of 1:3 (ca. 90 versus 270 g m^{-2} d.m.; Table 2), the corresponding NSC pool of *Soldanella* at the onset of winter is 44 g m^{-2} NSC above ground (some leaves may have been senescent, only a part of foliage is overwintering green) and ca. 146 g m^{-2} below ground. After 5 months at 0 °C these pools drop to ca. 30 g m^{-2} in foliage to 122 g m^{-2} in below-ground tissues that is from 190 to 152 g m^{-2} (1.9–1.5 t NSC ha^{-1}), a draw-down by 38 g m^{-2} for maintaining autotrophic respiration and minor growth activities such as flowering stalk development.

The detailed chromatographic analysis of the soluble sugar fractions revealed that glucose, fructose, and raffinose play a minor role, with the bulk of soluble sugars belonging to sucrose and stachyose, the latter responsible for ca. half of all soluble sugars (Table 3). While glucose and raffinose are rare in all organs, fructose makes a small but significant contribution to leaf sugars only. Sucrose is more abundant in leaves, while stachyose is by far the most prominent below-ground sugar, reaching peak concentrations in rhizomes and coarse roots. The decline in leaf sugars between the late autumn peak and mid March is largely driven by a halving of stachyose, a loss in sucrose by one-third and a gain in fructose. The over-winter changes in root sugars are a halving of fructose and glucose, a doubling in raffinose (all three contributing little to the overall pool), and a halving in sucrose plus a 25% loss in stachyose, the two major storage sugars. Unfortunately, rhizomes were not analysed separately in October (only bulk below-ground biomass). The separate analysis of belowground organs (in March only) indicates a much higher stachyose concentration (nearly twofold) in the rhizome than in the roots.

Stable isotopes and N concentration

We report here the stable isotope signals for *S. pusilla* leaves from our test region that had been obtained as part of parallel works, but were not explicitly presented in the related publications or are still unpublished (Yang et al. 2015;

Table 3 Concentration of different soluble sugars in *Soldanella* organs at the end of the growing season and after 5 months of darkness at 0 °C (% d.m., mean ± SE)

Collecting date	Organ	Fructose	Glucose	Sucrose	Raffinose	Stachyose	Total	Change % Total
13th Oct 2015	Leaves	0.67 ± 0.06	0.33 ± 0.03	15.82 ± 0.84	0.68 ± 0.05	18.30 ± 0.20	35.81 ± 0.88	
15th March 2016	Leaves	3.19 ± 0.52	0.45 ± 0.08	9.55 ± 0.52	1.20 ± 0.23	8.77 ± 0.38	23.16 ± 0.88	−35.3
13th Oct 2015	Roots/rhizome	1.18 ± 0.12	0.50 ± 0.04	12.05 ± 0.76	0.61 ± 0.07	16.26 ± 0.71	30.60 ± 1.37	
15th March 2016	Roots/rhizome	0.53 ± 0.06	0.18 ± 0.03	6.67 ± 0.27	1.14 ± 0.10	17.16 ± 0.65	25.68 ± 0.65	−16.1
15th March 2016	Rhizome[a]	0.57 ± 0.06	0.18 ± 0.03	7.07 ± 0.37	0.90 ± 0.07	22.80 ± 0.86	31.58 ± 0.84	n.a.
15th March 2016	Roots[a]	0.50 ± 0.06	0.18 ± 0.03	6.27 ± 0.24	1.32 ± 0.14	11.52 ± 0.90	19.78 ± 0.97	n.a.

[a]Analysed separately

Körner et al. 2016; E. Hiltbrunner unpublished data). With $\delta^{13}C$ −27.2‰ in replicated leaf samples from the snowbeds studied here, and a mean of 27.0‰ for ten locations of *Soldanella* occurrences in late melt-out *Carex curvula* grassland of the same region, the signals point at the absence of water shortage (for the given elevation). The mean for $\delta^{15}N$ of −4.2‰ is also very robust across a wide spectrum of habitats (from snowbed to grassland) and also matches a signal of −4.7‰ obtained from a herbarium voucher from the test area (Körner et al. 2016). Highly replicated peak season, current year leaf N concentration is around 2.4% d.m. for *Soldanella* and foliar C concentration is 46–47% d.m.

Reproductive system

Mesh bags placed around groups of flowers before release from snow revealed that *Soldanella* is a 100% out-breeder (matching the prediction by Molau 1993). All fruits in bagged plants remained empty (ESM Fig. 6). Successfully pollinated flowers produce capsules that commonly mature in late September, this is 10–12 weeks after release from snow. Seedlings can be found, so there are viable seeds despite the dominance of clonal growth. Pollination seems to be dominated by bumblebees. Bumblebees (during the observation period only *Bombus lucorum* sensu lato) are patrolling 10–20 flowers in patches of 10 × 10 m in very rapid bell-to-bell visits. They stay around 5 s in each flower with their body tightly filling the bell (YouTube 2019).

Discussion

Soldanella pusilla is one out of more than 300 angiosperm species in the test area, 300 m above treeline, and one of ca. ten species specialized to live in the centre of snowbeds. It is the only species that emerges from snow with fully developed flowers, rising the question when and how these structures have been developed. We show that the ca. 60 mm long flowering stalk and flower bell are largely produced between January and the release from snow in late June/July in a 0 °C environment. Long duration (ca. 5 months of growth, since its start in January) can compensate for the very low growth rates of growth of around 0.33 mm day^{-1} (mean across 150 days) under these conditions. Microscopy data evidence that the process of shoot formation at 0 °C is not just cell enlargement, but also includes structural investment. Yet, critical compounds, including fully polymerized lignins, seem to be missing at least in the sclerenchyma ring of the stalk at the time of release from snow, but appear to establish within a day once stalks experience summer weather, which indicates that non-staining precursors are most likely available.

There are no indications of carbohydrate shortage at any time of the year. In fact, *Soldanella* is so packed with non-structural carbohydrates (mainly soluble sugars) that clones could survive 2 years without complete snow melt. Scaled from the 38 g m^{-2} loss of NSC over the 5 months period at 0 °C, a 12 months period would consume ca. 91 g NSC m^{-2} from of an estimated end of season pool of 190 g m^{-2}, so this pool would suffice for 2 entire years under snow at 0 °C. Years without summer may indeed occur in snowbeds and may have selected for such a safety-margin. Either, record cold years such as 1816, the famous year without summer (no snow melt at elevation >2000 m) after the Tambora eruption in 1815 (Luterbacher and Pfister 2015), or a peculiar combination of a snow rich winter with massive wind-driven snow allocation towards depressions as occurred in patches near our field station in 1999 (pers. observation C. K.) may challenge snowbed plants and explain evolutionary selection for such huge reserves.

Why does *Soldanella* store so much soluble sugar, stachyose in particular? Very unlikely this relates to freezing tolerance, given that *Soldanella* belongs to the least freezing tolerant alpine taxa (a likely consequence of its protection from severe frost by snow pack, Larcher et al. 2010). Stachyose is a soluble tetrasaccharide (molar weight 666.6 g mol^{-1}) composed of one glucose and fructose and two galactose molecules; hence, its osmolality is four times smaller than in glucose. It is assumed that stachyose is synthesized from raffinose and is stored in the vacuole. Stachyose is well known to hydrolyse rapidly and is transported by the phloem to growing tissues (Dey and Haborne 1997). One may speculate that it is more readily available for metabolism during life under snow than starch and other insoluble sugar polymers, but exerts less osmotic pressure than monosaccharides or sucrose. Wright (2001) suggested that besides the function as compatible intracellular osmolytes, stachyose and raffinose may stabilize protein quaternary structures and lipid bilayer integrity in membranes, especially with declining free water activity, thus presumably enabling growth process at low temperature. The overall high concentrations of NSC are co-explained by the low dry matter density of tissues (high water content). Yet, the fact that half of tissue dry matter of *Soldanella* consists of NSC (Fig. 11) is quite remarkable.

What is the signal that induces the onset of length growth in January? Principally, there are two possible avenues, (a) a light signal (increase of day length after the longest night) and (b) an internal clock. Plants may respond to single photons passing deep snow, as Richardson and Salisbury (1977) demonstrated with a photo-multiplier and lettuce seed germination trials under 2 m of snow in the Rocky Mountains. Yet, we found no difference in stalk growth dynamics (onset or speed) either under a complete dark cover under snow in the field (tablets) or in dim light versus darkness in the

phytotron. In the phytotron, we cannot rule out that the short census at dim light synchronized both test cohorts. Such a signal would, however, represent a yes or no rather than day-length signal, and would not explain the actual timing, which in fact matches the field observations (almost fully grown stalks and flowers under deep snow). Since we find the same light independence under a complete dark cover in situ, this leads us to assume that a certain duration of low temperatures (comparable to chilling requirements in phenology) induces stalk growth. Given the constant 0 °C temperature at the soil–snow interface, combined with darkness (deep snow cover), we postulate the existence of an internal clock that causes the timely beginning of development, so that shoots are nearly complete by the time of earliest snow melt. One such mechanism may operate via fatty acid metabolism and membrane fluidity (see Penfield 2008 for a review; Inoue et al. 2018). An alternative trigger for the termination of dormancy found in 'mega-buds' (onion bulbs) could be fructo-oligosaccharides (fructans), the polymerisation state of which decreases with time under cool storage until a critical state is reached at which dormancy is terminated (Benkeblia et al. 2005). Third, the ratio between simple sugars and fructans was found to shift during dormancy transition (Chope et al. 2012). Although we observed an increase in simple sugars (fructose, glucose) and a decrease in sucrose, fructan concentrations increased, both in leaves and belowground structures between October and March, thus, we cannot delineate a potential trigger mechanism from the observed shifts given that the ratio between simple sugars and oligosaccharide concentrations remained roughly the same.

Whatever the actual 'clock' is, we noticed a substantial temporal variation (over ca. 6 weeks) among the emerging stalk cohorts (Fig. 8; see also Fig. 1, bottom), leading to a varied degree of maturity at snow melt, which may secure success under unpredictable weather, similar to what is known for seed germination in wild plants (Fenner 1985).

The rates of stalk growth at 0 °C are so slow that they would not be detected in a conventional census during the growing season. Accordingly, earlier studies of root extension growth under controlled conditions revealed no significant growth below ca. 5 °C (Alvarez-Uria and Körner 2007; Nagelmüller et al. 2016) or postulated zero growth at 0 °C (Körner and Woodward 1987), with more refined analysis revealing some minute extension growth between 1 and 3 °C (Schenker et al. 2014; Li et al. 2016) or even 0.8–1.4 °C (Nagelmüller et al. 2017). In essence, these are scale and resolution problems along an asymptotic cell production response to decreasing temperature as shown in Körner (2006) that will approach an absolute zero point when tissues freeze. Under normal growing season conditions, the amount of tissue produced at such low temperatures is functionally irrelevant. This is why temperature responses of crops or tree xylogenesis had been considered to reach 'practical' zero points of life at 5–6 °C. *Soldanella* opens a window to the ultimate constraints of growth at low temperature and the likely physiological mechanisms. These do obviously not include the provision of photo-assimilates, but relate to tissue formation as such (Nagelmüller et al. 2017).

Plant growth at 0 °C had been observed before. Monocellular or trichomatous green algae can grow on melting snow, some even reach peak rates of cell production at a 2–4 °C (Müller et al. 2001). Similarly cold adapted planktonic algae had been shown to achieve 45% of maximum growth rate at 0 °C (maximum at 6–8 °C; Lovejoy et al. 2007). Pollen tubes of alpine taxa have been shown to grow well at 0 °C (Steinacher and Wagner 2012) verified in situ by Steinacher and Wagner (2013). Among higher plants, root tips on the surface of the peak season permafrost layer in the high Arctic indicate root growth at or close to 0 °C (pers. observations by C. K. in Svalbard, 79°N) supporting experimental data by Billings et al. (1976) with arctic *Eriophorum*. So-called snow roots, that is a web of thin and fragile roots penetrating melting snow have been observed in *Corydalis conorhiza* in the alpine belt of the Caucasus (Onipchenko et al. 2014). These roots have very soft aerenchymatous tissue and disintegrate once the snow is gone and their role was ascribed to foraging for dissolved nitrogen in melt water (Onipchenko et al. 2009).

Is there a unifying set of traits that separates the growth of cold adapted algae or pollen tube growth (see above) from shoot growth in angiosperms? In algae and pollen tubes, the cell wall is very thin or reduced to the gametophytes membrane, and there are no complex tissues with supporting structures, no structures that have to balance turgor pressure. Finally, there are no secondary compounds involved such as lignin, suberin, or cutin. In contrast, angiosperm tissue commonly needs to balance a cellular turgor pressure of 1.5–2 MPa, that needs a strong cell wall, and they require phloem and xylem networks, with lignification essential for the latter. While even algae and mosses possess lignin-like polymers (Ligrone et al. 2008), their sealing function becomes essential only in tracheophyta. From earlier works, we assume that the thermal limitation of growth is not associated with cell division (Körner 2003), but relates to tissue differentiation, in essence cell-wall formation and lignification (Nagelmüller et al. 2017).

Lignification seems to be a key issue at very low temperatures. It results from the enzyme-mediated polymerization of mono-lignols initiated by unknown factors (Donaldson 2001). There seems to be no consensus as to when exactly the enzyme mediated phase ends (if it does), and autopolymerization takes over, given that lignified cell walls are dead by the time this process is completed. Post-mortem lignification of xylem tracheary elements has been debated for decades (Pesquet et al. 2013). These authors confirmed

that lignification does indeed occur after cell death, as one would expect. They showed that cells of *Zinnia elegans* were able to transport lignin monomers to the cell walls of dead tracheal elements. The source of these lignin precursors are surrounding parenchyma cells. Since the cell-wall matrix plays a critical role during lignin deposition (Donaldson 2001), it can be both, the state of that matrix, as well as the lignin deposition process that exerts greatest sensitivity to low temperatures. The fact, that our PEG embedded, deep-frozen samples collected from deep under snow fully disintegrated after thawing the microtome cuttings (not just the xylem part), may either have resulted from the non-lignified sclerenchyma ring, or indicate that the cell wall itself (or the cell–cell contact) retained an immature state as long as tissues grew at 0 °C only (pectin, incomplete hemicellulose networks, hydrogen bounds, and structural proteins).

We cannot resolve the fact that in the samples collected from deep snow, vessels were lignified, although more weakly according to the dye signals than the Raman signal, whereas the massive sclerenchyma ring was not, across the applied different methods. This lack of sclerenchyma lignification clearly weakens the stalks. Yet, it requires a few hours of positive temperatures only as stalks emerge from snow, to arrive at full cell-wall strength and lignification and achieve operative strength. Since structurally, tissues arrived at normal anatomy, constraints of cell formation as such (cell division and cell expansion) seem unlikely, as was concluded previously (Körner and Pelaez Menendez-Riedl 1989; Körner 2003). This also means that limitations related to the cytoskeleton (micro-tubuli) as was proposed for *Arabidopsis* or winter wheat are unlikely candidates for the observed lack in tissue/cell-wall strength (Abdrakhamanova et al. 2003; Plohovska et al. 2016). Poor or lacking lignification of cell walls was also observed in root tips of alpine plants that approached their low temperature growth limit in thermostated root environments under alpine field conditions (Nagelmüller et al. 2017).

With around 100 g d.m. m^{-2} above-ground production per year (accounting for a small fraction of overwintering leaves), closed, mono-specific stands of *Soldanella* are about half as productive as adjacent alpine grassland. Remarkably, almost half of this above-ground dry matter by the end of the growing season falls in the non-structural carbohydrate fraction. However, accounting for the short snow-free season in snowbeds, *Soldanella* is as productive per day as alpine plant communities are that are released from snow much earlier at similar elevation. The main constraint of *Soldanella* is just time.

In conclusion, we show that the ultimate limit for plant tissue formation at low temperature occurs at or slightly below 0 °C, but at 0 °C, the rates are so slow that it needs several months before any significant amount of tissue is produced. This process is not limited by the availability of photo-assimilates; hence, it is not carbon limited. However, at such low temperature, the tissues produced represent a 'soft' matrix that needs a short period of above zero temperatures to complete the ultra-structural network in the cell wall and/or lignification (a 'hardening' step). The fact that the few vessels of such stalks were clearly lignified at constant 0 °C, but the mass of the sclerenchyma ring was not remains puzzling. It also remains unclear how such poorly lignified flowering stalks can be 'spring-loaded' to gain an upright position during the snow melt process. Finally, it is a miracle if and how tracheal tissue can expand, without stretching lignified spiral wall thickening, if our preliminary counts of helix–loop density are correct.

Quite clearly, *Soldanella* does not grow through the snow, but is nearly fully developed when the snowpack is thinning in late spring/early summer. Snow releases almost complete stalks with fully developed flowers, but these influence the melting pattern by absorbing and re-radiating long-wave radiation. The biological clock that triggers the onset of stalk expansion, while plants are under a deep snow pack in January is unknown. It seems that this clock is not light related (e.g., day length controlled), but operates in an autonomous way. We have shown that such a tiny snowbed plant species accumulates large amounts of carbohydrates within a short growing season that would permit basic metabolism for at least two complete years under snow. The poor lignification in tissues grown at 0 °C and the extremely rapid completion of this process after release from snow await further exploration as does the setting of the internal clock. The *Soldanella* case touches upon the ultimate thermal limitation of plant growth and bears relevance for winter crops (Körner 2008) and for roots in cold soils such as on permafrost.

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Author contributions CK designed the study, conducted the field- and phytotron-work, and wrote the manuscript. EH helped with field work, provided macro-photographs, and contributed to the manuscript. SR conducted the light microscopy work and contributed the artwork. TK provided Raman spectroscopy scans, which would not have been possible unless FS managed to obtain microtome cuts of unembedded stem tissue and he verified lignification histochemically. AR and JW provided chromatography data for Table 3.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The work for this paper meets all ethical standards.

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