Ice barriers promote supercooling and prevent frost injury in reproductive buds, flowers and fruits of alpine dwarf shrubs throughout the summer

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A B S T R A C T

Over-wintering reproductive buds of many woody plants survive frost by supercooling. The bud tissues are isolated from acropetally advancing ice by the presence of ice barriers that restrict ice growth. Plants living in alpine environments also face the risk of ice formation in summer months. Little knowledge exists, how reproductive structures of woody alpine plants are protected from frost injury during episodic summer frosts. In order to address this question, frost resistance of three common dwarf shrubs, *Calluna vulgaris*, *Empetrum hermaphroditum* and *Loiseleuria procumbens* was measured and ice formation and propagation were monitored in twigs bearing reproductive shoots during various stages of reproductive development (bud, anthesis, and fruit) throughout the alpine summer. Results indicated that, in the investigated species, ice barriers were present at all reproductive stages, isolating the reproductive shoots from ice advancing from the subtending vegetative shoot. Additionally, in the reproductive stems ice nucleating agents that are active at warm, sub-zero temperatures, were absent. The ice barriers were 100% effective, with the exception of *L. procumbens*, where in 13% of the total observations, the ice barrier failed. The ice barriers were localized at the base of the pedicel, at the anatomical junction of the vegetative and reproductive shoot. There, structural aspects of the tissue impede or prevent ice from advancing from the frozen stem into the pedicel of the reproductive shoot. Under the experimental conditions used in this study, ice nucleation initially occurred in the stem of the vegetative shoot at species-specific mean temperatures in the range of −4.7 to −5.8 °C. Reproductive shoots, however, remained supercooled and ice free down to a range of −7.2 to −18.2 °C or even below −22 °C, the lowest temperature applied in the study. This level of supercooling is sufficient to prevent freezing of reproductive structures at the lowest air temperature occurring at the altitude of the upper distribution boundary of the natural habitat of the investigated species which is between −8 and −10 °C in summer. Frost resistance assays indicated that reproductive shoots are much less frost resistant than vegetative stems, and in contrast to vegetative shoots, are not ice tolerant. Supercooling of reproductive shoots in alpine, woody plant species is an effective mechanism that protects developing offspring from potential frost damage resulting from episodic summer freezing events.

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1. Introduction

Freezing temperatures can occur in alpine environments throughout the whole growing season (Körner, 2003) and frost severity and frequency increase significantly with gains in elevation (Taschler and Neuner, 2004; Larcher and Wagner, 2009). While air temperature minima in the timberline ecotone do not fall below −6.0 °C during the summer months, the situation changes dramatically at higher elevations. Around the elevation limit of closed vegetation in the European Alps, air temperature can drop down to −14.0 °C (measured in the alpine-nival ecotone at 2850 m a.s.l.; Ladinig et al., 2013; Neuner and Buchner, 2012). Additionally, the frequency of frost events increases significantly with increasing elevation. Night frosts were recorded on 20% of 174 snow free days at 1950 m, 69% of 74 snow free days at 3450 m (plant canopy temperature records; Larcher and Wagner, 2009), and daily above 4000 m a.s.l. (Körner, 2011). Hence, plants living in alpine environments may regularly face the risk of ice formation in their tissues, even during the period of active growth.

Vegetative parts of alpine plants typically exhibit some degree of ice tolerance (Taschler and Neuner, 2004), however, ice formation is the immediate cause of frost damage to reproductive organs in most alpine species. Previous studies have demonstrated that the reproductive organs of alpine species, with the exception of *Ranunculus glacialis*, are not ice tolerant (Hacker et al., 2011; Ladinig et al., 2013). The stigma and style are the most freezing sensitive reproductive tissues (Neuner et al., 2013). Hence, the supercooling of reproductive organs as a means to avoid ice formation must be considered as an ecologically significant adaption for safeguarding reproductive success in woody alpine plants.

In some alpine plant species, particularly cushion plants, freezing sensitive reproductive shoots (Ladinig et al., 2013; Neuner et al., 2013) are isolated from ice intrusion by thermal gradients (Hacker et al., 2011). In order for a thermal ice barrier to be active, an adjoining plant part needs to be warmer than 0 °C allowing attached plant parts to remain supercooled for a period of time as temperatures decrease. This temperature differential is often evident in below-ground shoots and buds, and woody stems, but can also be present in species with a densely packed above-ground plant stature, as found in the cushion growth form (Wisniewski et al., 2014).

Structural ice barriers, in addition to thermal gradients, may also protect bud meristems from ice intrusion. The presence of structural barriers have been mainly reported in reproductive over-wintering buds of woody plants (*Rhododendron* species: Ishikawa and Sakai, 1981, *Prunus* and *Forsythia*: Ashworth and Davis, 1984; Ashworth et al., 1992; *Cornus officinalis*: Ishikawa and Sakai, 1985; *Ribes nigrum*: Stone et al., 1993; *Juniperus virginiana*: George et al., 1982) and in meristems of vegetative conifer buds (reviewed in Sakai and Larcher, 1987; Zwiazek et al., 2001). In the few seasonal studies on supercooling capacity of reproductive buds and shoots (Quamme, 1978; Graham and Mullin, 1976; Andrews and Proebsting, 1987), ice barriers were found to persist only until flower bud expansion in spring (Burke et al., 1976). No barriers to the spread of ice were observed during and after bloom in peach (Anderson and Smith, 1989) and apple flowers (Pramsohler and Neuner, 2013).

There is little evidence that structural barriers to ice intrusion are active during anthesis or when fruits are present. Individual flowers in the complex inflorescence of black currant (*Ribes nigrum*) can freeze independently and survive by supercooling down to temperatures of about −5 °C (Carter et al., 1999, 2001). Since dormant black currant buds survive by deep supercooling (Stone et al., 1993), ice barriers may remain active (Carter et al., 1999). Ripening fruits in cranberry (*Vaccinium macrocarpon*) were able to supercool which was attributed to the presence of internal barriers to ice propagation from the stem or pedicel and the lack of extrinsic nucleation from the fruit surface (Workmaster et al., 1999). Alpine woody plant species face an imminent risk of ice formation throughout the whole summer, yet little knowledge exists about the freezing response of these alpine species during different reproductive stages. Freezing response may have a strong impact on reproductive success since reproductive organs have been found to be freezing sensitive, i.e. have little ice tolerance (Neuner et al., 2013; Ladinig et al., 2013). Initial results for *Rhododendron ferrugineum*, obtained by infrared differential thermal analysis (IDTA; Hacker and Neuner, 2007, 2008; Hacker et al., 2008), indicated that ice can propagate nearly unhindered into flowers during anthesis but not into individual florets of winter buds (Neuner and Hacker, 2012). These initial observations (Neuner and Hacker, 2012), and the findings reported for cranberry and black currant, suggest that ice barriers may be active in some woody species during anthesis and subsequent stages of fruit development.

Generally, little is known about ice barriers that influence the rates or avenues of ice propagation in plants (Wisniewski et al., 2009). In the present study, detailed studies of freezing response in three commonly distributed alpine dwarf shrub species was investigated in order to determine whether ice barriers exist between vegetative and reproductive shoots and to what extent reproductive tissues are protected from ice intrusion and frost damage in summer months when plants are actively growing and reproducing. Ice propagation was visualized using IDTA. This method of analysis was selected so the exact localization of the tissue responsible for blocking ice propagation into reproductive shoots could be determined. Ice nucleation temperatures were recorded and the level of frost resistance was assessed for reproductive and vegetative shoots in response to simulated frost events. Observations of freezing response were conducted on reproductive structures during the entire reproductive process, from flower bud to mature fruit, in order to determine if structural barriers to ice propagation were continually present.

2. Materials and methods

2.1. Plant material and collection site

Investigations were carried out on *Calluna vulgaris* (L.) HULL., *Empetrum nigrum* subsp. *hermaphroditum* (HAGERUP.) BÖCHER, and *Loiseleuria procumbens* (L.) DESV. All three species are evergreen, woody dwarf shrubs that are commonly distributed in the alpine zone of the Central European Alps. Their characteristics are summarized in Table 1. The upper distribution boundary of all three species is at elevations between 2500 and 2800 m.

Plant samples were collected approximately 6 km south of Innsbruck, Austria on the summit of Mt. Patscherkofel (2248 m) which is 200–500 m below the elevation limit of the species. At this site, all three species grow in close vicinity to each other, despite different habitat preferences. *C. vulgaris* was sampled on the south exposed slope of the summit (47°12′29.81″ N, 11°27′42.37″ E), while *L. procumbens* and *E. hermaphroditum* were collected on the north exposed slope (47°12′33.42″ N, 11°27′44.93″ E). Samples were taken randomly from at least 10 individuals and a total of 30 twigs per species were collected at each sampling. Single twigs (12 cm) were detached with a pruning shear, placed on wetted paper towels, and stored during the transport to the laboratory inside sealable plastic bags in a cooler box. The total time for transport of the samples to the laboratory environment was approximately 1.5 h. Plant material was stored at 4 °C in a dark cold room and used in experiments within 7 days after collection.

The presence of ice barriers, patterns of ice propagation, and frost resistance were evaluated at three different stages of reproductive development (b = unopened floral buds, a = anthesis, and f = mature fruit) throughout the growing period. Flower buds of *C. vulgaris* form in spring, and flowering time is usually midsummer, while fruits ripen in autumn. *E. hermaphroditum* and *L. procumbens* have over-wintering reproductive buds, are early-flowering, and have fruits that develop in summer. Since the species differ with respect to the timing of reproductive development, the stage of development and sampling design for each species is provided in Table 2.

2.2. Freezing treatment

Freezing of the plant samples was conducted in computer-controlled commercial chest freezers (Hacker and Neuner, 2007). The freezing compartments of these freezers have a volume of 141.9 L (43 cm × 50 cm × 66 cm). The freezing chambers used in this study allow for the programming of user defined cooling protocols which enable linear cooling and thawing rates. Temperature set points and the duration at each temperature can be freely set and designed to maintain temperature oscillations at a