Non-structural carbohydrate status in Norway spruce buds in the context of annual bud structural development as affected by acidic pollution

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Abstract

The present study focused on changes in the annual dynamics of the contents of non-structural saccharides (NSS) of Norway spruce vegetative buds related to their structural development under the effect of acidic pollution during the year 1995. Two types of material were analysed: (1) 4-year-old trees treated for 2 years by simulated acid rain (SAR; pH 2.9 and 3.9), and (2) 40–60-year-old trees growing in natural mountain stands exhibiting different degrees of macroscopic damage. Our study revealed that the dynamics of the NSS content reflected the major morphogenetic and developmental changes occurring during the annual bud developmental cycle. No systematic changes in the annual dynamics of NSS content were observed in buds from both mountain sites, or as a consequence of the SAR. The total sugar content of bud tissues was composed of a combination of five main sugar components: sucrose, glucose, fructose, raffinose family oligosaccharides (RFO; combination of raffinose and stachyose), and a pinitol fraction (PF) probably of cyclitols with pinitol as a main member. The dynamics of individual sugar components also reflected possible carbohydrate mediated bud frost protection. Interesting results were obtained from buds in dormant state. In dormant buds of the SAR experiment the higher value of the ratio PF:RFO of the pinitol fraction and raffinose family oligosaccharides followed the higher dose of SAR treatment. When evaluating the ratio from both types of material we assumed that changes in PF:RFO ratio corresponded to early stages of damage or acute metabolic reaction. Thus, we suggest the ratio PF:RFO as a possible non-specific metabolic marker of early bud stress reaction which is, among other stress factors, sensitive to increasing load of acidic pollutants. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Acid rain; Picea abies; Pinitol; Raffinose; Starch; Sugars

1. Introduction

Within the last 25–35 years air pollution has become a potential threat to forest production world-wide. Sulphur dioxide and nitrogen oxides are known as major pollutants in industrial areas,
primarily as causative agents of acid rain. The effect of acidic pollution expressed by anatomical and/or morphological changes is initially recognised at the level of metabolic detection. Several studies of Norway spruce have shown that disturbances caused by SO₂ in the biochemical function (Hampp, 1992) and the cell ultrastructure (Kärenlampi, 1986) of plants appear before visual symptoms or growth reduction. To enable the necessary measures to be taken in time, metabolic markers for the detection of early damage are of great importance for forest management.

The carbohydrate status of plant organs is quite a sensitive indicator of the physiological responses to natural and anthropogenic stresses, such as dust pollution (Mandre and Kloseiko, 1997), soil acidification and water availability (Kleinschmidt et al., 1998), acid mist and frost drought (Esch and Mengel, 1998). In addition, the effects of air-borne pollutants on the saccharide metabolism of coniferous needles have been investigated, however, the reported results were controversial. For example, a decrease in the contents of hexoses, sucrose and starch was observed as a consequence of the simulated effect of SO₂ in combination with O₃ (Hampp et al., 1990; Peace et al., 1995). In contrast Küppers and Klumpp (1988) reported elevated levels of the contents of starch and sugars when needles were exposed to a similar stress load. Data on the effect of indigenous acidic deposition on natural mountainous stands are still scarce because of stand heterogeneity and interactions of acting abiotic and biotic factors. Oren et al. (1988) analysed healthy, dark green needles and apparently yellowing ones macroscopically, and found that yellowing needles contained a higher amount of total soluble saccharides when compared with healthy needles, whilst differences in starch accumulation were not observed.

In conifer needles, changes in partitioning between starch and sugars favouring the accumulation of soluble sugars before the winter period were supposed to be involved in frost hardening. These changes are an important contributor to the increased frost tolerance of conifers in winter when sugars can act as cryoprotectants (Kandler and Hopf, 1982; Hampp, 1992; Ogren, 1997; Ogren et al., 1997). The increase in total sugar content during cold hardening, however, may not to be of primary importance as the ratio between particular sugars might prove to be crucial for cryoprotection (Obendorf, 1997; Liu et al., 1998). For example, sucrose is known to be able to retain the liquid-crystalline state of membranes under osmotic stress. Conifers frequently experience subfreezing temperatures as well as substantial water stress (Tranquillini, 1979) which might promote sucrose crystallisation and thus a loss of the cryoprotective effects of this sugar. Raffinose is supposed to inhibit the tendency of sucrose to crystallise and therefore to conserve the protective quality of sucrose (Caffrey et al., 1988). Other substances from plant carbohydrate spectrum, such as cyclitols, are also believed to have roles as cryoprotectants, desiccation protectants, and hydroxyl radical scavengers (Obendorf, 1997; Nelson and Bartels, 1998).

Bud apical meristems are very important sinks as they determine further growth and development of tree species. Bud physiological state including saccharide metabolism will determine their growth capacity, and thus the development of the whole crown architecture. Only limited information is available about changes in the dynamics of non-structural saccharide (NSS) content in buds of Norway spruce under stress conditions (Lux et al., 1997). According to our knowledge nothing is known about the effect of acidic pollution. Thus, the aim of this study was to determine the effect of acid rain on the content of NSS in buds of Norway spruce (Picea abies) during 1 year of their development. We tested the hypothesis that changes in contents and composition of non-structural carbohydrates in buds of Norway spruce are indicators of early damage or acute metabolic reaction caused by the effect of acid rain as a main stressor.

2. Materials and methods

2.1. Plants

Two types of plant material were chosen for the analyses: buds of Norway spruce Picea abies (L.) Karst sampled either from 4-year-old seedlings
included in the model experiment of simulated acid rain, or from adult, 40–60-year-old individuals from natural sites located in mountain areas showing different degrees of macroscopic damage.

2.1.1. Model experiment of simulated acid rain (SAR)

The SAR experiment was established in the field station of the Institute of Botany of the Academy of Science located in Lužnice by Třeboň (South Bohemia; longitude 14°44′ E, latitude 49°05′ N; Table 1). The station, being located in the Protected Landscape Area of Třeboňsko (UNESCO Biosphere Reserve) with negligible indigenous acidic deposition, ensured low interference with experimental treatment. A total of 150 3-year-old nursery-grown trees were potted and randomly divided into five experimental treatments (i.e. 30 seedlings per treatment). Trees of all treatments were placed into laminate basins and treated from January 1994 to December 1995 according to the following experimental design. Trees from the control treatment (K) were watered and sprayed with distilled water. Four other treatments A, B, C, D differed in the pH of applied solution reflecting the concentration of pollutants (either pH 3.9: treatments A, B; or pH 2.9: treatments C, D), and in the method of SAR application (either watering of the roots: treatments A, C; or root watering and spraying of the parts above ground: treatments B, D). In addition, the treatments A and C included spraying with an equivalent amount of distilled water. A solution with pH 3.9 simulated the quality of precipitation in the Krkonoše Mountains (Vávra, 1992) and a solution with pH 2.9 contained 3.3 times higher ion concentrations. The solution of pH 2.9 contained (in mg l⁻¹): SO₄²⁻ 73.3, NO₃⁻ 20.7, NH₄⁺ 5.3; pH 3.9 contained: SO₄²⁻ 22.0, NO₃⁻ 6.2, NH₄⁺ 2.0. Solutions were applied once a

<table>
<thead>
<tr>
<th>SAR experiment</th>
<th>Field site</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S</td>
</tr>
<tr>
<td>Location</td>
<td>PLA Třeboňsko, Sumava Mountains, NP</td>
</tr>
<tr>
<td>Region or mountain</td>
<td>South Bohemia, Třeboň Basin</td>
</tr>
<tr>
<td></td>
<td>South-West border, Malá Mokrůvka Mountains</td>
</tr>
<tr>
<td></td>
<td>North border, Medvedín Mountains</td>
</tr>
<tr>
<td>Elevation (m)</td>
<td>420</td>
</tr>
<tr>
<td>Aspect</td>
<td>NW</td>
</tr>
<tr>
<td>Slope (%)</td>
<td>1–10</td>
</tr>
<tr>
<td>Growing season</td>
<td>March–October</td>
</tr>
<tr>
<td>Stand damage class based on evaluation of loss of foliage</td>
<td>0 (healthy)</td>
</tr>
</tbody>
</table>
| Atmospheric deposition rates
  | Total annual deposition of H⁺ (mg m⁻² year⁻¹) | ≤50 |
  | Total annual sulphur deposition (g m⁻³ year⁻¹) | ≤1 |
  | Wet annual deposition of SO₄²⁻ (g m⁻³ year⁻¹) | ≤0.7 |
  | Dry annual deposition of SO₂ (g m⁻³ year⁻¹) | <0.5 |
  | Total annual deposition of NO₂ (g m⁻³ year⁻¹) | 0.5–1.0 |
  | Wet annual deposition of NO₃⁻ (g m⁻³ year⁻¹) | 0.6–0.8 |
  | Dry annual deposition of NOₓ (g m⁻³ year⁻¹) | <0.2 |
  | Wet annual deposition of NH₄⁺ (g m⁻³ year⁻¹) | ≤0.5 |

a Data from CHMI (1995); –, none.
Fig. 1. Average daily air temperatures and daily precipitation during the year 1995 recorded in the Lužnica station where the SAR experiment was conducted. The arrow indicates 5 days at the end of August when night temperatures decreased below 8°C.

week to the root systems (100 ml per tree) and sprayed three times per week (20 ml per tree) on the tree parts above ground during the growing season. Removable transparent plastic basin covers were used a couple of hours after the application of solutions to ensure the prolonged influence of the SAR. The watering of root systems was stopped with the beginning of frosts. Average daily temperature and precipitation were recorded for the whole year (Fig. 1).

During the whole 1995 growing season sampling of buds from all treatments was repeated regularly in 1-week intervals from the beginning of April 1995 until the middle of June and then in 2-week intervals until the end of October. Dormant state was studied in buds sampled before the growing season (16 January 1995 — dormancy 1) and after it (23 October and 20 November 1995 — dormancy 2). The terminal buds of branches of the first or second order were processed immediately after sampling. Buds were sampled for each variant of the SAR experiment in the following way. For anatomical observation and starch localisation five and three buds per sampling date were processed during the dormant and growing seasons, respectively. For non-structural saccharide determination, five samples, each prepared from 5–15 buds, were analysed during growing season. During dormant season three samples per tree and one sampling date, each prepared from 15–25 buds, were analysed. In 1995 17 collections were made in total.

2.1.2. Natural mountain sites

Sampling sites were selected in two Czech mountainous regions with indigenous Norway spruce populations (Table 1). Sampling site K (trees K1–K5) was located in the Krkonoše National Park (NP) in North Bohemia (longitude 15°40’ E, latitude 50°40’ N) and was heavily affected by acidic deposition in addition to natural stress factors (long winter period with deep frosts,
heavy icing, frequent gales, temperature fluctuations, frequent periods with limited water availability). Sampling site S (trees S1–S8) was located in the Šumava NP in South Bohemia (longitude 13°30' E, latitude 49°05' N) and was stressed mainly by natural stress factors similar to site K with relatively low interference by atmospheric pollution.

Visible macroscopic symptoms of tree damage were evaluated using visual evaluation of the degree of defoliation. The evaluation was done by an assessment of the percentage of crown defoliation using a series of comparative photographs (Müller and Stierlin, 1990). Then the degree of damage was classified: 0, no damage (0–10% of crown defoliation); 1, light damage (10–30% of defoliation); 2, medium damage (30–55% of defoliation); 3, heavy damage (55–90% of defoliation); and 4, dying tree (90–100% of defoliation) according to the scale currently used in forestry practices in the Czech Republic. The degree of stand damage was classified according to a percentage of dying or dead trees in the stand (Cudlín et al., 1995).

Buds were sampled during the growing season of 1995 at regular 1-month intervals. The dormant state was studied before (February 1995) and after the growing season (November 1995). The terminal buds of branches of higher order (second or third) ~8 m above ground level and on the southern side were sampled. Each time samples of branches were taken in early afternoon, and stored on ice in a thermo-box until they were put in a refrigerator when returning to the laboratory in the evening. The next day bud samples were processed. For anatomical observation and starch localisation five and three buds per sampling date were processed during the dormant and growing seasons, respectively, for each tree from mountain site. For non-structural saccharide determination two samples per tree and one sampling date, each prepared from 5–15 buds, were analysed during growing season. During dormant season three samples per tree and one sampling date, each prepared from 15–25 buds, were analysed. In 1995 ten collections were made in total.

2.2. Anatomical study and starch localisation

The anatomical description and histochemical detection of starch was done on longitudinal median sections (thickness 12 μm) embedded in paraffin prepared according to Johansen (1940). Samples were fixed with 70% FAA (formaldehyde/acetic acid/ethanol/water: 1/1/9/9, v/v/v/v); infiltration was improved by the application of lowered pressure. Lugol solution (iodine/potassium iodide) was used to localise starch grains. Starch histochemical localisation was verified by polarised light. The terminology of developmental stages of the bud apical meristem was taken from the study of Owens et al. (1977).

2.3. Non-structural saccharide content determination

The detailed results on NSS dynamics described in the following paragraphs were obtained from the buds sampled from the SAR experimental trees, frequent sampling being necessary for a detailed analysis.

2.3.1. Soluble NSS sugars

For sugar analysis, samples of dissected meristematic parts (during the dormant stage), or whole developing buds (during bud break and early shoot growth) with young needles removed, were used. Samples were frozen in liquid nitrogen and freeze-dried. Specimens were homogenised and boiled with 80% methanol (0.5 ml) at 75°C for 10 min, then evaporated to dryness. The residue was dissolved in re-distilled water in an ultrasonic bath for 10 min. The samples prepared for sugar determination were stored after filtration (using Whatman membrane filters, 0.45 μm) at −18°C.

The content of extracted soluble NSS was determined using high-pressure liquid chromatography (HPLC) with refractometric detection (temperature 80°C; column: Ostion LGKS Ca²⁺; Watrex, Czech Republic; eluent: re-distilled H₂O). Preliminary identification of raffinose and pinitol on the above described system was further verified by using high pH anion exchange chromatography with pulse amperometric detection (Dionex,
Sunnyvale, USA; column: CarboPac PA1 (4 × 250 mm) with companion guard column (4 × 50 mm); eluent: 100 mM NaOH).

2.3.2. Non-soluble NSS starch

Whole terminal buds were analysed with the young needles removed. Three parallel samples were taken. Two samples were homogenised and purified with 70% ethanol. A third sample was taken to determine dry weight. Further steps used were modified from the protocols of starch determination using colorimetric anthrone method (Viles and Silverman, 1949; Yemm and Willis, 1954) with the following modifications leading to a higher reproducibility: (1) inclusion of two acetone steps during specimen purification before starch extraction to remove high content of compounds of secondary metabolism (mainly phenolic compounds); and, (2) inclusion of specimen incubation with chloralhydrate during starch extraction to achieve better starch evolvement into the solution before filtration (Bourne and Weigel, 1965). Plant material was ground with 4.5 ml of 80% acetone. Obtained mixtures were centrifuged for 5 min at 200 × g. The supernatant was removed and the pellet was shaken in 6 ml of 80% acetone, centrifuged, the supernatant removed and the pellet shaken in 50% ethanol. After a further centrifugation the supernatant was again removed.

2.4. Statistics

The evaluation of results was performed only on data sets including five samples per variant using one-way ANOVA. Because only a limited amount of data was available, the non-parametric Kruskal–Wallis test was also conducted. Differences among treatments were considered significant at \( P < 0.05 \) only if both ANOVA and the Kruskal–Wallis test revealed significant differences.

3. Results

3.1. Anatomical changes during growing season

To achieve a better understanding of the development-related changes in carbohydrate pools, the anatomical changes of buds during annual developmental cycle were followed. The timing of developmental events was not affected by the experimental treatment in the SAR experiment, thus the timing given below corresponded generally to buds from the SAR experiment. The timing of developmental events in the mountain material differed (Table 2) based on shifts in the onset of different developmental phases.

3.2. Starch content and histochemical localisation

Two types of starch grains were identified during the annual cycle of spruce buds (Table 2). (1) Well stained larger grains, identified as reserve starch described by Stitt and Steup (1985), were generally localised, particularly during the dormant state, and at the beginning of bud break in cells of the primary cortex of subjacent last-year stem or in basal parts of the bud scales. (2) Smaller grains (identified as transitory starch) were localised during the whole year in young bud tissues, i.e. leaf primordia or young developing bud scales, and rarely in pith meristem. This pattern was general for all buds studied. Only the timing of starch grain deposition differed in the SAR experiment in comparison with mountain field sites, corresponding with the above-mentioned shifts in the onset of the developmental changes (Table 2).

The annual accumulation pattern of starch in buds was as follows (Fig. 2a). Before a phenologically detectable bud break, starch gradually accumulated and reached its first maximum value. Then during bud break starch was partially mobilised (April). In this period a bud underwent remarkable structural and developmental changes demanding high energy supply, and starch content reached its minimum value. Immediately afterwards starch accumulated again (beginning of May) to the second maximum value. Slow continuous mobilisation took place from the end of May until the beginning of August, and then accumulation to the amount characteristic for a dormant state took place.

Any systematic changes in starch amount or accumulation pattern were not apparent as a consequence of experimental treatment by SAR. No
remarkable changes in the pattern of starch storage either quantitative or qualitative were identified between plant material taken from trees of the SAR experiment and mountain sites independent of tree damage. The only exception was a smaller accumulation of starch before bud break in buds of trees from mountain sites (Fig. 2b, c). Thus, we may consider the above pattern as general for buds of Norway spruce.

3.3. Sugars

The annual pattern of total sugar content in buds taken from trees of the SAR experiment was as follows (Fig. 3a). The total sugar content was at its maximum in the dormant state. Before bud break, a rapid lowering of reserves was recorded to almost one third of the initial value recorded in dormancy. The sugar content was at its minimum in early August. During that time apical meristem produced new leaf primordia, thus it could be implied that at that period carbohydrates were utilised for this differentiation process. From the end of August accumulation slowly increased to the values characteristic for the beginning of winter dormancy.

The SAR treatment did not induce any systematic changes in the dynamics of total sugar content during the annual developmental cycle (Fig.

### Table 2
Timing of developmental events of bud development and localisation of two described types of starch grains during annual cycle 1995 of buds of Norway spruce

<table>
<thead>
<tr>
<th>Bud developmental stage</th>
<th>Dormant bud</th>
<th>Early scale initiation</th>
<th>Late scale initiation</th>
<th>Early leaf initiation</th>
<th>Late leaf initiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Timing for SAR experiment</td>
<td>January–March</td>
<td>April–mid May</td>
<td>Mid May–end of June</td>
<td>July</td>
<td>August-end of September</td>
</tr>
<tr>
<td>Timing for mountain sites</td>
<td>January–April</td>
<td>May–mid June</td>
<td>Mid June–mid July</td>
<td>Mid July–mid August</td>
<td>Mid August–end of September</td>
</tr>
</tbody>
</table>

**Histochemical localisation of types of starch grains (big:small)**

<table>
<thead>
<tr>
<th>Type</th>
<th>Dormant bud</th>
<th>Early scale initiation</th>
<th>Late scale initiation</th>
<th>Early leaf initiation</th>
<th>Late leaf initiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bud rib meristem (RM)</td>
<td>–/–(+)</td>
<td>–/+</td>
<td>–/+</td>
<td>–/+</td>
<td>–/+</td>
</tr>
<tr>
<td>Leaf primordia (LP)</td>
<td>–/–</td>
<td>–/+</td>
<td>–/+</td>
<td>–/+</td>
<td>–/+</td>
</tr>
<tr>
<td>Basal part of old bud scales (BS)</td>
<td>++/–</td>
<td>++/–</td>
<td>++/–</td>
<td>++/–</td>
<td>++/–</td>
</tr>
<tr>
<td>Cortex of subjacent last year shoot (SC)</td>
<td>++/–</td>
<td>++/–</td>
<td>++/–</td>
<td>++/–</td>
<td>++/–</td>
</tr>
</tbody>
</table>

*AM, apical meristem; CP, collenchymatic plate. Semi-quantitative evaluation of starch deposition: –, none present; +, small amount; ++ large amount; none recorded, structure is not present during bud developmental stage.*
The annual dynamics of individual sugar components showed different regularities. Sucrose was a prevalent sugar component of the sugar spectrum (almost 50%) during the whole year and its dynamics had a similar course as the dynamics of the total sugar content. Both glucose and fructose had a very similar pattern of annual dynamics and composed only a small portion of the total sugar spectrum, 5–15% during shoot growth and active growing (May–August), and 2–3% in the dormant state. The occurrence and amount of raffinose and stachyose in the total sugar spectrum were important only during the dormant state. During shoot growth and the active growing season they almost disappeared (Fig. 3b). The pinitol fraction showed annual dynamics resembling that of the total sugar content (Fig. 3c) composing 15–30% of the total sugar amount.

Interesting results on the effects of the SAR treatment were apparent only in the dormant state, which is the most easily detected and longest phenological phase. In both dormant periods studied, total sugar content corresponded to a trend of higher values for variants B and D treated with an experimental solution on the parts above ground (Fig. 3a). Nevertheless, statistical analysis (P < 0.05) confirmed a significant difference only between B and C variants. In the PF:RFO ratio the higher value of the ratio followed the higher dose of acidic treatment (Fig. 4). The sum of those two sugars accounted for 40% of the total sugar content in dormant buds. Starting with a growth period, this detected change among variants began to be less apparent. The increased PF:RFO ratio for more treated variants was repeatedly recorded in dormant states. The only exception to this trend was the control variant K which will be discussed later.
Fig. 3. Annual dynamics of soluble NSS (sugar) content in buds of Norway spruce. SAR experiment. K: control treatment (distilled water), Treatments A, B, C, D differed in pH of applied solution (A, B: pH 3.9; C, D: pH 2.9), and in the method of application of SAR (A, C: watering of the roots only; B, D: root watering and spraying of the part above ground). D.W., dry weight. Dormancy 1 and 2: n = 5; growing season: n = 2. Bars above curves correspond to LSD values. Removal of young needles corresponds to the sampling system described in Fig. 2. (a) Total soluble NSS content. (b) Raffinose family oligosaccharide (RFO) content. (c) Pinitol fraction content.

The ratio PF:RFO was approximately stable for the majority of trees from both mountain sites. There were quantitative differences between individual trees, but trends in those differences seemed to be more or less stable and characteristic for individual trees, but no relation was revealed between the P:R ratio and the degree of macroscopic tree damage (Fig. 5a, b). For trees from the less damaged site, S, the ratio slightly increased in dormancy 2 and was very stable. For the more damaged site, K, it showed a non-systematic decrease.

Fig. 4. Ratio PF:RFO of the pinitol fraction and raffinose family oligosaccharides in buds of Norway spruce of SAR experiment in two successive dormant seasons. Dormancy 1: January 1995 (n = 5); dormancy 2: October and November 1995; bars indicate S.D. (n = 10). K: control treatment (distilled water), Treatments A, B, C, D differed in pH of applied solution (A, B: pH 3.9; C, D: pH 2.9), and in the method of application of SAR (A, C: watering of the roots only; B, D: root watering and spraying of the part above ground). Data with common letters are not statistically different; differences among treatments were considered significant at P < 0.05 only if both ANOVA and the Kruskal–Wallis test revealed significant differences.

4. Discussion

Structural and histochemical analyses enabled the precise matching of the changes in structure and starch localisation to the changes in annual dynamics of non-structural carbohydrate contents. Our results showed that the trees located in two different altitudes (420 m and 1200–1250 m a.s.l.) exhibited an apparently altitude-driven timing of developmental events (Table 2).

In contrast to Hejnowicz and Obarska (1995) we did not find a large amount of starch in pith meristem during the dormant state. The observed difference in localisation could be explained by the interference of colour histochemical reactions of tannins and starch, which we overcame by using polarised microscopy, which distinguished both substances reliably. The SAR treatment did not affect starch content similar to the results of Peace et al. (1995) on Norway spruce needles.

We observed remarkable RFO accumulation in buds before dormancy and their presence during
the dormant state. Conifers are known for a remarkable accumulation of raffinose, instead of sucrose, during a period of frost hardening (Hinesley et al., 1992; Lux et al., 1997). The start of RFO accumulation in September (Fig. 3b) corresponded to the decrease in average daily temperatures to under 10°C, which was recorded at the end of August (Fig. 1). It is known that at least in some plants two distinct pools of RFO exist: (1) a large storage pool which is also probably involved in stress tolerance; and (2) a transport pool (Bachman et al., 1995). We suppose that in our material all RFO belonged to the first type of pool, as during the period of shoot growth and active growing season, RFO almost disappeared. Perhaps raffinose in buds could play a similar role in frost tolerance, preventing sucrose crystallisation, as is known from desiccation tolerance of seeds (Bernal-Lugo and Leopold, 1995). We concluded that glucose and fructose serve mainly as transitory metabolic compounds. Sucrose was found to be a prevalent sugar constituent determining the annual dynamics of total sugar content.

The attempt to confirm the identity of pinitol with the help of Dionex HPLC system revealed that pinitol was present in the detected fraction, but there were several other co-migrating compounds. We suppose they are other cyclitols (inos-
itol derivatives). For Norway spruce buds, a similar spectrum of sugars was identified by Lux et al. (1997) with the exception of the identification of only pinitol instead of a larger group of likely related compounds. During a major part of the year the pinitol fraction belonged to the second most abundant sugar component (Fig. 3c). Its dynamics pattern resembled that of sucrose. As cyclitols are supposed to be involved in stress tolerance (Lux et al., 1997; Obendorf, 1997), their presence in our material might be important. Nevertheless, in some plants pinitol can mainly play a metabolic role (Ichimura et al., 1998). Based on the dynamics during the annual cycle, we cannot precisely conclude what is the role of the pinitol fraction in spruce bud physiology.

Taking into account that no consistent morphological (colour changes of foliage, etc.) or anatomical changes (qualitative changes of bud structure or their timing) have been detected in the SAR experiment, we may assume that our simulation demonstrated only early tree damage. We propose the increasing ratio of PF:RFO (pinitol fraction:raffinose family oligosaccharides), which increased with the increasing stress load in variants in the order $A < B < C < D$ (Fig. 4), as a possible indicator of early damage of dormant Norway spruce buds. The higher value of the ratio for the control variant K in dormancy 2 may be explained by a probable nitrogen deficit because no additional nitrogen was supplied to the K variant during the 2-year experimental treatment with distilled water. It is known that limited nitrogen supply can cause shifts in carbohydrate status, e.g. increase in soluble NSS (Wainhouse et al., 1998). Thus, we regard the increased ratio PF:RFO to be a non-specific indicator of early damage of spruce buds, which is, among other stress factors, sensitive to increasing load of acidic pollutants.

In mountain sites macroscopic tree damage has already been exhibited. No dependence of the PF:RFO ratio on the degree of macroscopic tree damage was observed in either mountain site, which is in accordance with the hypothesis that changes in PF:RFO ratio correspond to early stages of damage or acute metabolic reaction. The PF:RFO ratio for the site S was generally lower and was stable when compared to the heavily polluted and more damaged site K, where acute metabolic reactions could be suspected. Similarly to our study, studies focusing on markers of early damage of Norway spruce (Godbold et al., 1992; Wild and Schmitt, 1995) frequently have difficulty in identifying a common pattern of stress markers, a situation often explained by the high genotypic variability.

In the present study we confirmed that not only qualitative but also quantitative changes expressed as a ratio of individual components of the NSS spectrum are of great importance when investigating the effect of increasing stress load on the carbohydrate status of a plant organ.

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