Dehydration and osmotic adjustment in apple stem tissue during winter as it relates to the frost resistance of buds

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In deciduous trees, measurement of stem water potential can be difficult during the leafless period in winter. By using thermocouple psychrometry, osmotic water potentials ($\Psi_o$; actual $\Psi_o^{(act)}$; $\Psi_o$ at full saturation: $\Psi_o^{(sat)}$) of expressed sap of bark and bud tissue were measured in order to test if the severity of winter desiccation in apple stems could be sufficiently assessed with $\Psi_o$. Water potentials were related to frost resistance and freezing behaviour of buds. The determination of $\Psi_o$ reliably allowed winter desiccation and osmotic adjustments in apple stem tissue to be assessed. In winter in bark tissue, a pronounced decrease in $\Psi_o^{(act)}$ and $\Psi_o^{(sat)}$ was found. Decreased $\Psi_o^{(sat)}$ indicates active osmotic adjustment in the bark as observed earlier in the leaves of evergreen woody plants. In terminal bud meristems, no significant osmotic adjustments occurred and dehydration during winter was much less. Osmotic water potentials, $\Psi_o^{(act)}$ and $\Psi_o^{(sat)}$, of bud tissue were always less negative than in the bark. To prevent water movement and dehydration of the bud tissue via this osmotic gradient, it must be compensated for either by a sufficiently high turgor pressure ($\Psi_t$) in bark tissue or by the isolation of the bud tissue from the bark during midwinter. During freezing of apple buds, freeze dehydration and extra-organ freezing could be demonstrated by significantly reduced $\Psi_o^{(act)}$ values of bud meristems that had been excised in the frozen state. Infrared video thermography was used to monitor freezing patterns in apple twigs. During extracellular freezing of intact and longitudinally dissected stems, infrared differential thermal analysis (IDTA) images showed that the bud meristem remains ice free. Even if cooled to temperatures below the frost-killing temperature, no freezing event could be detected in bud meristems during winter. In contrast, after bud break, terminal buds showed a second freezing at the frost-killing temperature that indicates deep supercooling. Our results demonstrate the applicability of thermocouple psychrometry for the assessment of winter desiccation in stem tissues of deciduous trees and corroborate the finding that dormant apple buds survive by extra-organ freezing and do not deep supercool. In addition, they indicate that significant changes of the frost-survival mechanism can occur during the apple bud development in spring.

Keywords: frost hardiness, infrared thermography, Malus domestica, thermocouple psychrometry, water relations, winter desiccation stress.

Introduction

Winter desiccation and dehydration tolerance in deciduous trees are difficult to determine with the commonly employed methods. Tissue water content can be reliably determined during the leafless period, but water content does not describe the availability of water in cells as well as the various components of water potential. The standard method to determine water potentials in plants is the pressure bomb technique. However, in deciduous trees, it is difficult to apply in winter, as the formation of embolisms in the conduits and a small portion of living tissues may lead to erroneous results.

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using thermocouple psychrometry, total and osmotic water potentials of plant tissues or of expressed tissue saps can be determined (Kikuta and Richter 1987, Rinne et al. 1998). \( \Psi_{o(sat)} \) has additionally been shown to be a good indicator of species drought tolerance (Bartlett et al. 2012, Scholz et al. 2012). While thermocouple psychrometry has been used to measure seasonal changes of components of water potential in leaves of a few evergreen woody plants (Neuner et al. 1999, Callister et al. 2008, Rodriguez et al. 2012), comparatively little information exists about stem tissue. For buds of deciduous trees, seasonal changes of water potential components during winter were reported (Richards and Bliss 1986, Cottignies 1990). There have also been attempts to measure components of water potential of secondary phloem in tissue discs of Norway spruce (\textit{Picea abies} (L.) Karst) during summer (Rosner et al. 2001, Rosner and Kikuta 2002). However, to our knowledge this method has not been applied to document changes of osmotic water potential during winter in bark tissues of woody plants. Therefore, by employing thermocouple psychrometry, we intended to measure osmotic water potentials in expressed sap of bud and bark tissue in order to assess winter desiccation and osmotic adjustments in living stem tissues of apple trees during winter.

The various stem tissues of apple trees survive freezing temperatures by different frost-survival mechanisms. While xylem parenchyma cells in apple trees are known to undergo deep supercooling (for a recent review see Pramosohler et al. 2012), the bark tissue does not (Ashworth et al. 1988, Malone and Ashworth 1991). Apple bark tissue survives freezing by extracellular ice formation and freeze dehydration (Burke et al. 1976, Ashworth et al. 1988, Ashworth and Wisniewski 1991). Dehydration tolerance of the cells during freezing appears as an important trait, as with decreasing temperature the extent of dehydration stress increases (Yelenosky and Guy 1989). By the measurement of \( \Psi_{o(sat)} \), we intended to assess dehydration tolerance of bark and bud tissues during winter.

Apple bud primordia appear to survive freezing by extra-organ freezing (Wiegand 1906, Quamme 1991). During extra-organ freezing of bud primordia, a temperature-dependent migration of water from the bud primordial tissue towards the sites of ice formation in the stem and in the bud scales occurs (Sakai and Larcher 1987, Ishikawa et al. 1997). Hence, for extra-organ freezing of bud tissue during winter, a sufficiently high dehydration tolerance at the cellular level must be achieved. Increased dehydration tolerance generally seems to be a prerequisite for frost survival of woody species (Sakai and Larcher 1987, e.g., conifers Zwiazek et al. 2001), and this might also be seen in reduced \( \Psi_{o(sat)} \) values in bud meristems during winter.

Water loss of bud meristems during freezing should be recognizable in decreasing \( \Psi_{o(sat)} \) values. By \( \Psi_{o(sat)} \) measurements of bud tissue before, during and after a controlled freezing treatment, we wanted to experimentally demonstrate the migration of water out of the apple bud primordial tissue. Additionally, the recently invented infrared differential thermal analysis (IDTA) technique (Hacker and Neuner 2007, Hacker and Neuner 2008, Hacker et al. 2008) allows very small freezing events (Neuner et al. 2010, Pramosohler and Neuner 2012), which may not be seen with the classical method of differential thermal analysis (DTA, Burke et al. 1976) to be visualized. By employing IDTA, we intended to reconfirm that dormant apple buds do not deep supercool, as reported in earlier studies. Further, we wanted to investigate whether the freezing pattern would change during the structural changes occurring in spring during bud development. Hence, several IDTA studies were performed on buds of apple stems that were in different developmental stages after bud break.

## Materials and methods

### Study site and plant materials

Samples were taken from apple trees (\textit{Malus domestica} Borkh. cultivar ‘Golden Delicious’ and ‘Nicoter’ grafted on M9 rootstocks) growing in an apple orchard in Tarsch, South Tyrol, Italy (46°36’N, 10°53’E, 860 m a.s.l.). Apple trees were 5–6 years old. The 1-year-old long shoots with terminal buds were used. Twig samples were taken at a height of ~1.5 m. The time of sampling was always around 10.00 am. The apple trees were sampled over a time period of 2 years, from December 2009 till May 2011, covering two winter periods. Twig samples were detached from the trees and transported in plastic bags from the field site to the laboratory. Measurements were either conducted immediately or samples were stored in a cold room at +4 °C for a maximum of 12 h.

### Determination of osmotic water potentials of expressed sap of plant tissues

**Actual osmotic water potential (\( \Psi_{o(sat)} \))**

Osmotic water potentials were determined using eight calibrated C-52 chambers (Wescor Inc., Logan, UT, USA) that were connected to a PSYPRO water potential system (Wescor Inc.). Water potentials were determined using the psychrometric operation method of the instrument. The C-52 chambers were used with sample holders with a diameter of 6 mm and a depth of 2.5 mm. Calibration of the C-52 chambers was done...
with NaCl solutions (Wescor Osmolality Standards with concentrations of 100, 290, 1000 and 2000 mmol kg$^{-1}$) absorbed by filter paper discs with a diameter of 6 mm. Similarly, expressed saps of apple tissues were measured using 6-mm diameter filter paper discs. Expressed saps were collected as follows. Terminal buds were detached from the twig at the insertion point of the bud scales, dissected longitudinally with a razor blade and a filter paper disc was inserted between the two halves. The two halves containing the filter paper were then inserted into a vice. By applying a moderate mechanical pressure, sufficient expressed sap from the bud primordial tissue could be absorbed by the filter paper. To collect expressed sap from bark tissues, the cortical tissue was removed at the cambium. Again, the filter paper disc was inserted between two cortex pieces and a moderate pressure was applied by the vice to squeeze out the expressed sap until the filter paper disc was saturated with the expressed sap of the bark tissues.

Osmotic water potential at full saturation ($\Psi_{o(sat)}$)

Full water saturation in bud and bark tissues was induced by submersing 1-year-old apple twig samples, 15 cm in length, in a water bath for 16 h at room temperature. Time until full saturation was tested in preliminary experiments. A range of exposure times, from 4 to 24 h, was used; by 16 h, full water saturation had been achieved. After saturation, superficial water was removed from the twigs. Expressed sap from watersaturated bud and bark tissues was collected as described above and the osmotic water potential at full saturation was measured.

Determination of bud frost resistance

Frost resistance of terminal apple buds was assessed on detached twigs of 15-cm length. The samples were inserted into polythene plastic bags and placed inside the cooling compartment of six computer-controlled commercial freezers (described in Hacker and Neuner 2007). The twig samples were cooled at a rate of 4 K h$^{-1}$ to target temperatures ranging from −2 to −30 °C. After an exposure time of 4 h at the target temperature, controlled thawing at a rate of 4 K h$^{-1}$ till +5 °C took place. After the freezing treatment, the samples were stored inside the plastic bags for 5–7 days at room temperature to allow frost damage to become visible. Then the buds were dissected with a razor blade and tissue damage was assessed visually with a stereo microscope (Olympus SXZ12, Tokyo, Japan). Additionally, tissue viability was tested with the topographic tetrazolium (TTC) test (Ruf and Brunner 2003, Larcher et al. 2010). Buds were rated undamaged if all bud tissues looked healthy and were coloured red by TTC staining. Frost damage rating based on scales was not practical as various tissues could be affected to different extents and this could also change with season. Therefore, the highest temperature producing initial frost damage ($LT_i$) to buds was assessed. $LT_i$ was taken to be the highest frost exposure temperature at which the red staining by TTC as observed in the controls was replaced by a brownish discoloration in the sample. For frost resistance determination, 10 replicates for each target temperature were used.

Determination of translocated ice

Terminal pieces of apple twigs, 15-cm length, were placed inside the cooling compartment of a computer-controlled commercial freezer (described in Hacker and Neuner 2007). Samples were cooled at a rate of 4 K h$^{-1}$ down to a temperature of −6 °C. After a 4-h period of time, at −6 °C twigs were thawed at a rate of 4 K h$^{-1}$ till +4 °C was reached. Actual sample temperatures during the experiment were recorded every 10 s with type-T thermocouples (solder junction diameter 0.13 mm) connected to a CR10 data logger (Campbell Scientific, Logan, UT, USA) that were fixed to the periderm of the twigs with adhesive tape (Transpore, 3M Health Care, St. Paul, MN, USA). The actual osmotic water potential $\Psi_{o(act)}$ of the terminal bud was measured as described above before the onset of the freezing test (control), after 4 h exposure at −6 °C (frozen) and after the twig samples had thawed. The terminal bud was detached from the twigs at the insertion point of the bud scales. All bud scales down to the true leaves were removed. If the buds were abscised in the frozen state, they were allowed to thaw at +4 °C for 30 min before expressed saps were collected and the measurement of $\Psi_{o(act)}$ started; 8–24 replicates were used in each experiment.

Infrared differential thermal analysis

To study the freezing pattern in twigs and buds, ice nucleation and propagation were measured with infrared video thermography. A ThermaCAM S60 (FLIR Systems, Danderyd, Sweden) was used, which was equipped with a close-up lens (LW64/150) to achieve a spatial resolution of 200 μm. Infrared images were recorded at a measurement interval of 100 ms. Further analysis of the infrared images was performed by IDTA with the software ThermaCAM Researcher (FLIR Systems, Danderyd, Sweden) as described in Hacker and Neuner (2007). Detached apple twigs, either entire or dissected longitudinally, were used. For infrared thermography, they were fixed on a plastic plate to keep them in the focus plane. Then, the samples were exposed to controlled cooling experiments with cooling rates of 4 and 8 K h$^{-1}$ down to a target temperature of −25 °C with a computer-controlled commercial freezer (described in Hacker and Neuner 2007). Additionally, 6 type-T thermocouples (solder junction diameter 0.13 mm) were placed on the surface of the twigs to provide reference temperatures at a 10-s interval with a CR10 data logger (Campbell Scientific).

In Experiment 1, terminal apple shoots were collected on 16 March 2009 and the entire terminal shoots were used. Buds
were still dormant but already in the process of frost dehardening. In Experiment 2, terminal apple shoots were collected on 31 January 2010, and only the longitudinal sections were used. The buds in Experiment 2 were in a state of winter dormancy and still had maximum winter frost resistance. Experiments with terminal buds after bud break were performed with buds sampled at the developmental stages commonly described as ‘silver tip’, ‘green tip’, ‘tight cluster’ and ‘full bloom’. A classification of floral bud phenology was done according to the descriptions given in the BBCH scale (Lancashire et al. 1991, Meier 1997).

**Statistical data analysis**

Values are presented as means ± standard error of the means (SE). Replicate numbers are given in the figures or in the figure legends. Significant differences between mean values of normally distributed data with equal residuals were tested using one-way analysis of variance and the Bonferroni post hoc test (\( P < 0.05 \)). If homogeneity of variances was not given, the Tamhane post hoc test was used. All analyses are carried out using PASW Statistics 18 (formerly SPSS, IBM Corporation, New York, NY, USA).

**Results**

**Actual osmotic water potential** \( \Psi_{o(actual)} \)

\( \Psi_{o(actual)} \) measured in bark tissue showed a seasonal course with significantly lower values during winter in both years (Figures 1a and 2a). Lowest values were measured in December of both years, i.e., \(-4.3 \) and \(-3.1 \) MPa, respectively. In May of both years, \( \Psi_{o(actual)} \) values were around \(-1.3 \) MPa. In bud tissue, a significant decrease of \( \Psi_{o(actual)} \) was found only in winter 2009/2010. Throughout the measurement period, \( \Psi_{o(actual)} \) values measured on bud tissue were less negative than those determined in bark tissue. A maximum difference of \( \Psi_{o(actual)} \) between the two tissues was observed in December 2009, when \( \Psi_{o(actual)} \) of the bark and that of the bud differed by 2.6 MPa. In December 2010, the difference in \( \Psi_{o(actual)} \) between the tissues was less and amounted to 1.1 MPa.

**Osmotic water potential at full saturation** \( \Psi_{o(sat)} \)

\( \Psi_{o(sat)} \) measured in bark tissue showed a significant decrease during winter of both years (Figures 1b and 2b) indicating osmotic adjustment. The seasonal minimum during both measurement years amounted to \(-2.9 \) and \(-2.4 \) MPa, respectively. In May, after the commencement of growth, \( \Psi_{o(sat)} \) values increased to \(-1.2 \) MPa. The values of \( \Psi_{o(sat)} \) of the terminal bud tissue showed no significant seasonal change, even though values tended to increase slightly in spring. \( \Psi_{o(sat)} \) values of buds were in both years less negative than those of the bark tissue.

In spring of both measurement years, there was no significant difference between \( \Psi_{o(actual)} \) and \( \Psi_{o(sat)} \) of the bark tissue. However, values differed significantly in the winter of both years. This difference (\( \Delta \Psi_{o(sat)} \)) reached a maximum of 1.6 MPa in January 2010. In winter 2010/2011, \( \Delta \Psi_{o(sat)} \) was less pronounced showing a maximum of 0.9 MPa in December. In the bud tissue, the maximum difference \( \Delta \Psi_{o(sat)} \) between \( \Psi_{o(actual)} \) and \( \Psi_{o(sat)} \) values amounted to 1.3 MPa in February 2010, and 0.6 MPa in December 2010, while in spring there was no significant difference. Similar results were obtained on the apple cultivars ‘Nicoter’ and ‘Braeburn’ (see Appendix 1 and Appendix 2 available as Supplementary Data at Tree Physiology Online).

**Determination of bud frost resistance**

Frost resistance (temperature of initial frost damage, LT) of terminal buds varied seasonally in both measurement years. The maximum frost resistance of \(-30 \) °C was measured during midwinter (Figures 1a and 2a). During the frost-dehardening period in spring, frost resistance of buds decreased to a value of \(-5 \) °C (see Figure 1a). This low value was measured during bud break.
Determination of translocated ice

The actual osmotic water potential $\Psi_{o(act)}$ of terminal buds detached from twigs in the frozen state (after 4 h exposure at $-6$ °C) averaged $-3.0$ MPa (Figure 3). This value was significantly lower ($P < 0.01$) than in unfrozen buds measured before the onset of the freezing test ($-1.9$ MPa). Also in thawed buds detached from the twigs after the freezing treatment, $\Psi_{o(act)}$ was significantly different ($-2.0$ MPa). This change of $\Psi_{o(act)}$ in buds excised from frozen twigs indicates a significant water migration out of the bud tissue during freezing of twigs.

Infrared differential thermal analysis of dormant buds

Infrared images (Figure 4a) of the surface of twigs and buds (terminal and axillary buds) showed that after ice nucleation, the ice spreads throughout the xylem of the twig, however, without reaching the bud primordial tissue. After the first freezing event (high-temperature exotherm, HTE) also on buds dissected longitudinally, no further freezing event could be detected down to a temperature of $\sim-25$ °C.

Infrared differential thermal analysis of buds after bud break

On terminal apple buds during flushing in spring, HTE occurred at temperatures between $-3.1$ and $-5.6$ °C. Additional freezing events at lower temperatures were easily detectable (Figure 5). Under the experimental conditions, these low-temperature exotherms (LTEs) were observed at temperatures between $-3.4$ and $-8.1$ °C. These freezing events could be measured on entire terminal buds and on buds dissected longitudinally. The LTEs seemed to originate from intracellular ice formation inside the bud tissue that had remained unfrozen and supercooled during the HTE. Low-temperature exotherms were measured only on terminal buds, which on the twig samples used were usually flower buds. The separate freezing events were very likely caused by discontinuous freezing of different parts of reproductive structures in the bud. The other explanation, freezing of supercooled fine films of surface water, cannot be completely excluded. In later developmental stages (‘tight cluster’), supercooling diminished again and all single flower...
buds froze together with the twig (data not shown). Also in experiments with apple flowers (developmental stage ‘full bloom’), no supercooling of the various flower organs could be detected, but all flower parts froze at once (Figure 6).

**Discussion**

Assessment of water potentials in deciduous trees during the leafless period in winter is difficult. Our data show that the measurement of osmotic water potentials in bark tissue can be used to assess osmotic adjustments and indicate the severity of desiccation in deciduous trees during winter. While for evergreen conifer shoots and for leaves of other evergreen tree species, an osmotic adjustment over winter has already been reported (Tyree et al. 1978, Auge and Stodola 1989, Yelenosky and Guy 1989, Gross and Koch 1991, Colombo and Teng 1992, Neuner et al. 1999, Norisada et al. 2005, Callister et al. 2008), to our knowledge, there are no observations of winter values of \( \Psi_{o(sat)} \) for bark tissues available. Osmotic water potentials at full saturation \( \Psi_{o(sat)} \) of bark tissue showed a seasonal decrease with significantly lower values in winter. This indicates active osmotic adjustment in apple bark tissue in winter, which can be achieved by the accumulation of osmotically active substances (Sakai and Larcher 1987). Osmotic adjustment has a functional role in maintaining the cell turgor by improvement of dehydration tolerance (Jones and Turner 1978) and can be understood as an adaptation to winter-specific drought stress. Also the actual osmotic water potential of bark tissue in apple trees decreased during both winter periods, which is in addition to osmotic adjustments caused by an overall dehydration of the bark tissue of the apple trees during winter. In comparison with critical threshold \( \Psi_{o(act)} \) values for drought damage in the bark, \( \Psi_{o(act)} \) measurements could be employed as a sensitive indicator of drought stress.

The only reported \( \Psi_{o(sat)} \) values for bark tissue are in the range of our values obtained on the expressed sap of bark tissue in May of both measurement years. Rosner and Kikuta (2002) determined \( \Psi_{o(sat)} \) of secondary phloem of Norway spruce during summer using tissue discs. The authors report an August value for \( \Psi_{o(sat)} \) varying between \(-1.3\) and \(-0.9\) MPa,
depending on the measurement method employed. In the current study, osmotic water potentials were obtained on expressed tissue saps. In the expressed sap of tissues, a dilution of symplastic with apoplastic liquids occurs; therefore, the values can be less negative than if intact plant tissue is used (Wenkert 1980, Kikuta and Richter 1992). Further, Kikuta and Richter (1992) indicated that in expressed saps osmotically active solutes may not be completely extracted. Therefore, expressed saps may additionally underestimate the osmotic potential of the symplastic solution. In contrast, it was shown recently that osmotic water potentials obtained on expressed saps—also if less negative—are comparable with values obtained by pressure–volume analysis (Callister et al. 2006). The authors did a comparative study on the different methods to measure osmotic water potentials of leaves from trees. The pressure–volume technique is time consuming and not applicable for use on all tissues. The osmometry of expressed saps from leaves is therefore proposed as a suitable method for large-scale investigations (Callister et al. 2006), which is corroborated by our results on the seasonal dynamic of osmotic water potentials in stem tissues of deciduous apple trees. Additionally, in a recent meta-analysis, Bartlett et al. (2012) showed that \( \Psi_{o(sat)} \) is the main driver for the water potential at the turgor loss point. Shifting the turgor loss point to more negative water potentials helps maintaining turgor down to lower water contents and may therefore be a suitable drought tolerance parameter. In the current study, osmotic adjustment and therefore the increased dehydration tolerance were found only in bark tissue and not in bud tissue.

In bud tissue, only the actual osmotic water potential \( \Psi_{o(act)} \) in winter 2009/2010 showed a significant seasonal change. A similar winter decrease of \( \Psi_{o(act)} \) to what we found for apple bud tissue was reported for terminal buds of Fraxinus excelsior L. (Cottignies 1990). Osmotic water potentials \( \Psi_{o(act)} \) and \( \Psi_{o(sat)} \) of apple bud tissue were always less negative than those of the bark tissue. Differences in the concentration of solutes among the two tissues are also reported for Juglans regia L. having higher sucrose contents in bark than in bud tissue during winter (Bonhomme et al. 2010). This corroborates our results of a lower osmotic water potential in the bark tissue of apple trees than in bud tissue. Gradients in total water potential between buds and the xylem during winter are reported for Larix lyallii Parl. growing at the timberline (Richards and Bliss 1986). Because of the large differences in water potential found between the two tissues, the authors conclude that buds were isolated from the xylem during winter. This isolation would allow buds of L. lyallii to avoid winter desiccation.

The question is how a bud tissue can hold water against a much more negative osmotic water potential in the bark tissue. We assume that this could be due to a lower cell wall pressure in bud tissues due to lower rigidity of cell walls than in the bark. In this way, the total water potential of the cells in the bark—due to a higher cell wall pressure potential in bark tissue—could be equal to that of the bud primordial cells, which allows them to keep water inside the tissue. This difference in cell wall pressure potential is evident, as bud tissue consists of widely undifferentiated cells with only primary cell walls. Additionally, this difference might be brought about by active changes of cell wall elasticity during winter as reported, for instance, for conifers (Zwiazek et al. 2001) or for leaves of desert shrubs (Scholz et al. 2012).

An additional hypothesis for the marked differences in \( \Psi_{o(act)} \) measured in midwinter (up to 2.6 MPa in December 2009) could be that, due to dehydration and embolism formation in the xylem in midwinter, the water in bud primordial tissue does not have direct contact with the water in bark tissue. This disconnection as already discussed for buds of L. lyallii (Richards and Bliss 1986) would allow winter desiccation of bud tissue to be avoided. The formation of a water-free zone between the two tissues prevents water migration following a water potential gradient. Dry zones between buds and other stem tissues have been reported to form during winter. In frozen peach flower buds, a dry region between the bud primordia and the bud scales and axis has been described, and was thought to
function as an ice barrier protecting the bud primordial tissue from ice entrance (Quamme 1978). Peach buds perform deep supercooling, and staying ice free is therefore essential. Similarly, the interruption of vascular continuity seems to play a role for frost survival of buds of some Prunus species performing deep supercooling (Ashworth 1982, Ashworth 1984). In contrast, in non-deep supercooling buds of Prunus species, vascular continuity remained in winter. Wisniewski and Davis (1995) indicated in a paper that the cell walls at the base of the bud scales were very thick and contained a lot of pectin. The authors speculated that this pectin was hygroscopic, but pectins can also release water very readily; so if water freezes in the bud scales, it is then pulled from these cell walls and they in turn would pull water from the bud tissues. Additionally, aquaporins appear to be key players in water removal (Barigah et al. 2013) and may influence the rate at which water is removed from the cells.

In buds of various woody plant species, ice formation was observed at the site of the bud axis and in the bud scales (Wiegand 1906, Quamme 1978, Sakai 1979, Ashworth et al. 1989, Ishikawa et al. 1997, Ide et al. 1998, Endoh et al. 2009). This freezing behaviour of buds was defined as extra-organ freezing (Sakai 1979, Sakai and Larcher 1987). Two mechanisms can be found to explain extra-organ freezing: one, where buds perform deep supercooling and the other where the buds do not perform supercool but allow freeze dehydration (Ishikawa et al. 1997). Dormant apple buds seem to belong to the latter (Quamme 1991).

By the use of IDTA, we could show that in dormant buds during the high-temperature exotherm (HTE), where extracellular water freezes in the apoplast, the ice is not spreading into the bud primordial tissue. Hence, there must be an ice barrier at the bud base preventing ice propagation into the bud primordial tissue. At temperatures below the HTE in dormant buds, no further freezing event could be detected. This corroborates earlier findings that dormant apple buds do not show deep supercooling (Quamme 1991). However, in buds during flushing in spring, supercooling could be demonstrated in the current study. The measured freezing events occurred after apoplastic freezing and seemed to originate from supercooled parts of reproductive structures. At this developmental stage, frost events in the field may still occur. Supercooling might be a strategy to protect reproductive structures from frost damage during certain stages after bud break. Supercooling was not detected in the developmental stages of ‘tight cluster’ and ‘full bloom’. Intrinsic ice nucleation in a detached apple flower occurred at low temperatures (~10.4 °C) and all flower parts froze at once.

Our results indicate water movement from the bud primordial tissue towards ice masses in the stem and/or scales during freezing. A movement of water along a water potential gradient towards the site of ice formation outside of the bud primordial tissue upon extra-organ freezing has been demonstrated several times for woody species (Rhododendron: Ishikawa and Sakai 1981, Cornus officinalis Sieb-et-Zucc.: Ishikawa and Sakai 1985, Acer japonicum Thunb.: Ishikawa et al. 1997).

Frost resistance of bud tissue, similarly to other frost-resistant cells, could depend on a sufficiently high water-retaining power of cells, as extracellular ice formation may remove ‘vital water’ from the symplast resulting in lethal desiccation stress (Weiser 1970). Survival at low temperature seems to be a function of tolerance rather than resistance to desiccation (Gusta et al. 1975, Burke et al. 1976, Levitt 1980). As \( \Psi_{o(sat)} \) values of bud tissue remained unchanged, our results do not suggest an increased dehydration tolerance of bud tissue during frost hardening. Decreased \( \Psi_{o(act)} \) values may indicate increased dehydration during winter. Studies on the amount of remaining liquid water in bud tissues by differential scanning calorimetry and nuclear magnetic resonance in relation to the level of frost resistance yielded contradictory results, as either increases or no relationships were reported (reviewed by Vertucci and Stushnoff 1992). Nuclear magnetic resonance studies in apple buds showed that the condition of freezeable liquid water can change with changing frost resistance (Vertucci and Stushnoff 1992). In apple buds, it was shown that water in dormant buds had restricted mobility (Faust et al. 1991), but dormancy was not directly related to frost resistance. Similar results on the relationship between water mobility and dormancy were also reported for axillary bud and vascular bud trace regions of poplar trees (Kalcsits et al. 2009). Still, our results suggest no increase in dehydration tolerance during frost hardening in apple bud tissue (by unchanged \( \Psi_{o(sat)} \) values), but a certain dehydration of bud tissue in winter as illustrated by reduced \( \Psi_{o(act)} \).

Supplementary data

Supplementary data for this article are available at Tree Physiology Online.

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