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Seasonal variation in the nitrogen metabolism of young Scots pine

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TIIVISTELMÄ: MÄNNYN TAIMIEN TYPPIAINEENVAIHDUNNAN VUODENAIKAISVAIHTELUSTA

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Seasonal changes in total nitrogen, protein, amino acid, ammonia, nitrate and nitrite concentrations, and nitrate reductase and γ-glutamyltransferase activities in the needles, buds and shoots of young Scots pine (*Pinus sylvestris* L.) were studied. A relationship between the variation in the nitrogen metabolism and both winter dormancy and its breaking was proposed. Pine tissues stored soluble nitrogen over the winter largely in the form of arginine which, in addition to a high nitrogen content, can neutralize acidic cytoplasmic constituents such as nitrates and nitrites. Specific nitrate reductase and γ-glutamyltransferase activities were highest in late summer or autumn, and is apparently connected to the mobilization of nitrogen reserves for the winter.

Kokonaistypen, proteiinien, aminohappojen, ammoniakin sekä nitraatti- ja nitriittionien pitoisuuksien ja nitraattireduktaasin ja γ-glutamyylitransferaasin aktiivisuuksien vuodenaikaisvaihtelua männyn neulasissa, silmuissa ja versoissa selviteltiin. Tulosten perusteella etsittiin yhteyttä typpiaineenvaihdunnan vaihtelun ja talvilevon sekä sen päättymisen välillä. Männyn solukot varastoivat talveksi liukoista typpeä pääasiassa arginiinina, mikä korkean typpipitoisuutensa lisäksi voinee vielä neutraloida sytoplasman happamia aineita, esimerkiksi nitraatteja ja nitriittejä. Nitraattireduktaasin ja γ-glutamyylitransferaasin spesifiset aktiivisuudet olivat korkeimmillaan loppukesällä tai syksyllä. Nämä aktiivisuuksien huiput liittyvät ilmeisesti typpivarastojen mobilisaatiotapahtumiin talvea varten.

Keywords: amino acids, arginine, proteins, nitrate reductase, γ-glutamyltransferase, pine buds and needles
ODC 174.7 *Pinus sylvestris* +161.4+182.2/.3

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1. Introduction

Plants have several physiological and biochemical mechanisms for controlling cold acclimation: 1) The cytoplasm has the ability to reversibly change from a gel to sol state (Tumanov 1967; the gel state increases when winter arrives, and is then followed by a reconversion of the cytoplasm to the more fluid state in the spring (Jirgensons 1958, Tumanov 1967). 2) Biosynthesis of certain protector proteins takes place in the cyto-

plasm in the autumn (Siminovitch 1963, Siminovitch et al. 1968). 3) The intracellular concentration, both relative and absolute of water decreases (Tumanov 1967, McKenzie et al. 1974, Kaku et al. 1981). This can occur through extracellular ice formation leading to intracellular dehydration (Mazur 1963, Weiser 1970, Christersson 1971, Steponkus 1984). 4) The amount of bound-hydrate water on the surface of the cytoplasmic protein molecules increases in the winter (Tanford 1965, Tumanov 1967). This electrostatically bound water does not form ice crystals. 5) A number of cryoprotectant, low-molecular weight metabolites (sugars, amino acids and other organic acids, amides, alcohols, phenols etc.) accumulate, which prevent ice lattice formation (Sakai and Yoshida 1968, Durzan 1971, Senser et al. 1971, Sagisaka 1974, Kandler et al. 1979, Sagisaka and Araki 1983). 6) The velocity of metabolic processes and enzyme activities needed to produce the above cryoprotectant metabolites increases (Hellergren et al. 1983). 7) There appears solubility changes in membraneous proteins, associated with the stability of the membranes (Ziegler and Kandler 1980). 8) There occur changes in the membraneous and cytoplasmic lipid phase which increase fluidity of the lipids through oxidation (increase of unsaturated fatty acids) (Senser 1982, Senser and Beck 1982), thus leading to increased elasticity and fluidity of the membranes. Changes in the group of phospho- and galactolipids, in particular, have been observed. 9) Increases in cell volume and surface area ratio, and increased organ size (Sakai and Eiga 1983) may allow increased water transport out of the cell. One should bear in mind that the acquisition of frost resistance is a continuous procedure involving the majority of the processes mentioned earlier.

The nitrogen supply and various nitrogenous compounds have an important role in controlling the cold resistance of plants. The status of the nitrogenous constituents is controlled by the availability of nitrates, their reduction to ammonia, the binding of the latter to amino acids and their incorporation into proteins and other biosynthetic products. Too high a level of nitrates may disturb water binding and gel formation of the cytoplasm leading to freezing of cytoplasmic water and causing thus tissue killing and winter damage (Tumanov 1967). The apical buds and shoots seem to be particularly sensitive, and this results in the disappearance of apical dominance leading to increased branching (Will 1971).

Availability of nitrates, the activity of nitrate reductase (Hellergren 1981, Girs et al. 1982), the biosynthesis of amino acids and the control of the concentrations of intra-and extracellular amino acids may be among the first factors affecting the cold acclimation process. This paper summarized observations on different phases of nitrogen metabolism in the needles, buds and shoots of young Scots pine. A functional relationship is proposed between these seasonal observations, and both the wintering processes and the breaking of winter dormancy.

2. Material and methods

Bud and shoot samples were collected from some 20-year-old Scots pine (*Pinus sylvestris* L.) trees growing on a peatland area at Muhos (65°52'N, 25°07'E) as detailed in Pietiläinen and Lähdesmäki (1986). The site was originally a small sedge bog that was drained in 1967 using a ditch spacing of 40 m. The stands were thinned in 1974 to 1500 stems per ha. The mean height growth of the stand had been 29 cm per annum during the last 5-year period (1979–1984). The mean age of the trees was 20 years and height 2–4 m. Most of the chemical analyses were carried out in 1984 and 1985, but the total nitrogen determinations are from a longer period (1982–1985). Samples from about 15 randomly selected trees were combined and stored in plastic bags at –20 °C. At least three determinations were performed from each combined sample. In addition two year old seedlings, grown in a greenhouse under controlled conditions (Pietiläinen and Lähdesmäki 1988) were used for nitrate reductase activity determinations. Young adult trees (30–35 years) were used as control material in some total nitrogen determinations.

Total nitrogen was determined by the Kjeldahl distillation method (Halonen and Tulkki 1981), nitrate, nitrite and ammonium nitrogen using standard methods (Lähdesmäki and Pietiläinen 1988). The protein content was mesured as described by Lowry et al. (1951) employing precipitation with trichloroacetic acid to remove soluble phenolic substances. Free amino acids in tissue homogenates (Pietiläinen and Lähdesmäki 1986) were analyzed in an automatic amino acid analyzer (Kontron Liquimat III).

Nitrate reductase activity was measured with an *in vivo* method developed by Jaworski (1971) in which the amount of nitrite formed, when 0.2 mol/l KNO_3 reacts with the endogenous enzyme, is analyzed. 2 g samples of needles and 0.5 g samples of buds and shoots, cut into approximately 2 mm sections were kept in a vacuum for 10 min to allow the buffer (80 mmol/l potassium phosphate, pH 7.5) to penetrate the tissue. Nitrate reductase activity was determined by first measuring the evolution of NO_2^- immediately after vacuum infiltration in order to obtain the endogenous NO_2^- concentration. Then NO_2^- evolution after 60 min incubation at 25 °C was determined to obtain the enzyme activity. The procedure was carreid out in the dark, and the reaction was stopped by boiling the samples. NO_2^- evolution was determined from 1 ml of the incubation solution mixed with 1 ml 1 % (w/v) sulfanilamide in 3 mol/l HCl solution and 1 ml 0.02 % N-naphtyl-l-ethylene diammonium dichloride in distilled water. The incubation time for the colour reaction was 20 min, and absorbance was measured at 540 nm. Nitrate reductase activity was calculated by subtracting the endogenous NO_2^- from that evolved after 60 min incubation.

γ-glutamyltransferase activity, an enzyme responsible for the transfer of amino acids from the extracellular space to the intracellular one (Meister 1973), was measured according to Meister et al. (1981) using L-γ-glutamyl-p-nitroanilide as the substrate and 2 mmol/l glycylglycine as an acceptor at pH 8.0 (for details, see Pietiläinen and Lähdesmäki 1986).

3. Results

The total nitrogen content of the needles was between 1.0 and 1.3 % fresh weight (Fig. 1). The highers concentrations were obtained in the spring (April-May) and late autumn (October). The contents in June and winter months were approximately the same (about 1.0–1.1 %), but a little higher in July and August. Since enzyme activities and extraction of most of the nitrogen fractions had to be performed with fresh tissues, all the concentrations were calculated per tissue fresh weight. The water content of the pine tissues showed slight seasonal variations, but this had no influence on calculations of the concentrations of the different nitrogen fractions.

The pattern observed in total nitrogen in needles (Fig. 1) was reflected in the pattern of the sum of the two nitrogen fractions in buds and shoots (Fig. 2). The spring-time peak in total nitrogen largely reflected the high level of free, low-molecular weight amino compounds and the autumn peak the protein level (Fig. 2).

Arginine was the most abundant free amino acid in pine tissues, especially in the winter buds (determined in the buds from March, Fig. 3). The concentration of arginine decreased significantly in the shoots in spring and summer, while that of glutamine, in particular, increased at the same time and decreased later. The concentrations of some other amino acids (see a detailed list in Pietiläinen and Lähdesmäki 1986) remained relatively constant. The levels of arginine and glutamine largely determined the concentrations of the summed free, low-molecular weight amino compounds. Arginine, ornithine, citrulline and urea belong to the urea

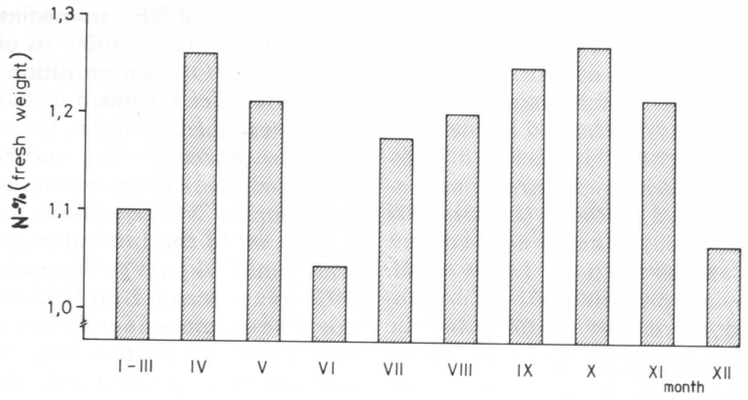

Fig. 1. The mean total nitrogen content of the needles of Scots pine (% of fresh weight) in monthly samples collected throughout the year. Results are means for 6–8 determinations using one year old needles from 20–30 year old trees. SD varied between 3 and 5 %.

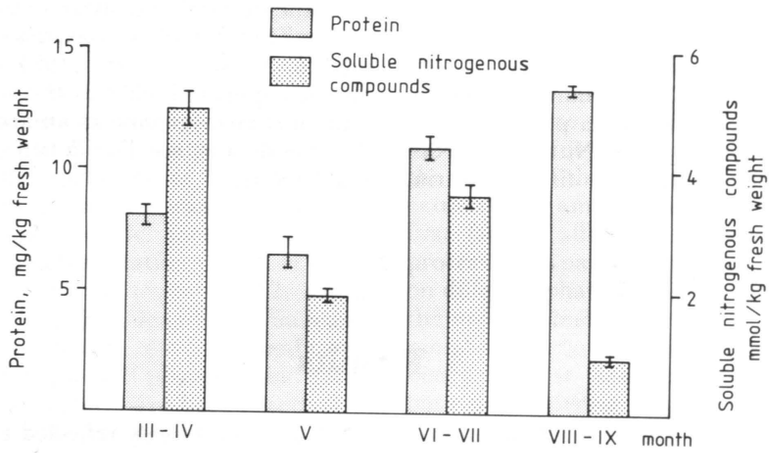

Fig. 2. Seasonal variation in the concentration of proteins and free, low-molecular weight amino compounds in Scots pine buds and shoots. Means (protein as mg, low-molecular weight compounds a s mmol/kg fresh weight) ± SD values are for 3–7 determinations.

cycle nitrogen reserve, and aspartic and glutamic acid plus their amides (asparagine and glutamine) to another metabolically close nitrogen reserve. These two nitrogen reserves dominated the pool of free, low-molecular weight amino compounds (Fig. 4). The concentration of total basic amino acids (arginine, lysine and histidine plus their derivatives) was three times as high in the winter buds as in the shoots in summer.

The levels of ammonium, nitrate and particularly nitrite were lower than those of free amino acids or proteins, calculated as molar or mass concetrations (Figs. 3, 5). The highest nitrate concentrations were measured in the needles during the winter (between October and March), while ammonium nitrogen was highest in the spring (May) and late summer in August (Fig. 5). Nitrite clearly accumulated in the needles in the autumn.

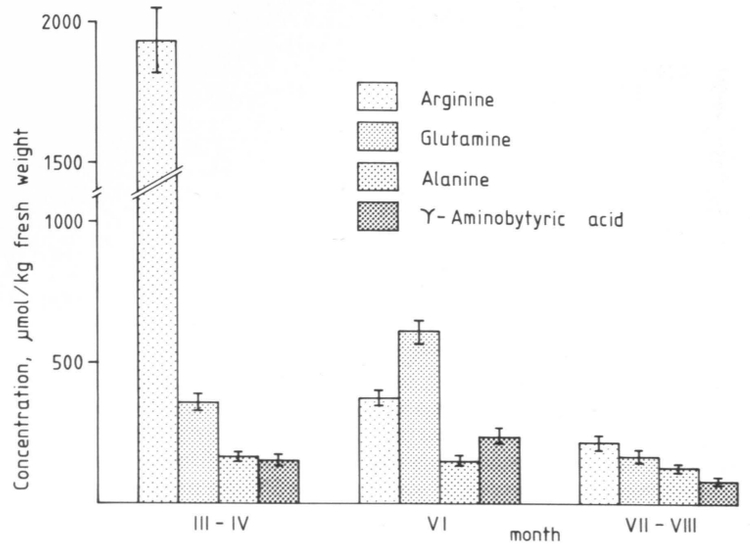

Fig. 3. Examples of amino acids whose concentrations showed considerable seasonal variation (arginine, glutamine) or stability (alanine, γ-aminobutyric acid) in Scots pine buds and shoots, presented during the period from March to August. Means (µmol/kg fresh weight) ± SD values are for 4–7 determinations.

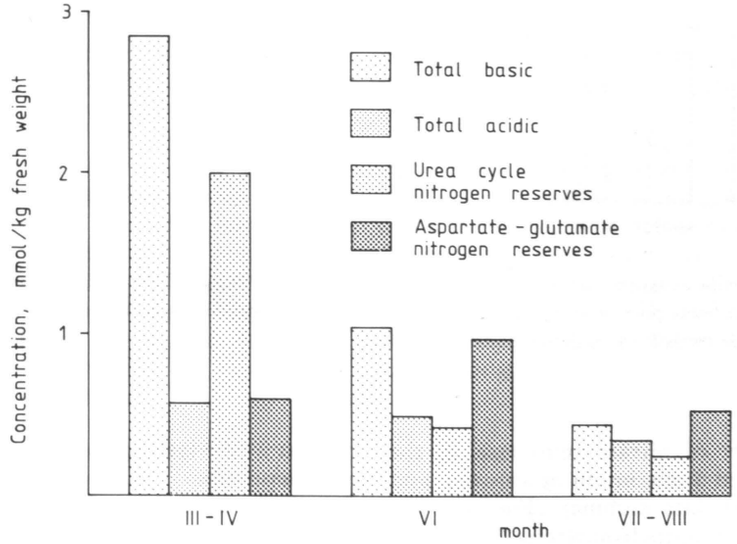

Fig. 4. Summed concentrations of certain amino acid groups in Scots pine buds and shoots at three seasonal points from March to August. Total basic amino acids include arginine, lysine and histidine plus their derivatives, total acidic aspartic and glutamic acids, urea cycle nitrogen reserves arginine, urea, citrulline and ornithine, and aspartateglutamate nitrogen reserves aspartic and glutamic acids plus asparagine and glutamne. Results are summed from 4–7 determinations.

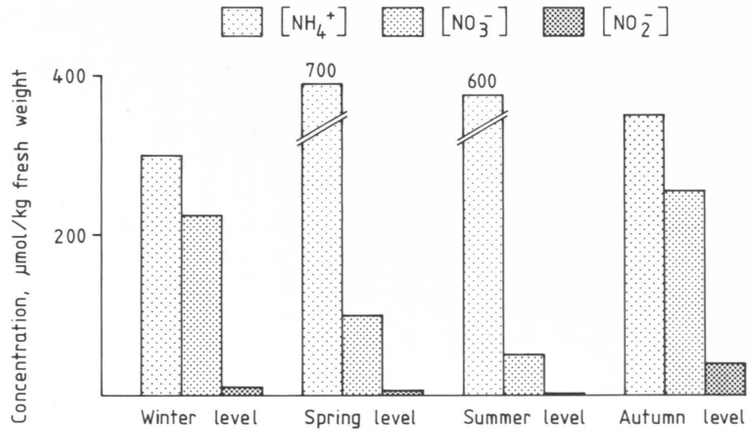

Fig. 5. Seasonal concentrations (µmol/kg fresh weight) of ammonium, nitrate and nitrite in Scots pine needles. Average levels for 3–5 determinations are given.

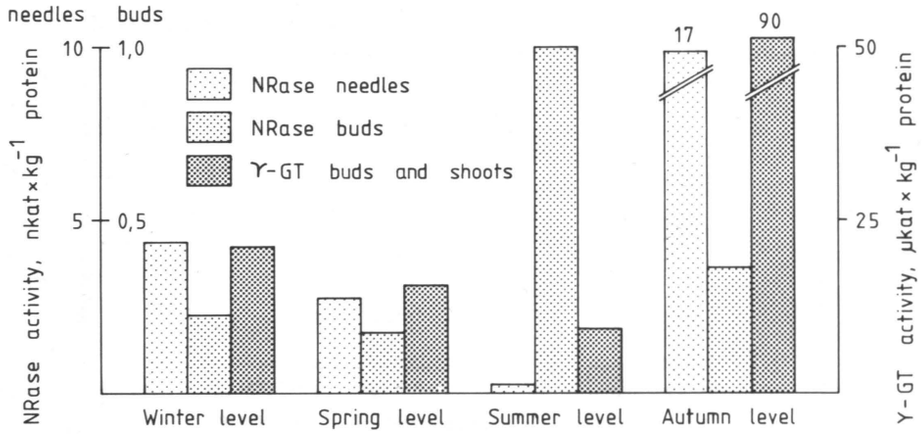

Fig. 6. Specific activities (kat = mol × s^{-1}) of nitrate reductase (NRase) and γ-glutamyltransferase (γ-GT) in Scots pine needles, buds and shoots (separate scales for buds and needles in NRase). Average levels for 4–6 determinations are given.

The activity of nitrate reductase in the needles reached a maximum in autumn, and in the buds in late summer (Fig. 6). The activity of γ-glutamyltransferase was also highest in the late summer and autumn (August-September), but the activity was low in the winter buds and particularly in the shoots during the elongation phase (Fig. 6).

4. Discussion

The nitrogenous compounds at different biochemical levels in the pine tissues showed considerable seasonal quantitative variation. Although the highest proportion of the total nitrogen in the pine tissues was always present as protein (given in mass concentration in Fig. 2), the concetrations of free amino acids in the winter buds (given in molar concentration in Fig. 2) were remarkably high. Arginine, in particular, dominated the free amino acid pool. Since free arginine is strongly cationic, it certainly gives a positive net charge to the cytoplasm during the winter. Since arginine is present at millimolar levels, it has a considerable neurtalizing effect in the cytoplasm. It could neutralize the relatively high nitrate ion concentration in winter time, and is thus probably present in the form of argininium nitrate. In this way free arginine would eliminate the strong nitrate ion, which is otherwise an effective remover of hydrate water and can cause frost damage during winter (Tumanov 1967). Both arginine and nitrate are potential low-molecular weight nitrogen reserves, which can be transported to the growing points and remetabolized in the spring when growth starts. Thus the plant has nitrogen for growth even though the ground is frozen and nitrogen uptake is minimal. The considerable concentration of nitrite that is stored in the tissue during the winter (Fig. 5) may also be neutralized by arginine.

The activity maxima of nitrate reductase in late summer or autumn may ensure that nitrate does not build up to dangerously high concentrations during the winter. Since nitrite clearly accumulated in the autumn, it appears that the reduction of nitrite to hydroxylamine and ammonia is blocked. The physiological significance of this is not known. In any case, it has been postulated that excessive nitrite concentrations may be toxic for many organisms.

The activity of γ-glutamyltransferase was about ten times higher in late summer and autumn than during other seasons, although the concentration of free amino acids was then at its lowest. It is therefore probable that the largest amount of free amino acids occurs at that time inside the cells, but in winter time a considerable proportion of the free amino acids would be present in the extracellular spaces (apoplastic spaces) or vacuolar spaces where they provide a continuous nitrogen supply to the developing bud and leader (Girs et al. 1982). This may be favourable situation for the mobilization processes of amino acids in different tissues. The accumulation of soluble, low-molecular weight nitrogen reserves (eg. nitrate, nitrite and arginine) may be both metabolically and energetically profitable, since they can easily be transported and metabolized at various growth sites in early spring when the ground is frozen and nutrient uptake minimal (Sagisaka 1974).

It can thus be concluded that the pine tissues store nitrogen for the winter mainly in the form of arginine. Its accumulation starts in the late autumn. Arginine forms the primary endogenous nitrogen source in the spring when the growth starts meanwhile the ground is still frozen. The levels of arginine thus largely reflect both winter dormancy and its breaking.

References

Christersson, L. 1971. Frost damage resulting from ice crystal fromation in seedlings of spruce and pine. Physiol. Plant. 25: 273–278.

Durzan, D. J. 1971. Free amino acids as affected by light intensity and the relation of responses to the shade tolerance of white spruce and shade intolerance of Jack pine. Can. J. For. Res. 1: 131–140.

Girs, G. I., Zubareva, O. N. & Elagin, I. N. 1982. Influence of low soil temperature on the content of nitrogenous substances in Pinus sylvestris

needle. Lesoved. 4: 62–66.
Halonen, O. & Tulkki, H. 1981. Ravinneanalyysien työohjeet. Metsäntutkimuslaitoksen tiedonantoja 36: 1–23.
Hellergren, J. 1981. Frost hardiness development in Pinus silvestris seedlings in response to fertilization. Physiol. Plant. 52: 297–301.
— , Widell, S., Lundborg, T. & Kylin, A. 1983. Frost hardiness development in Pinus sylvestris: The involvement of a K^+-stimulated Mg^{2+}-dependent ATPase from purified plasma membranes of pine. Physiol. Plant. 58: 7–12.
Jaworski, E. G. 1971. Nitrate reductase assay in intact plant tissues. Biochem. Biophys. Res. Commun. 43: 1274–1279.
Jirgensons, B. 1958. Organic colloids. Elsevier, Amsterdam. 655 p.
Kaku, S., Iwaya, M. & Jeon, K. B. 1981. Suprecooling ability, water content and hardiness of Rhododendron flower buds during cold acclimation and deacclimation. Plant Cell Physiol. 22: 1561–1569.
Kandler, O., Dover, C. & Ziegler, P. 1979. Kälteresistenz der Fichte. I. Steuerung von Kälteresistenz, Kohlenhydratund Proteinstoffwechsel durch Photoperiode und Temperatur. Ber. Dtsch. Bot. Ges. 92: 225–241.
Lähdesmäki, P. & Pietiläinen, P. 1988. Seasonal changes in the nitrate reductase activity and the concentrations of inorganic nitrogen compounds in Scots pine buds and needles. Aquilo Ser. bot. (in press).
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275.
Mazur, P. 1969. Freezing injury in plants. Annu. Rev. Plant Physiol. 20: 419–448.
McKenzie, J. S., Weiser, C. J., Stadelmann, E. J. & Buke, M. J. 1974. Water permeability and cold hardiness of cortex cells in Corpus stolonifera Michx. A preliminary report. Plant Physiol. 54: 173–176.
Meister, A. 1973. On the enzymology of amino acid transport. Sceince 180: 33–39.
— , Tate, S. S. & Griffith, O. W. 1981. γ-Glutamyltranspeptidase. In colowick, S. P. & Kaplan, N. O. (eds.). Methods in enzymology, Vol. 77. Academic Press, New York. pp. 237–253.
Pietiläinen, P. & Lähdesmäki, P. 1986. Free amino acid and protein levels, and γ-glutamyltransferase activity in Scots pine apical buds and shoots during the growing season. Scand. J. For. Res. 1: 387–395.
— & Lähdesmäki, P. 1988. Effect of various concentrations of NO_3^- and NH_4^+ on nitrate reductase activity in the roots and needles of Scots pine seedlings. Ann. Bot. Fenn. (in press).
Sagisaka, S. 1974. Transition of metabolism in living poplar bark from growing to wintering stages and vice versa. Plant Physiol. 54: 544–549.
— & Araki, T. 1983. Amino acid pools in perennial plants at the wintering stage and at the beginning of growth. Plant Cell Physiol. 24: 479–494.
Sakai, A. & Eiga, S. 1983. Physiological and ecological aspects of cold adaption of boreal conifers. In: Kaurin, Å., Junttila, O. & Nilsen, J. (eds.). Plant production in the north. Norwegian University Press, Tromsö. pp. 157–170.
— & Yoshida, S. 1968. The role of sugars and related compounds in variation of freezing resistance, Cryobiology 5: 160–174.
Senser, M. 1982. Frost resistance in spruce (Picea abies (L.) Karst.). III. Seasonal changes in the phospho- and galactolipids of spruce needles. Z. Pflphysiol. 105: 229–239.
— & Beck, E. 1982. Frost resistance in spruce (Picea abies (L.) Karst.). IV. The lopid composition of frost resistant and frost sensitive spruce chloroplasts. Z. Pflphysiol. 105: 241–253.
— , Dittrich, P., Kandler, O., Thanbichler, A. & Kuhn, b. 1971. Isotopenstudien über den Einfluss der Jahreszeit auf den Oligosaccharidumsatz bei Coniferen. Ber. Dtsch. Bot. Ges. 84: 445–455.
Siminovitch, D. 1963. Evidence from increase in ribonucleic acid and protein synthesis in autumn for increase in protoplasm during the frost-hardening of black locust bark cells. Can. J. Bot. 41: 1301–1308.
— , Rheaume, B., Pomeroy, K. & Lepage, M. 1968. Phospholipid, protein and nucleic acid increases in protoplasm and membrane structures associated with development of extreme freezing resistance in black locust tree cells. Cryobiology 5: 202–225.
Steponkus, P. L. 1984. Role of the plasma membrane in freezing injury and cold acclimation. Annu. Rev. Plant Physiol. 35: 543–584.
Tanford, C. 1965. Physical chemistry of macromolecules. John Wiley & Sons Inc., New York. 710 p.
Tumanov, I. I. 1967. Physiological mechanism of frost resistance of plants. Soviet Plant Physiol. 14: 440–455.
Weiser, C. J. 1970. Cold resistance and injury in Woody plants. Science 169: 1269–1278.
Will, G. M. 1971. Nitrogen supply, apical dominance and branch growth in Pinus radiata. Plant & Soil 34: 515–517.
Ziegler, P. & Kandler, O. 1980. Tonoplast stability as a critical factor in frost injury and hardening of spruce (Picea abies (L.) Karst.) needles. Z. Pflphysiol. 99: 393–410.

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