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FATTY ACID AND ALKANE CHANGES IN WILLOW DURING FROST-HARDENING

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Key Word Index—*Salix viminalis*; Salicaceae; willow; fatty acids; *n*-alkanes; freezing tolerance; frost-hardening.

Abstract—Frost-hardening of 10 basket willow (*Salix viminalis*) clones was studied by visual scoring of twig recuperation after controlled freezing treatments, together with measurements of total fatty acids, total *n*-alkanes (gas chromatography) and dry wts (% of fresh wt) of the twigs during the fall of 1994 in central Finland. The initial frost-tolerance of the clones was −5°. Hardening of the clones began at the end of September and proceeded similarly until mid October, when clonal differences became observable. The final frost-tolerances of the clones at the end of November varied from −37° to −80°, as 50% killing temperatures. Twig dry wts showed some increase prior to hardening, but an abrupt increase was observed during the first four weeks of hardening. After this, the increase in dry wts ceased. The total amount of twig *n*-alkanes (mg g^{-1} dry wt) decreased constantly prior to hardening until October. The total amounts of fatty acids (mg g^{-1} dry wt) decreased prior to hardening but doubled after hardening had begun. This increase in total fatty acids was accompanied by an abrupt increase in the ratio of unsaturated to saturated fatty acids. The amounts of linoleic (18:2) acid began to increase at the expense of linolenic (18:3) acid simultaneously with the beginning of hardening. No significant changes in fatty acids, *n*-alkanes or dry weights were observed during the late hardening stage. The differences in fatty acid profiles of the clones could not be attributed to clonal differences in frost-tolerances in the late hardening stage.

INTRODUCTION

The poor overwintering capability of willows with high biomass productivity is one of the major obstacles to the broader application of willows for bioenergy production in northern latitudes. One of the main goals for breeding is to develop short-rotation willow clones with high overwintering capacity together with high productivity [1]. Although, in general, most willows have a genetic ability to achieve extreme freezing-tolerance [2], the best biomass producers, usually of southern origin, often have poor overwintering capability due to their late hardening [1, 3, 4].

Overwintering ability of willows depends on various factors and processes. The most important processes are hardening and dehardening. Hardening is a complex process which includes physiological, physicochemical and chemical changes, and it enables plants to achieve high frost-tolerance, which is essential for overwintering in northern hemispheres. Even southern willows with poor capability to overwinter in cold climates have a genetic capacity to achieve an extreme freezing-tolerance, if the hardening process can be initiated [2, 5]. Timing of hardening and dehardening is one of the most critical factors in overwintering [3]. The poor overwintering of high producer willows in northern hemispheres is often caused by the late initiation of the hardening process and the damage caused by the first night frosts [1]. The prerequisite for hardening of willows is growth cessation, which is usually triggered by cool nights, shortening days and sub-zero temperatures, like the first night frosts. Desiccation-stress may also induce hardening [2, 5].

The most studied biochemical changes in plant cells during frost-hardening occur in cytoplasm and in cell membranes [6]. The changes in cytoplasm include an increase in soluble proteins and ribonucleic acids, an

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accumulation of sugars and sugar alcohols, and degradation of polysaccharides [5–9]. In plasma membranes, the total amount of membrane lipids, contents of phospholipids and unsaturation of fatty acids have been observed to increase with hardening [6, 10]. The increase in the unsaturation of fatty acids during hardening has been well established for herbaceous plants [10–12]. It has been suggested that the increase in the unsaturation of membrane fatty acids lowers the phase transition temperature of the membranes, thus maintaining an adequate permeability of the cells at low temperatures [6]. Several reports exist which suggest that changes in the total amount of lipids, especially in the amounts of polar lipids, are more important in the hardening of woody plants than the changes in fatty acid unsaturation [8, 13–15]. The increase in total lipids is connected to the increase in membrane area and flexibility, and further to an improved tolerance of cell collapse during freezing dehydration [6]. The increase in phospholipids, especially unsaturated phosphatidylcholine, and an increase in the phospholipid : sterol or phospholipid : cerebroside ratios, both prevent micellar separation from plasma membranes and phase-transitions during cell dehydration [10].

According to recent studies [16, 17], low amounts of *n*-alkanes in nonhardened stems of willow clones correlate well with the high overwintering survival of the clones but their fatty acids do not show any relation to their overwintering capability. It was suggested that *n*-alkanes were connected to timing of hardening via control of water relations in willow [4, 16, 17]. As large changes in fatty acids are common in woody plants during hardening [8, 13–15], it became of interest to study hardening-related changes in willow *n*-alkanes and water contents, together with changes in fatty acids. For this we analysed the changes in fatty acids, *n*-alkanes and dry wts of stems of 10 clones of field-grown basket-willow (*Salix viminalis*), with respect to stem frost-hardening, studied by visual scoring of recuperation of twigs after controlled freezing treatments.

RESULTS AND DISCUSSION

Frost-hardening

During July and August, the 50% killing temperatures (LT_{50}) of all clones, determined by the visual scoring, were ca $-5°$ (Fig. 1). Hardening of all the studied clones began at the end of September. During the period from Sept 20 to Oct 4 the LT_{50} values of all clones dropped to below $-10°$ and, during the following 2 weeks, to below $-20°$. Frost-hardening was similar among the clones until mid-October (Oct 18), when the hardening process became slower in clones 6, 8 and 9. Some retardation was also observed in the hardening of clones 2, 7 and 10, between Nov 1 and Nov 28, while the hardening of clones 1, 3, 4 and 5 continued to the end of the sampling period.

In order to analyse clonal differences in dry wt content, *n*-alkanes and fatty acids, a clonal categorisation was made based on the late hardening phase, i.e., the final LT_{50} values on Nov. 28. The first category represents extreme frost-tolerant clones (clones 1, 3, 4, 5: final LT_{50} $-70°$), the second intermediate tolerant clones (clones 2, 7, 10: final LT_{50} $-46.2°$–$-54.4°$) and the third category least tolerant clones (6, 8, 9: final LT_{50} $-36.7°$–$-45.5°$).

Twig dry weights

The initial dry matter contents (dry wt, % of fr. wt; *DW*%) of the clones in July varied between 4.1 and 20 %. Between June 19 and Sept 20, the overall trend in twig dry wts was increasing but not significant. An abrupt and significant increase in *DW*% appeared between Sept 20 and Oct 18, during the first weeks of hardening (Fig. 2). During this period, *DW*% increased from 18.5–30.9% to 43.4–45.9%. After this, the overall trend in *DW*% was decreasing, but not significant. An increase in plant dry matter content has often been observed during hardening [5] and it may enhance the ability of the twigs to supercool [8]. Desiccation-stress applied to willows has also been shown to be capable of triggering the hardening process [3]. The observed increase in twig *DW*% (decrease in twig water contents) during the first weeks of hardening may play an important role in the hardening process of willow, either by directly affecting freezing-tolerance or by triggering the hardening.

Twig n-*alkanes*

The major *n*-alkanes of the willow twigs were *n*-heptacosane (55–70%), *n*-nonacosane (30–47%) and *n*-pentacosane (1–5%). The *n*-alkane composition showed no significant correlation with the freezing tolerances of the clones. The *n*-alkane contents of the clones decreased notably in September and levelled off at ca 1.5 mg g^{-1} dry wt on Oct. 4, just after the beginning of hardening (Fig. 3). As *n*-alkanes are major epicuticular wax components in willow [4], the decrease of the *n*-alkane content at the end of growing season may reflect a decrease in wax synthesis, and also decrease in overall metabolic activity in the willow stems during prehardening. Although the clonal differences in *n*-alkanes did not show any significant correlation with final frost-tolerances of the clones examined, the present observation of the decrease in the willow twig *n*-alkanes (mg g^{-1}) during the prehardening phase, together with previous observations of correlations between low twig and leaf *n*-alkane, low leaf wax contents and the high overwintering survival of willow clones [4, 16, 17], suggests a connection between waxes and hardening. One of the main functions of epidermal waxes is to reduce cuticular transpiration and, thus, prevent water loss [18]. It may be suggested that the wax load on twig cuticles reaches a minimum, which is not sufficient to prevent water loss.

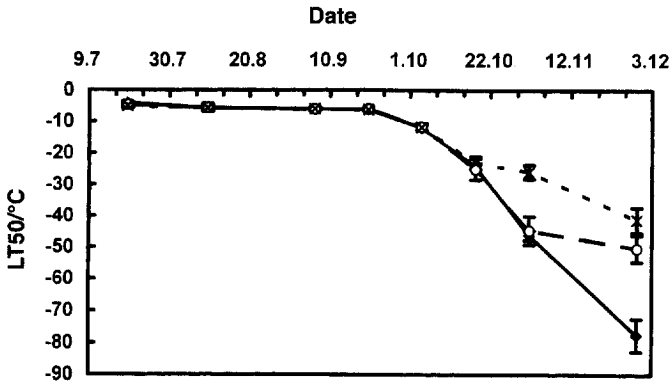

Fig. 1. Frost hardening of willow clones growing in field conditions in 1994 in eastern Finland determined by visual scoring of twig recuperation after freezing treatments. Solid line (◆): category 1 clones (1, 3, 4, 5); Dashed line (○): category 2 clones (2, 7, 10); dotted line (x): category 3 clones (6, 8, 9). Bars represent clonal variation within each category.

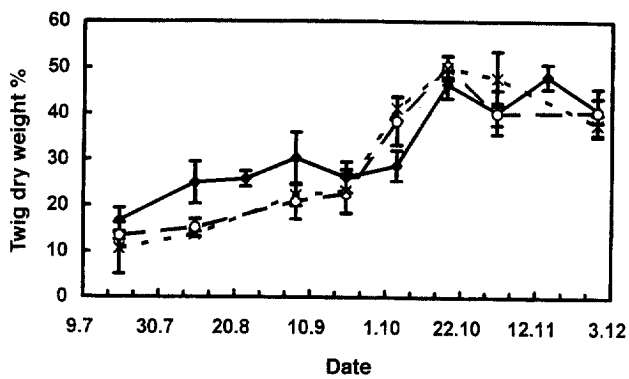

Fig. 2. Changes in dry wt content (% fr. wt) of willow twigs during hardening. Solid line (◆): category 1 clones (1, 3, 4, 5); Dashed line (○): category 2 clones (2, 7, 10); dotted line (x): category 3 clones (6, 8, 9). Bars represent clonal variation within each category.

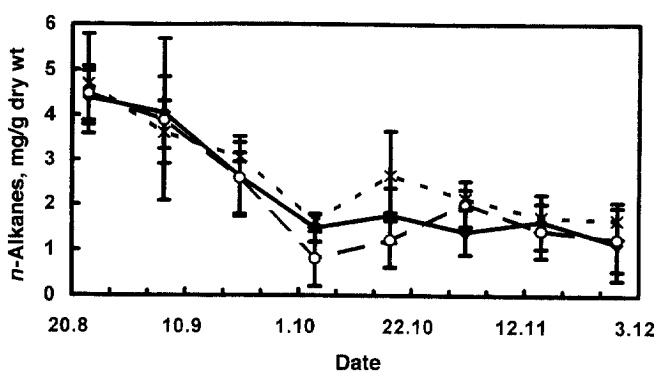

Fig. 3. Changes in total amounts of *n*-alkanes (mg g^{-1} dry wt) during hardening. Solid line (◆): category 1 clones (1, 3, 4, 5); Dashed line (○): category 2 clones (2, 7, 10); dotted line (x): category 3 clones (6, 8, 9). Bars represent clonal variation within each category.

On the other hand, water uptake from soil derives its energy from foliar transpiration [18]. Foliar senescence cuts the primary energy source of water uptake and may also explain the rapid increase in twig dry weights.

Fatty acids

The most notable changes associated with hardening were observed in the amounts of total fatty acids (Fig. 4), in the contents of linolenic and linoleic acids

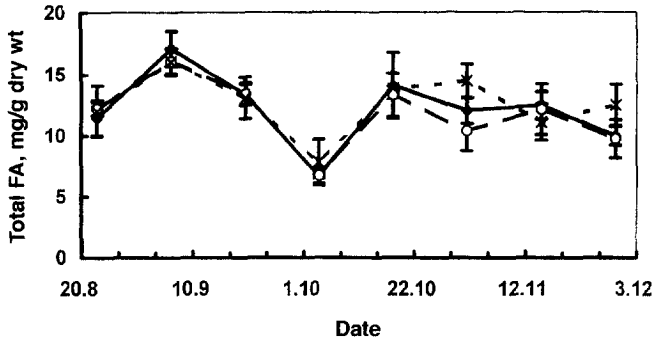

Fig. 4. Total fatty acids (FA) (mg g^{-1} dry wt) in willow stems during hardening. Solid line (◆): category 1 clones (1, 3, 4, 5); Dashed line (○): category 2 clones (2, 7, 10); dotted line (x): category 3 clones (6, 8, 9). Bars represent clonal variation within each category.

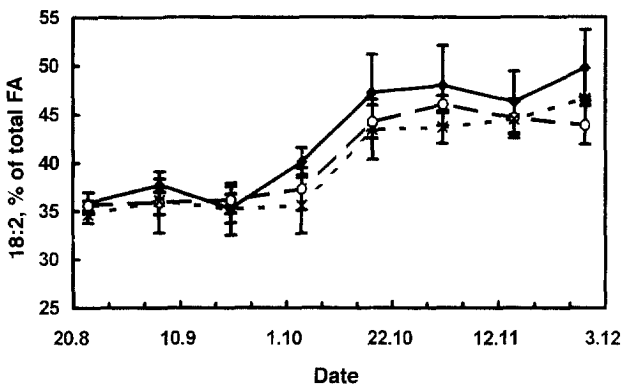

Fig. 5a. Amounts of linoleic acid (18:2) in willow stems during hardening. Solid line (◆): category 1 clones (1, 3, 4, 5); Dashed line (○): category 2 clones (2, 7, 10); dotted line (x): category 3 clones (6, 8, 9). Bars represent clonal variation within each category.

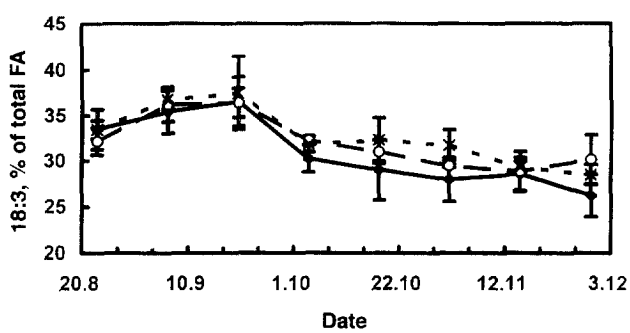

Fig. 5b. Amounts of linolenic acid (18:3) in willow stems during hardening. Solid line (◆): category 1 clones (1, 3, 4, 5); Dashed line (○): category 2 clones (2, 7, 10); dotted line (x): category 3 clones (6, 8, 9). Bars represent clonal variation within each category.

(Fig. 5) and in the ratio of unsaturated to saturated fatty acids (Fig 6). However, no significant correlation between individual fatty acid profiles of different clones and the different final frost-tolerances of the clones was observed.

The major fatty acids in the stems of willow were palmitic (16:0) (15.4–21.1% (w/w) of total fatty acids), linoleic (18:2) (33.2–54.6% (w/w)) and linolenic (18:3) (23.8–39.2%). Minor fatty acids included stearic (1.0–3.1% (w/w)), oleic (1.6–6.0), eicosanoic (0.3–9.5 %) and docosanoic acid (0.6–3.6% (w/w)). The total amount of fatty acids varied from 6.4 to 18.7 mg g^{-1} dry wt. The main changes in the amount of total fatty acids observed in this study included a decrease in total fatty acids during a period from Sept 6 to Oct 4 and a sharp increase during the following two weeks (Fig 4). The amounts of 18:3 began to decrease and those of 18:2 began to increase after Sept 20 (Figs 5a and b). Furthermore, the ratio of unsaturated acids (18:1, 18:2, 18:3) to saturated

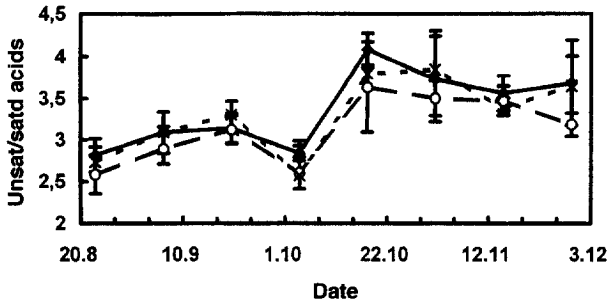

Fig. 6. Ratio of unsaturated to saturated fatty acids (18:1+18:2+18:3)%/(16:0+18:0+20:0+22:0)% in willow stems during hardening. Solid line (◆): category 1 clones (1, 3, 4, 5); Dashed line (○): category 2 clones (2, 7, 10); dotted line (x): category 3 clones (6, 8, 9). Bars represent clonal variation within each category.

acids (16:0, 18:0, 20:0, 22:0) decreased between Sept 20 and Oct 4 but showed a sharp increase during the following two weeks (Fig. 6).

The first decrease in the amount of total fatty acids in September prior to hardening could be associated with growth cessation and maturation of phloem and xylem cells [19, 20]. The sharp increase at the beginning of October when hardening commenced, on the other hand, suggests an increase in protoplasmic membranes. The increase in total lipids and polar lipids has also been observed during the hardening of mulberry bark and poplar [13, 15]. It has been suggested that the increase in membrane lipids may enhance the flexibility of the protoplast membranes and, thus, increase the freezing dehydration tolerance of cells [6, 10].

The concentrations of 18:2 and 18:3 showed a close correlation with the initiation of hardening (Figs 5a and b). 18:2 began to increase at the expense of 18:3 at the end of September, when hardening was initiated. As 18:3 is the major fatty acid in chloroplasts [21], the changes in 18:3 may be connected to aggregation of chloroplasts, which has been observed to occur in stem cortex during hardening of deciduous trees [6]. The increase in 18:2 may be associated with the increase in total fatty acids and, further, with changes in cell membranes. The increase in 18:2 content during hardening has also been observed in mulberry bark [15], red pine (*Pinus resinosa*) and Austrian pine (*Pinus nigra*) needles [22].

The ratio of the unsaturated fatty acids (18:1, 18:2 and 18:3) to saturated fatty acids (16:0, 18:0, 20:0 and 22:0) illustrates the hardening-associated changes (Fig. 6). The ratio decreased slightly at the end of September before and during the initiation of hardening and was followed by an abrupt increase between Oct 4 and Oct 18. Double bond index (DBI) showed a similar development, but it was not as significant due to the higher relative effect of the decrease in 18:3 and was not presented here as a figure. The first decrease in unsaturation was connected with the decrease in 18:3, while the subsequent increase illustrates the increase in the contents of 18:2, suggesting an increase in the unsaturation of cell membranes. Similar changes were observed in the fatty acids of protoplast phospholipids of mulberry bark cells [15], i.e. a parallel increase of freezing tolerance and the ratio of unsaturated to saturated fatty acids. The increase in membrane unsaturation has been suggested to improve cell permeability and, thus, prevent cell damage during freeze-dehydration [6].

In conclusion, the progress of hardening could be divided into three stages. The prehardening stage was associated with a decrease in *n*-alkanes, in the total amount of fatty acids and the ratio of unsaturated to saturated fatty acids. These changes indicate growth cessation and a decrease in metabolic activity. The early hardening stage was accompanied by an increase in fatty acid unsaturation, amounts of 18:2, total fatty acids and twig dry wts. These changes suggest both quantitative and qualitative changes in cell membranes and cell water-status. During late hardening, no biochemical changes were clearly attributed to progress of the hardening. This suggests that early hardening is related to changes in membrane fatty acids and water relations, while extreme hardening is mediated by physical and physicochemical processes [6]. Thus, it is not suprising that fatty acids showed no relation to clonal differences in frost-tolerances, because these differences only became evident at the late hardening stage. Furthermore, these results indicate the importance of the control of water relations in willow before and during hardening. Changes in fatty acids at the beginning of the hardening, including an abrupt increase in total fatty acids and fatty acid unsaturation, together with an increase in 18:2 and a decrease in 18:3, may be used as markers for predicting initiation of hardening in willows.

EXPERIMENTAL

Plant material

Stems of 10 fast-growing clones of basket willow (*S. viminalis*) were collected from a field experiment established at Tohmajärvi (eastern Finland) (62°14N, 30°21E, 90 m a.s.l.) by the University of Joensuu. Clones were originally obtained from Sweden and

their clone numbers were 832502 (clone 1); 810217 (clone 2); 78112 (clone 3); 78183 (clone 4); 832803 (clone 5); 821624 (clone 6); 78021 (clone 7); 811110 (clone 8); 812509 (clone 9); 832501 (clone 10) [23]. The work numbers used in this study are presented in brackets. The experiment was established in 1992 on a fertile site with high N (total N 0.8% dry wt) and organic matter (38% dry wt) contents. The site was formerly used as pasture land. Stem sections of current year shoots were collected for dry wt and freezing-tolerance testing at two-week intervals between 18 July 1994 and 28 November 1994. The samples (ca 20 cm long) were taken from the upper part of the shoot (one sample per shoot, ca 15 cm from the top). At each sampling time, 56 samples per clone were collected for frost-hardiness assessment by controlled freezing tests. Sixteen samples per clone were freeze-dried for biochemical analyses. Sampling for biochemical analyses started at Aug 20.

Freezing treatment

At two-week intervals, the frost-hardiness of willow cuttings was assessed by visual damage scoring. For this purpose, samples were set in plastic bags and exposed to six different frost temps in air-cooled chambers. The number of replicates for each treatment temp. was eight for each clone; +5° always represented the control. In the freezing program, the start and final temp. of the treatment was 10°, the rate of cooling and warming was 5° h^{-1} and the minimum temp. was maintained for 4 h. The minimum temp. achieved by the equipment was −70°. After treatment, shoots were set in a small amount of H_2O in a beaker and moved to controlled greenhouse conditions (22/15° and 16/8 h for light/dark, respectively) where they were allowed to recuperate for 14 days. Shoots were then scored visually into 10 damage classes at 10% intervals, depending on the colour of the shoot and growth of new roots. A logistic sigmoid function was used to determine the temp. with 50% survival (LT_{50}), i.e. the inflection point of the function [24].

Determination of dry weight

The fr. wt of samples ($n = 16$) was examined and the samples were dried for 24 h in an oven (105°). Dry wts were examined at an accuracy of 0.01 g and dry wt content was calculated as the ratio between the dry and fr. wts.

Determination of total fatty acids and n*-alkanes*

Fatty acids and *n*-alkanes were determined from freeze-dried stem sections by GC [25]; ca 10 g of freeze-dried material was cut into small pieces, mixed and 100 mg was used for each duplicated trial. Samples were saponified with NaOH in MeOH, methylated in acidic MeOH, neutralised and analysed by GC. GC of fatty acid Me esters and *n*-alkanes was carried out by FID-GC with an automatic injection sampler. The column used was HP-FFAP WCOT (25 m × 0.2 mm × 0.3 μm); carrier gas 1 ml min^{-1} He; split ratio 1:20; inj. and det temp. 250°; temp. prog. from 70 to 200° at 25° min^{-1}. GC peak areas were recorded by integration and compounds identified by RR_ts to internal Me heptadecanoate and verified by mass-selective detector. Fatty acid Me esters and *n*-alkanes were quantitated by comparing their peak areas to that of the int. standard [16, 17, 25].

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REFERENCES

1. Lumme, I. and Törmälä, T., *Silva Fennica.*, 1988, **22**, 67.
2. Sakai, A., *Nature*, 1960, **185**, 393.
3. Sakai, A., *Ecology*, 1970, **51**, 485.
4. Hietala, T., Laakso, S. and Rosenqvist, H., *Phytochemistry*, 1995, **40**, 23.
5. Sakai, A. and Larcher, W., *Frost Survival of Plants, Responses and Adaptation to Freezing Stress* Springer-Verlag, Berlin, Heidelberg, 1987, pp. 97–137.
6. Levitt, J., *Responses of Plants to Environmental Stresses: Chilling, Freezing and High Temperature Stresses, Vol* 1. Academic Press, New York, 1980.
7. Siminovitch, D., Gfeller, F. and Rheaume, B., *Cellular Injury and Resistance in Freezing Organisms. Inst. Low. Temp. Sci. (series B)* Ed. E. Aschina, Hokkaido University, Sapporo, 1967, pp 93-118.
8. Siminovitch, D., Rheaume, B., Pomeroy, K. and Lepage, M., *Cryobiology*, 1968, **5**, 202.
9. Weiser, C. J., *Science*, 1970, **169**, 1269.
10. Steponkus, P. L., Uemura, M. and Webb, M. S., In *Advances in Low-Temperature Biology, Vol* 2. Jai-Press Ltd, 1993, pp. 211–312.
11. Christiansen, M. N., *Recent Advances in Phytochemistry*, 1984, **18**, 177.
12. Murata, M., Ishizaki-Nishizawa, O., Higashi, S., Hayashi, H., Tasaka, Y. and Nishida, I., *Nature*, 1992, **356**, 710.
13. Yoshida, S. and Sakai, A., *Plant and Cell Physiology*, 1973, **14**, 353.
14. Siminovitch, D., Singh, J. and De La Roche, I. A., *Cryobiology*, 1975, **12**, 144.
15. Yoshida, S., *Plant Physiol.*, 1984, **76**, 257.
16. Rosenqvist, H. and Laakso, S., *Phytochemistry*, 1991, **7**, 2161.
17. Rosenqvist, H. and Laakso, S., *Phytochemistry*, 1992, **31**, 97.

18. Martin, J. T. and Juniper, B. E., *The Cuticles of Plants* Edward Arnold, London, 1970.
19. Lawton, J. R., *New Phytologist*, 1976, **77**, 761.
20. Sennerby-Forsse, L., *Physiologia Plantarum*, 1986, **67**, 529.
21. Nobel, P. S., In *Biophysical Plant Physiology and Ecology*. W.H. Freeman and Company, San Francisco, 1983, pp. 1–45.
22. Sutinen, M. -L., *Acta Universitas Ouluensis A*, 1992, **240**.
23. Ager, A., Rönnber-Wästljung, A., Thorsen, J. and Siren, G., *Swedish University of Agricultural Sciences, Department of Ecological and Environmental Research Report*, 1986, **43**.
24. Repo, T. and Lappi, *Scandinavian J. Forest Research*, 1989, **4**, 67.
25. Suutari, M., Liukkonen, K. and Laakso, S., *J. Gen. Microbiol.*, 1990, **136**, 1496.