

Freezing exposure releases bud dormancy in *Betula pubescens* and *B. pendula*

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ABSTRACT

Bud dormancy in woody plants is released by long-term exposure to non-freezing chilling temperatures, whereas freezing temperatures have been considered to have little or no effect. However, the present results demonstrate that short-term exposure to freezing can release bud dormancy in *Betula pubescens* (Ehrh.) and *B. pendula* (Roth). Short-term freezing during the dormancy induction phase improved the release of bud dormancy only if an adequate level of dormancy had been reached. In fully dormant or chilled plants both the percentage and the speed of bud-burst increased, the more so the lower the temperature. Our results rule out the possibility that endogenous abscisic acid could be directly involved in the physiological control of bud dormancy release. The fast, easily applicable method presented here for bud dormancy release could further investigations into the biochemical and biophysical background to the process. The mechanisms of bud dormancy release and its relationship to cold acclimation are discussed in the light of these results, as also are the implications of the findings for modelling of bud dormancy.

Key-words: abscisic acid; bud dormancy; chilling; climatic adaptation; cold hardiness; dormancy release; freezing tolerance.

INTRODUCTION

The adaptation of trees native to temperate and cold regions consists of optimal synchronization of growth with the climate at the growing site. Low but non-freezing chilling temperatures have important, but apparently contradictory, effects in this synchronization, in that chilling facilitates growth cessation (Heide 1974) and initiation of dormancy at first (Powell 1987a), while later it promotes dormancy alleviation (Noodén & Weber 1978; Fuchigami *et al.* 1982). The growth-promoting role of chilling has been long recognized (Coville 1920), but there are still no explanations

available as to how it exerts this effect (Lang 1994). According to the available information, the transition from dormancy to active growth is accompanied by numerous molecular and biochemical changes, including changes in hormonal balance (Noodén & Weber 1978; Lang 1994). One potential regulator is abscisic acid (ABA), which inhibits growth and may mediate plant responses to environmental stimuli (Bray 1991). However, determining a clear role for ABA in dormancy release has proved to be difficult (see Trewavas & Jones 1991; Dennis 1994).

The optimal range of chilling temperatures for bud dormancy release is from 5 to 7 °C in most tree species (Powell 1987b). This has been demonstrated by monitoring the bursting of buds predisposed to a range of chilling temperatures. It is important to note that the bud-burst itself is facilitated by heat sum accumulation, and therefore the test favours temperatures that lie at the upper end of the range of temperatures promoting dormancy release. In addition, since dormancy release has been regarded as a physiological process (Powell 1987a), the role of lower temperatures or freezing under natural conditions in temperate and cold regions has remained unexplored, although some experiments point to such a role (Hasegawa & Tsuboi 1960; Tinklin & Schwabe 1970; Noodén & Weber 1978).

The purpose of the present work was to examine bud dormancy release in *Betula pubescens* (Ehrh.) and *Betula pendula* (Roth) after short exposure to freezing temperatures. Bud ABA levels were followed throughout the treatments in the case of *B. pendula*. The possible mechanisms involved and the relationship between cold acclimation and bud dormancy release are discussed in the light of the results, as also are the implications of the findings for the modelling of bud dormancy.

MATERIALS AND METHODS

B. pubescens experiment

Plant material

Seeds collected in northern Finland (65°10'N, 27°20'E) were sown on 1 July 1994 and germinated at 23 °C under continuous light. After 1 month the seedlings were transplanted into 500 cm³ pots containing commercial fertilized peat (Vapo B2, 1.2 kg m⁻³; N 12%, P 9%, K 18% and micronutrients)

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and sand in the ratio 4:1 (v/v). Seedlings were grown in a heated glasshouse, irrigated once a day and subjected to the natural photoperiod, with a mean daytime temperature of 21.0 (± 1.9) °C, a night temperature of 19.0 (± 0.4) °C and a water pressure deficit of -30 MPa. Additional light with a photon flux density of 90–100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at 400–740 nm was provided by fluorescent lamps (Philips TL65 W/83) between 0400 and 2000 h to ensure a long day photoperiod throughout. The experiments were started when plants were about 2 months old and 30–50 cm in height.

Induction of growth cessation and bud dormancy

Growth cessation and bud dormancy were artificially induced at the beginning of the experiment using three treatments: (i) 31 seedlings were subjected to a *short day* regime (SD, 8 h) by covering them with a 1.5-m-high cabinet between 1600 and 0800 h; (ii) 27 seedlings were subjected to *exogenous ABA* treatment under LD conditions by spraying shoots with 25 mmol m^{-3} (\pm)ABA (Fluka) in 0.5% (v/v) Tween 20 once a day for the first 2 weeks and twice a week thereafter, and (iii) another 23 seedlings under LD conditions were subjected to *water stress* (WS) by reducing the water content of the soil by 30% relative to the well-watered plants. A further 44 well-watered plants under LD conditions served as controls.

Freezing treatments

Freezing was applied 0, 1, 3 and 7 weeks from the beginning of each treatment, using a computer-controlled freezer as described by Welling, Kaikuranta & Rinne (1997). Samples were kept at each of the final temperatures, -4.6, -9.0, -13.5 and -20.0 °C, for 0.5 h before thawing in a cold room (5 °C). Three to five replicate plants (30 buds) subjected to each treatment were used to represent each of the freezing temperatures.

Observation and calculation of bud-burst

After freezing, the stem segments were cut into pieces comprising one internode, which were kept under forcing conditions in water culture in a climate chamber (21 °C, 16 h photoperiod, water vapour pressure deficit -39 MPa) as described previously by Rinne, Saarelainen & Junttila (1994a). After 22 d, the viability of the meristematic tissues was examined according to Ritchie (1991). 'Days required for bud-burst' (DBB) was recorded at the point when the first leaf had unfolded, even though more leaves or long shoots developed later (results not shown). Bud dormancy status was illustrated by 'burst buds as a percentage of all viable buds' (BB%).

B. pendula experiment

Plant material

Seeds produced at a seed orchard (69°09'N, 27°03'E), were sown in April 1994 and germinated in commercial

peat (Vapo F1K2) in a greenhouse. After 1 month they were transplanted into 580 cm^3 pots. They were placed outdoors on May 10 and given several doses of a commercial fertilizer (Kekkilä Superex-5: N 11%, P 4%, K 25% and micronutrients). Commercial fungicides (Berlatea, Bayleton) were applied from time to time. At the beginning of September the seedlings were moved to the Botanical Garden of the University of Joensuu (62°36'N, 21°48'E) and transferred into plastic bags of volume 1000 cm^3 containing commercial fertilized peat (Vapo XL: N 10%, P 18%, K 14% and micronutrients). They were then kept out of doors until the beginning of the chilling and freezing treatments, on 21 September 1994, when they were still in full leaf.

Chilling and freezing treatments

The chilling treatment was carried out in darkness in two walk-in growth chambers (5.1 ± 1.2 °C) for 12 weeks. Ten seedlings were sampled randomly at 2 week intervals and transferred to forcing conditions (see below) for bud-burst observations. At 4 week intervals 10 additional seedlings were randomly selected for controlled freezing treatment at each of the temperatures of -8, -16, -24 and -32 °C. These samples were cooled at a rate of 5 °C h^{-1} in air-cooled chambers, starting from 10 °C, kept for 4 h at the final temperature and thawed at a rate of 5 °C h^{-1} until 10 °C was regained. Freezing of the roots was prevented by insulation with polyurethane foam. The seedlings were transferred to forcing conditions for bud-burst observations.

Observation and calculation of bud-burst

Forcing took place in a plastic cabinet (250 cm × 300 cm × 205 cm) in a heated greenhouse (20.0 ± 1.5 °C). Illumination was with fluorescent tubes (Sylvania Studio 186, 58 W, 6200 K, CRI: Class 1B Daylight Deluxe), providing 15–35 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at 400–740 nm. The five uppermost buds in every 10 replicate seedlings were checked visually twice a week for determining BB% and DBB (described above for *B. pubescens*).

ABA analyses

In selected treatment groups, additional seedlings were sampled for ABA analysis. Samples usually comprising the 10 uppermost buds were collected 0, 4, 8 and 12 weeks after chilling from both non-frozen and frozen plants, in the latter case 0, 1 and 7 d after the freezing treatment. Samples were weighed and immediately frozen in liquid nitrogen. [$^2\text{H}_4$]ABA was used as an internal standard and the samples were extracted and purified by HPLC as described earlier (Welling *et al.* 1997). The fraction containing ABA was evaporated to dryness, dissolved in acetonitrile, methylated with diazomethane and quantified by gas chromatography-mass spectrometry (GC-MS) with selective ion monitoring. Chromatography was carried out on a 25 m × 0.25 mm CP-SIL 5CB column with a 0.25 µm

film thickness (Chrompack) using helium as the carrier gas at a flow rate of $1 \text{ cm}^3 \text{ min}^{-1}$. GC-MS was performed with a Carlo Erba 4160 gas chromatograph (Oriola) linked to a Perkin Elmer Ion Trap Detector. A m/z ratio of 190:194 was used to calculate the endogenous ABA content and a ratio of 162:166 was used to check the interference.

Statistics

BB% data from different freezing temperatures were tested against data for non-frozen plants by Fisher's exact test and DBB data from different freezing temperatures were tested against data for non-frozen plants by the Mann-Whitney U-test.

RESULTS

The seedlings of *B. pubescens* responded to SD after 5 d by slowing down their elongation growth, which ceased completely after 10 d. The plants treated with ABA under

LD conditions stopped growing after 10 d, and the water-stressed plants continued growing for a few days longer under LD conditions. SD and water stress induced a deeper dormancy than ABA, as indicated by the lower BB% from 3 weeks onwards (Table 1). All the treatments improved freezing survival similarly. Some of the seedlings died as a result of short-term freezing, but dormancy release was significantly enhanced among the survivors if BB% had been lowered to 35% or less (Table 1). ABA treatment did not decrease BB% sufficiently and in this case freezing accelerated dormancy. When the plants had been under SD for 7 weeks and BB% had decreased to zero, all the temperatures tested became rather less effective (Table 1).

The seedlings of *B. pendula* were fully dormant at the beginning of the experiment, as indicated by the zero BB% for the non-frozen plants during the first 4 weeks (Fig. 1). Thereafter, dormancy was gradually released as a result of long-term chilling until 12 weeks, although the speed of bud-burst was still accelerated by further chilling. Short-

Table 1. Effects of short term freezing on bud dormancy release in single-node cuttings of *Betula pubescens*. See 'Materials and Methods' for description of bud-burst % (BB-%) and days required to bud-burst (DBB). Contr. = control plants under long-day conditions; SD = short-day treatment; ABA = abscisic acid treatment; WS = water stress treatment; nd = not determined. Values are mean \pm SE, $n = 3-5$. Data from different freezing temperatures were tested against data for non-frozen plants (Fisher's exact test). Statistical significances: * $0.05 > P > 0.01$; ** $0.01 > P > 0.001$; *** $0.001 > P$

Treatment	Weeks	Factor	Non-frozen	-4.6°C	-9.0°C	-13.5°C	-20.0°C
Contr.	0	Survival-%	98.5 \pm 1.6	0.0	0.0	0.0	nd
		BB-%	77.9 \pm 8.7	—	—	—	—
		DBB	11.3 \pm 1.1	—	—	—	—
SD	1	Survival-%	100.0 \pm 0.0	0.0	0.0	0.0	0.0
		BB-%	35.6 \pm 19.3	—	—	—	—
		DBB	15.1 \pm 2.2	—	—	—	—
ABA	3	Survival-%	100.0 \pm 0.0	100.0 \pm 0.0	100.0 \pm 0.0	25.0 \pm 25.0	nd
		BB-%	9.1 \pm 9.1	18.9 \pm 13.1	31.0 \pm 3.8*	0.0	nd
		DBB	10.3 \pm 0.0	10.8 \pm 1.2	10.6 \pm 0.5	—	nd
WS	7	Survival-%	100.0 \pm 0.0	nd	100.0 \pm 0.0	100.0 \pm 0.0	100.0 \pm 0.0
		BB-%	0.0	nd	0.0	2.8 \pm 2.8	4.2 \pm 4.2
		DBB	—	nd	—	13.8 \pm 0.0	13.0 \pm 0.0
ABA	1	Survival-%	92.6 \pm 7.4	7.4 \pm 7.4	16.7 \pm 16.7	0.0	nd
		BB-%	50.0 \pm 28.9	50.0 \pm 0.0	0.0	—	nd
		DBB	10.9 \pm 0.5	11.0 \pm 0.0	—	—	nd
ABA	3	Survival-%	100.0 \pm 0.0	75.0 \pm 25.0	25.0 \pm 25.0	0.0	nd
		BB-%	10.8 \pm 7.9	85.0 \pm 7.6	0.0	—	nd
		DBB	12.5 \pm 1.5	11.1 \pm 0.4	—	—	nd
ABA	7	Survival-%	100.0 \pm 0.0	100.0 \pm 0.0	95.18 \pm 4.2	100.0 \pm 0.0	nd
		BB-%	8.1 \pm 4.8	0.0	45.2 \pm 24.9**	0.0	nd
		DBB	13.3 \pm 1.8	—	12.1 \pm 0.3	—	nd

term freezing was effective in causing dormancy release in those buds that were still dormant after receiving some chilling from 4 weeks onwards (Fig. 1). Characteristically, lower temperatures (-24 and -32 °C) significantly improved bud dormancy release at each freezing date, although their effect generally diminished as the plants received more chilling. Lower temperatures also significantly improved the speed of bud-burst (Fig. 1). Without chilling the lowest temperatures tested appeared to be too severe and most of these buds died. ABA levels remained constant in non-frozen ($3.6\text{--}5.1 \mu\text{g g}^{-1}$ dry weight at 95% confidence intervals) and frozen viable buds ($3.5\text{--}4.3 \mu\text{g g}^{-1}$ dry weight at 95% confidence intervals), and did not show any correlation with the duration of chilling or with freezing-induced bud dormancy release.

DISCUSSION

Effect of freezing on bud dormancy release

The present experiments demonstrate that short-term freezing efficiently releases bud dormancy in *B. pubescens* and *B. pendula* (Table 1 & Fig. 1). When the plants were

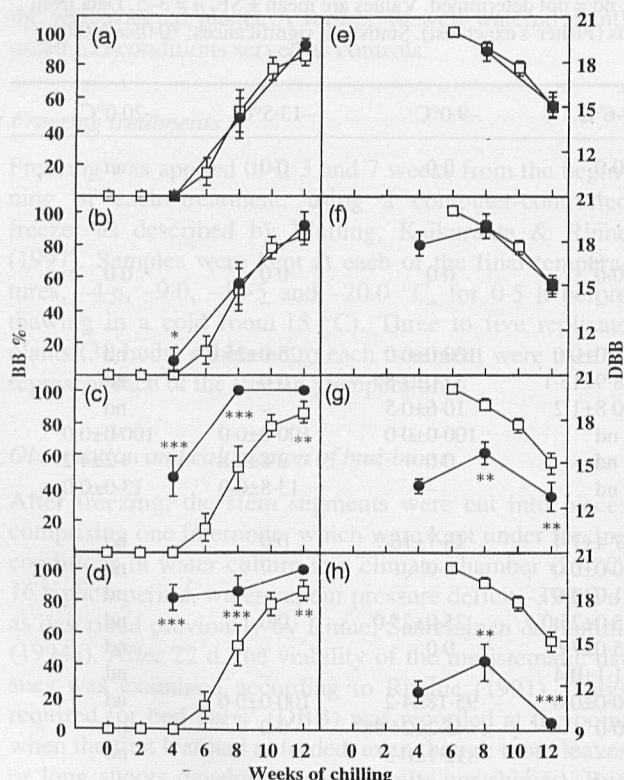


Figure 1. Effects of long-term chilling (□) at 5 °C and short-term freezing (●) at -8 °C (a, e), -16 °C (b, f), -24 °C (c, g) and -32 °C (d, h) on bud dormancy release in seedlings of *Betula pendula* as indicated by bud-burst percentage (BB%) (a-d) and by the speed of bud-burst (days to bud-burst, DBB) (e-h). BB% and DBB obtained at different freezing temperatures were tested against data for non-frozen plants by Fisher's exact test and the Mann-Whitney U-test, respectively. Statistical significances: * $0.01 < P < 0.05$, ** $0.01 < P < 0.001$, *** $P < 0.001$. Values are means \pm SE.

entering dormancy, however, an effect of short-term freezing was observed only if a sufficient level of dormancy had been reached, which appeared to be when bud-burst was 35% or lower for *B. pubescens*. Under SD and water stress this level was reached in 3 weeks, but exogenous ABA failed to promote an appreciable level at dormancy and in this case freezing rather accelerated than released bud dormancy (Table 1).

It has been observed that the rate of dormancy release follows a convex temperature response curve (Gilreath & Buchanan 1981; Erez & Couvillon 1987). According to Sarvas (1974), for instance, the maximum rate of dormancy release in *B. pubescens* is obtained at 3.5 °C, the rate decreasing towards lower temperatures, and no dormancy release takes place at temperatures below -3.4 °C. The effects of freezing temperatures on bud dormancy have been reported in a few cases: -15 °C was effective for *Ribes nigrum* (Tinklin & Schwabe 1970) and temperatures between -5 and -10 °C for the mulberry (Hasegawa & Tsuboi 1960). In all these cases the plants had received natural pre-chilling, and no non-chilled controls were tested. Although a few examples exist, freezing-induced dormancy release has seldom been regarded as being involved in the natural dormancy release mechanism of woody plants growing in cold regions. For example, plants with a high chilling requirement are thought to gain part of their chilling in the autumn and the rest in the spring, as the temperatures in the intervening time are below the optimum for physiological chilling (Powell 1987a). It remains to be seen whether short-term freezing has similar effects on dormancy release to long-term chilling. Regardless of the outcome, however, freezing may be a useful approach for studying the release of bud dormancy, as specific dormancy-releasing requirements are met within a few hours.

Mechanisms of bud dormancy release and cold hardiness

Although many metabolic and physiological changes can be connected with dormancy release during traditional chilling (Noodén & Weber 1978; Lang 1994), it has proved difficult to find any key factors for dormancy release, partly due to the long duration of the process and the presence of overlapping phenomena, i.e. freezing tolerance and bud-burst. There are many examples of the ABA level increasing in the autumn and decreasing during the next winter (Harrison & Saunders 1975; Lavender & Silim 1987; Rinne, Tuominen & Junntila 1994b). These alterations do not relate directly to dormancy induction and alleviation according to the present results, for ABA levels remained unchanged under chilling or after freezing although both chilling and freezing stimulated dormancy release. The abrupt release of dormancy induced by short-term freezing indicates that the mechanism operates briefly, and therefore structural and/or biophysical modifications might be more important in dormancy release than previously thought. Freezing usually follows chilling tem-

peratures, and could induce at least partially similar mechanisms in buds to those caused by traditional chilling.

The present results are consistent with the biophysical evidence presented by Faust *et al.* (1991), who showed that water in the bound and free states in buds characterizes endodormancy and eco-/paradormancy, respectively. These changes were not monitored in the present experiment, but it has been shown that buds desiccate during dormancy initiation so that 35–40% of their water content (fresh weight basis) will be retained (Rinne *et al.* 1994b; Welling *et al.* 1997). Freezing stimulates further dehydration of the cells, as water moves across the plasma membrane to the apoplast, but during subsequent thawing cellular water status is recovered (Palta & Weiss 1993). Therefore, it is likely that freezing causes alteration in the distribution and compartmentalization of cellular water. Some other sub-lethal stresses, i.e. heat and chemical treatment, are also able to release the dormancy of buds (Tanino *et al.* 1989; Shirazi & Fuchigami 1995; Wisniewski *et al.* 1996) and seeds (Cohn 1996), effects that are thought to result from removal of barriers that restrict the movement of water to the dormant buds (Wisniewski *et al.* 1996). Although water is not a regulator itself, an abrupt increase in the level of free water would allow the acquisition of metabolism and growth coordination in buds.

Bud dormancy and freezing tolerance can be regulated independently (Irving & Lanphear 1967; Fuchigami *et al.* 1982). One sign of this is that a frost-hardened state continues in many woody plants long after dormancy has been removed (Sakai & Larcher 1987). Some relationship between bud dormancy and freezing tolerance may exist, however, as initiation of freezing tolerance is determined by the timing of growth cessation, and freezing tolerance is lost more easily after release from dormancy than during it (Proebsting 1963; Colombo 1990; Valkonen *et al.* 1990; Leinonen, Repo & Hänninen 1997). We found that a gradual decrease in temperature was needed for bud dormancy release when plants were entering deeper dormancy. This response can be affected both by improved freezing tolerance and freezing avoidance, i.e. maintenance of unfrozen bound water in the cells (Vertucci & Stushnoff 1992), which reduces the dehydrative force of a given temperature. All the treatments used here improved freezing tolerance (Table 1), although not necessarily by the same mechanisms. ABA, for example, may not desiccate the tissues to the same extent as the other treatments (Welling *et al.* 1997). ABA was also less effective for dormancy induction compared to the other treatments (Table 1). The results suggest that water status and the distribution and cellular compartmentalization of water form an important biophysical link between dormancy and freezing tolerance.

Implications for modelling

Several mathematical chilling unit models have been presented in order to describe the temperature response of bud dormancy release in quantitative terms. When synthesized with different temperature sum models, these models pre-

dict the timing of bud-burst for a given location on the basis of climatic data (for reviews, see Hänninen 1990, 1995; Kramer 1994). Some assume that long-term exposure to freezing temperatures causes bud dormancy release in a similar manner to long-term exposure to non-freezing chilling temperatures (Landsberg 1974; Cannell & Smith 1983; Kramer 1994, 1995; Leinonen 1996), but none takes into account the dormancy-releasing effect of short-term exposure to freezing temperatures. According to the present experimental results, this phenomenon should be addressed when seeking to refine the models further. This is especially important if one is aiming at models with a high level of *biological realism*, i.e. models addressing all of the most important aspects of the environmental regulation of bud-bursting (Levins 1966; Hänninen 1995).

The prevailing models of bud dormancy release have been used to predict the effect of climatic change on the timing of bud-burst (Cannell & Smith 1986). The present experimental results suggest that a failure to address the dormancy-releasing effect of short-term exposure to freezing could (1) reduce the biological realism of the present models, and (2) lead to errors in scenario analyses carried out with the aid of these models (Hänninen 1995). Therefore the effect of short-term freezing on bud dormancy release has to be taken in account, as well as the interactions of photoperiod and temperature (Heide 1993a,b), in order to develop realistic models for climatic change impact studies.

ACKNOWLEDGMENTS

The authors thank Professor Olavi Junnila and Ilkka Leinonen, Msc for their constructive criticism of the manuscript; Ms Päivi Joensuu for help with mass spectrometric analysis; Ms Aino Hämäläinen, Ms Kaarina Veijola, Mr Tapio Mäkelä and Annikki Welling, Phil.Lic. for taking care of the plants, and the Academy of Finland (Research Council for the Environment and Natural Resources) for financial support.

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Received 7 February 1997; received in revised form 12 May 1997; accepted for publication 14 May 1997

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