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SEASONAL DYNAMICS OF SUGARS IN THE LEAVES OF *Salix dasyclados* AND THE EFFECT OF SOIL TREATMENT WITH CEMENT DUST

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Abstract. Cutting-derived *Salix dasyclados* Wimm. were grown on soil containing high amounts (2 kg m^{-2} in year 1 + 1 kg m^{-2} in year 2) of dust from filters of a cement plant. The pH of the treated soil was increased from 6.5 (control) to 7.6. Soluble sugars, starch, and chlorophylls in leaves of spring flush shoots were analysed during the vegetative growth of the second year of treatment. The lower hexose content in the leaves of treatment trees was approximately balanced by their higher sucrose content. Maltodextrins and starch were not affected significantly and no fructans were found. The effect of treatments on chlorophyll *a* and *b* was neglible. Total biomass was significantly lower than in control trees without any change in the partitioning of biomass between different parts of the trees.

Key words: alkaline soil, cement dust, chlorophyll, fructose, glucose, starch, sucrose.

INTRODUCTION

Activated sugars are regarded as a central metabolite pool (Dennis & Greyson, 1987; Trethewey et al., 1999). Free sugars form another important pool, which has basic interfaces to the central metabolite pool, sugar transport, sucrose cleavage, and the hydrolysis of storage polysaccharides. However, part of the carbon flow by-passes the pool of free sugars since triosephosphates from photosynthesis are often used directly for energy generation and synthetic processes. The framework of sugar metabolism in leaves is briefly outlined in Fig. 1. The main functions of free sugars in plants are temporary storage, osmotic adjustment (Büssis et al., 1997), regulation, and transport (Lalonde et al., 1999). Starch is known as a storage material.

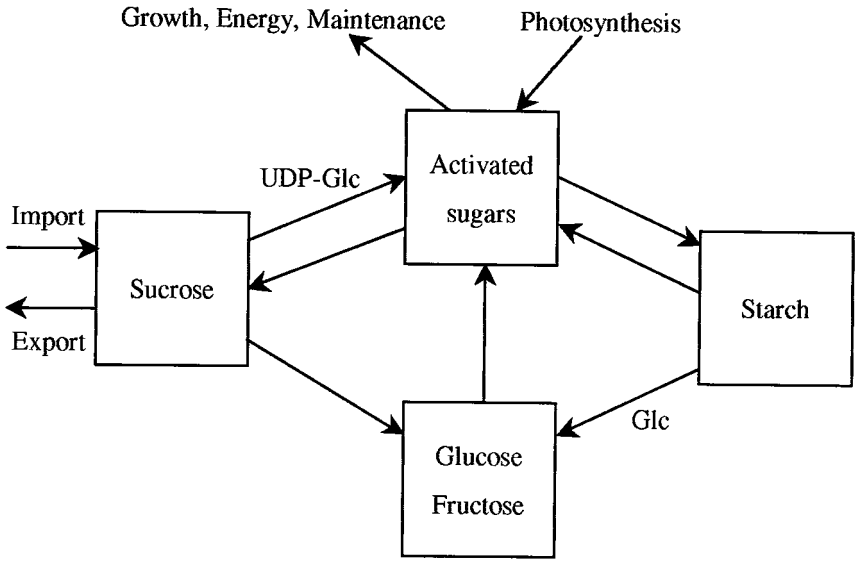

Fig. 1. Outline of relationships between the main carbohydrate pools and physiological processes in a plant leaf. Sucrose and starch have both two paths for breakdown. Sucrose cleavage by invertase yields equal amounts of glucose and fructose while cleavage by sucrose synthase adds glucose residue directly to the pool of activated sugars and only free fructose is yielded. In daytime, starch is broken down phosphorylitically, which immediately yields activated sugars, and at night, free glucose is formed from starch by amylolysis. The size of the pool of activated sugars never achieves the size of the pool of free sugars and is usually by several orders of magnitude smaller than the pool of free sugars. Glc = glucose; UDP = uridine diphosphate.

During the bud-break period, young leaves depend solely on reserves imported from the storage organs. The imported sucrose is usually rapidly cleaved and the products, hexoses, are readily phosphorylated. Sugars in sink leaves are processed by the phloem unloading, sucrolytic enzymes, and sugar kinases. Sucrose cleavage is catalyzed by invertases (EC 3.2.1.26) and sucrose synthase (EC 2.4.1.13). Invertases yield glucose (Glc) and fructose (Fru), but sucrose synthase yields Fru and UDP-Glc (activated sugar). The ratio of the activities of sucrolytic enzymes changes during the development and the ratio of Fru to Glc can be affected through the interface to sucrose cleavage (Hampp et al., 1994). Moreover, sucrose synthase is located only in the cytosol whereas invertases are located in the vacuole, cytosol, and cell walls. Hexoses (Glc + Fru) are found almost exclusively in the vacuole (Heineke et al., 1994). Experiments indicate a considerable role of the vacuolar space in the competition for sugars in the cytosol and intercellular space (Heineke et al., 1994; Hajirezaei et al., 2000). Thus even transport can affect the ratio of hexoses if the rates of translocation of different hexose forms are different.

The hydrolysis of storage polysaccharides as a reason for the reduced Fru to Glc ratio was proposed by Egger et al. (1996). Recently it was clarified that at night starch is decomposed by the amylolysis and Glc is transported out of plastids (Schleucher et al., 1998). Some species use fructans as storage compounds and a significant amount of fructans is present in the leaves of *Salix viminalis* (Bollmark et al., 1999). Fructans are a special case since the effect of their metabolism on the ratio of hexoses can be in both directions. Glc is released during the synthesis of fructans and predominantly Fru is released during the hydrolysis of fructans (Duffus & Duffus, 1984). Another process that can potentially influence the Fru/Glc ratio is the release of Glc from secondary compounds. For example, during lignin synthesis, the glycoside coniferin is hydrolyzed by β-glucosidase (Dharmawardhana et al., 1995).

Hexoses are allocated to the vacuole for temporary storage or irreversibly phosphorylated by the kinases. Leaves contain several forms of hexose kinases, which have different affinities for the hexoses (Schnarrenberger, 1990). In potato tubers, for example, high fructokinase (EC 2.7.1.4) activity decreases the Fru/Glc ratio (Hajirezaei et al., 2000). However, differences in the production and utilization of Fru and Glc are coordinated and the ratio of Fru/Glc can serve as a component in sugar sensing. The preferential utilization of Fru versus Glc substrates can be marked for a given sugar kinase, and this could translate into differential signals or sensing capacities (Martinez-Barajas et al., 1997; Fox et al., 1998; Hajirezaei et al., 2000; Koch et al., 2000). In the source organs sucro-neogenesis dominates over sucrolysis and changes in the specific forms of hexose kinases regulate carbon flow through Fru and Glc pools at different stages of development (Fox et al., 1998). The regulation of sugar metabolism, in turn, has several common properties to suit the development of plants and to respond to environmental or nutritional factors (Black et al., 1987).

Alkaline soil reduces the availability of iron and manganese to plants. Fe is needed for chlorophyll (Chl) synthesis and Mn is an essential component of the water-splitting unit involved in photosynthetic oxygen evolution. Often, when plants are unable to adapt to alkaline soil, interveinal chlorosis is observed (Graham et al., 1995; Ellsworth et al., 1997). Disturbed Chl synthesis may reduce photosynthesis and sugar production. Therefore, it was of interest to examine how the effect of alkaline soil on Chl content is related to the sugar content of leaves.

In this study, young cutting-derived willow trees had grown under stress conditions resulting from the treatment of soil with high amounts of cement dust (pH 12.6). The levels of free sugars and Chl*a* and *b* were measured in leaves during a vegetative period. *Salix* spp. are pioneer species used in short rotation forestry and require neutral or slightly alkaline soils. The current experiment is part of a larger project aimed at ascertaining the suitability of *Salix* for being grown on the extremely alkaline soils formed due to long-term pollution in North-East Estonia.

MATERIAL AND METHODS

Plant material and chemical analyses

Cuttings of *Salix dasyclados* Wimm. (clone 79097) were planted in May 1998 into soil treated with cement dust (2 kg m^{-2}). At the same time next year 1 kg m^{-2} cement dust was added together with peat (3 cm layer) and a complete fertilizer (N:P:K 10:7:16 and microelements; 50 g m^{-2}). The cement kiln dust was obtained from the electrostatic filters of the Kunda cement plant. The dust contained (on mass basis) 28% Ca, 5.5% K, 3.3% S, 1.6 % Mg, 0.2% P, and small amounts of Na, Fe, Cu, Co, Zn, and B.

The experiment was conducted on two sample plots (2.2 × 10 m) with 80 trees (at 60 × 60 cm space) on each plot. The soil of the treated plot was surrounded with a 15 cm plastic border to reduce the dilution of the dust treatment at the plot edges and to prevent any influence on the control plot. On the second year of the experiment, leaves from 6–10 trees were collected in the morning (9.00–10.00 a.m. local time) from May to October for analyses. The leaves were combined into three samples. Until July, leaves from trees 1 + 2, 4 + 5, and 7 + 8 were combined into first, second, and third samples respectively, later trees 1 + 2 + 3, 4 + 5 + 6, and 7 + 8 + 9 + 10 were used.

For the carbohydrate determination, the leaves were fixed in boiling ethanol (initially 99% and after fixation not less than 85%) for 3–4 min, oven-dried at 70 °C for 16 h, and ground in stainless steel jars with a Mixer Mill M200 (Retsch Gmbh, Germany) for 60 s at full speed. The ground material (50–100 μg) was analysed enzymatically applying the procedure described by Steen & Larsson (1986). Extraction conditions were slightly modified according to Hansen & Beck (1994) with 3 mM HEPES-NaOH (5 mL, pH 6.8) at 40 °C for 1 h used. Polyphenols, which can cause colloidal haze, were removed with Polyclar SB100 (BDH Inc., UK) (Müller et al., 1994). After centrifugation on a bench centrifuge (Hettich Universal 16A, Germany) during 10 min at 3500 rpm, the aliquot in the extract was divided into three portions (800, 200, and 200 μL) for the determination of Glc and Fru, sucrose and fructans, and maltodextrins. Sucrose and fructans were determined after acid hydrolysis (37 mM H_2SO_4 at 80 °C for 70 min) as the increase in Glc and Fru, respectively. Maltodextrins were determined after hydrolysis by amyloglucosidase (EC 3.2.1.3, 4.6 U mL^{-1}, 60 °C, 60 min) as the increase in Glc. Glc and Fru were determined by the hexokinase (EC 2.7.1.1)/glucose-6-phosphate dehydrogenase (EC 1.1.1.49) method (Bergmeyer, 1981), using a Heλiosα UV-visible spectrophotometer (UNICAM Ltd., UK) and Biohit Proline (BIOHIT OY, Finland) electronic pipettors. The enzymes were obtained from Sigma Inc. (US).

Starch and the unknown soluble fraction were analysed applying the anthrone method (Yemm & Willis, 1954; Arasimovich & Ermakov, 1987). Starch was hydrolysed with HCl. The unknown fraction was calculated by subtracting the sum of hexoses, sucrose, and maltodextrins from the soluble fraction measured

with anthrone. It should be emphasized that the anthrone method detects also glucosyl residue in glucosides, which are known to accumulate often in quite large quantities in plant tissues.

Chlorophylls were analysed according to Vernon (1960). Separate leaves were used for the determination of the dry matter content and for the expression of the content of pigments on dry weight basis (mg g^{-1} DW).

The soils were analysed in the Estonian Plant Protection Centre at Saku using ISO standard methods (ISO/10390, 1994; ISO/11260, 1995; ISO/11261, 1995). The content of mineral elements in the soil available to plants was determined and the pH of the soil was measured as the potential acidity in 1 N KCl.

Morphometry

The growth of shoots, stems, and leaves was measured at the end of August in both years of treatment (1998 and 1999). Foliage, stems, and roots were weighed. The length of spring flush shoots (cm) and stem height (cm) were measured. The length and width (cm) of leaves were measured and their dry and fresh weights (g) were determined.

Climate conditions

Weather conditions can substantially modify the seasonal effect of stress factors on plants. According to the nearest weather station (15 km SW), the vegetative growth period of the sampling year was unusually dry, warm, and sunny. May, July, August, and September were months of low precipitation (5–45 mm per month) and leaves showed signs of wilting in July and August. From May to September, the mean monthly amount of precipitation was 34 mm, the mean temperature 14.5 °C, and there were 316 h of sunshine, while the respective 30-year average monthly values were 67 mm, 13.3 °C, and 246 h.

Statistical analyses

Data were analysed by Systat version 7.0 (Systat Inc., US). As the samples were gathered from the same trees on different dates, the differences of treatment from control were tested with the repeated measures analysis (Girden, 1992). Parameter ratios were analysed after log-transformation to unify the scale of variances. For the analysis of differences in growth and soil data, a *t*-test was used. Correlation (*r*) was calculated with MS Excel 9.0. Significances (*p*) of differences were accepted at the 95% confidence level.

RESULTS

Soil

Cement kiln dust increased the pH of soil (max 8.1) and the levels of Ca, K, and Mg (Table 1). It is well known that a high pH of soil reduces the mobility and availability of several mineral elements: mainly metals (Fe, Mn, Cu), which are oxidized, and P, which forms insoluble Ca phosphates. Also, the availability of B is low in alkaline soils (Lehto, 1994), but in the current experiment this was most likely compensated by the B contained in the cement dust. Liming can increase or decrease the amount of available N. However, we did not observe any effect on the N content of soil.

Table 1. Available content (μg g^{-1}) of mineral elements and the pH of the soil treated with cement dust. Average (n = 5) during 1998 and 1999. (± SD; p value by two sided pair-wise t-test)

Parameter	Control	Soil with cement dust	p
pH_{KCl}	6.54 ± 0.3	7.58 ± 0.4	0.001
N	2020 ± 130.4	2120 ± 228.0	0.419
P	162.8 ± 20.1	144 ± 8.6	0.091
K	42.6 ± 10.0	322.0 ± 91.4	0.000
Ca	5200.0 ± 369.1	12332.0 ± 5114.8	0.014
Mg	465.6 ± 44.2	701.4 ± 210.3	0.040
Cu	8.39 ± 0.4	7.08 ± 1.6	0.117
Mn	36.4 ± 4.0	20.6 ± 0.9	0.000
B	0.76 ± 0.1	1.388 ± 0.4	0.005

Growth

On the treated soil, the biomass of trees (measured in the first year of the experiment) was notably lower than control (Table 2). Statistical tests for differences from control yielded highly significant ($p < 0.0003$) results for all organs and for the total dry weight. However, the indicator of carbon allocation – partitioning of biomass between organs – was not significantly affected ($p = 0.493$).

The treated trees had fewer leaves, and the dimensions of leaves were smaller than on the control trees. Differences from control in the length and width of

Table 2. The effect of soil treatment with cement dust on the dry weight of trees in the first year of treatment (n = 10; ± SD)

Organ	Dry weight, g		% of total	
	Control	Dusted	Control	Dusted
Foliage	3.8 ± 0.5	2.4 ± 0.4	49.7 ± 1.9	50.8 ± 2.0
Stem	3.4 ± 0.8	2.0 ± 0.4	43.6 ± 3.4	42.2 ± 1.8
Root	0.5 ± 0.1	0.3 ± 0.1	6.7 ± 1.9	7.1 ± 1.3
Total	7.7 ± 1.3	4.6 ± 0.9	100	100

leaves were larger in the second year. The effect of treatment was less pronounced on the dimensions of leaves (90–100% of control, $n = 20$ trees $\times$ 5 leaves) than on the number of leaves (60% of control). The length of the spring flush shoots was not different from control in the first year (on 5 July). Substantial differences in the shoot length appeared in the second year when a reduction by 30% from control ($n = 15$, $p < 0.001$) was observed.

Pigments and carbohydrates

Across the sampling dates, the mean Chl content was by 5% lower in the leaves of treated trees than in control ($p < 0.001$), with the largest differences observed in July and August (Fig. 2). On 1 July the Chl content was 25% lower in treatment trees. Still, the reduction in the Chl content of Fe-deficient pear trees could be much greater even before the appearance of chlorosis (Morales et al., 2000).

Sucrose made up a large part of the soluble sugars during the whole vegetation period (Fig. 3). Maltodextrins contributed significantly only in May and June. We did not find any substantial levels of fructans in contrast to the results reported by Bollmark et al. (1999) on *Salix viminalis*.

The relative influence of cement dust was greatest on the level of hexoses (38–101% of control). In terms of the absolute effect, the lower hexose content was approximately balanced by the higher sucrose content. Maltodextrins were not affected significantly. As a result, the total content of hexoses + sucrose + maltodextrins did not differ substantially from control while the (Glc+Fru)/sucrose ratio differed most. There was still some indication that the total content of hexoses + sucrose + maltodextrins in the treatment variant actually fluctuated around the control level (p value of interaction with time = 0.083). The unknown soluble fraction increased at the beginning of summer and later reached values approximately as high as sucrose. Before August, the unknown fraction was higher in the treatment variant than in the control but later a reverse relationship was observed. The content of starch was not affected by treatment.

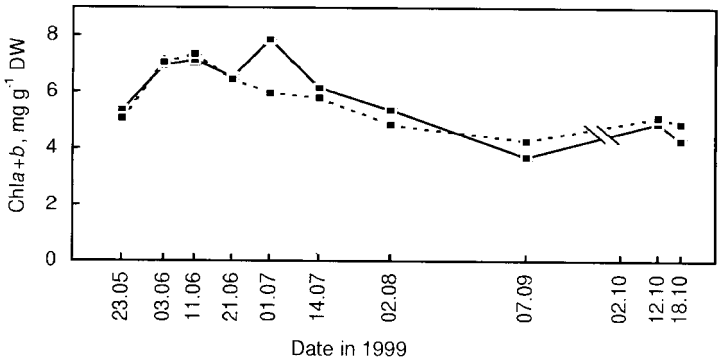

Fig. 2. Total chlorophyll (Chl*a* + *b*) in leaves of willow grown on soil treated with cement dust. Solid line, control; dashed line, treatment; $n = 3$; ±SD (if exceeding the size of markers).

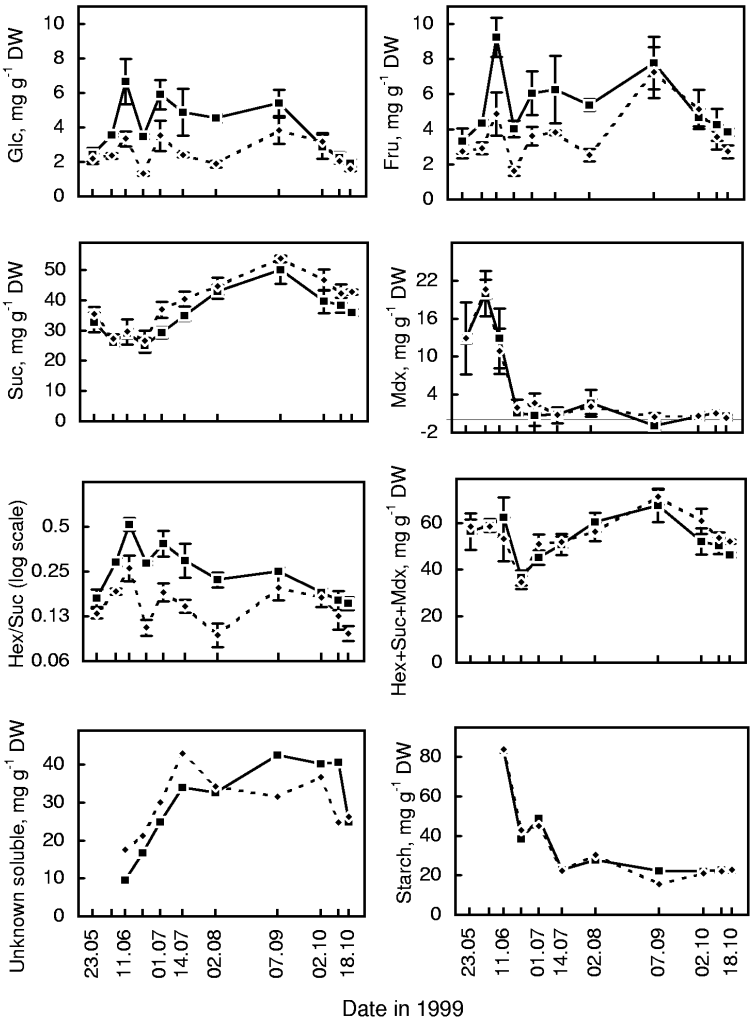

Fig. 3. Soluble sugars and starch in leaves of willow grown on soil treated with cement dust. Solid line, control; dashed line, treatment; $n = 1$ and 3; ±SD; Fru = fructose; Glc = glucose; Hex = glucose + fructose; Mdx = maltodextrins; Suc = sucrose.

The ratio of total hexoses to sucrose often corresponds to the relative activities between sucrolysis and sucroneogenesis, and thus it can show whether leaves were functioning more as sinks or more as sources. The correlation of (Glc+Fru)/sucrose ratio with time was negative in both control and treatment trees ($r = –0.61$ and –0.30, respectively), but the significance of the correlation was higher in the control ($p = 0.048$) than in the treatment ($p = 0.365$). Fluctuations in the (Glc+Fru)/sucrose ratio were similar to those in the content of Glc and Fru as the dynamics of sucrose was relatively smooth and hexoses fluctuated considerably.

The levels of Glc and Fru were correlated significantly in both control and treated trees. Differences from control were also correlated. From May to September, the effect of treated soil on the Fru/Glc ratio resembled largely the effect of dust on the Chl*a*/Chl*b* ratio (Fig. 4). The observed treatment effect on a ratio of parameters can depend on the relevant ratio in control (<1, =1, or >1). In the current experiment, differences from control did not depend on the value in control as the Fru/Glc and Chl*a*/Chl*b* ratios were higher than 1 throughout the vegetation period.

The ratios (Glc+Fru)/sucrose and Fru/Glc were negatively correlated in the control leaves ($r = -0.65$, $p = 0.029$) (Fig. 5). The differences from control of these two ratios had a relationship composed of three parts, which suggests the occurrence of different metabolic regimes with abrupt transitions.

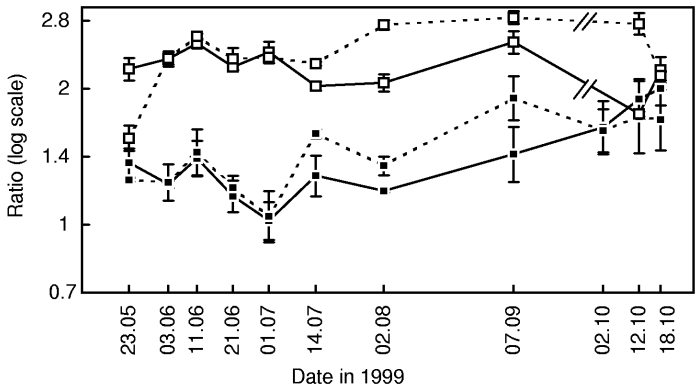

Fig. 4. Ratios of fructose to glucose (black squares) and chlorophyll *a* to chlorophyll *b* (open squares) in leaves of willow grown on soil treated with cement dust. Solid line, control; dashed line, treatment; $n = 3$; ± SD.

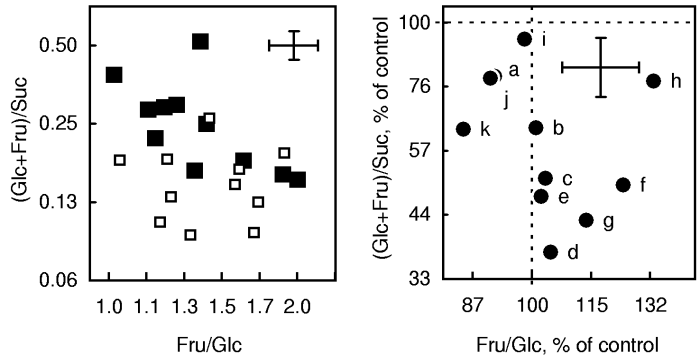

Fig. 5. Relationships between different sugar ratios within control (black squares) and within treatment (open squares), and between effects of treatment (black circles). Letters indicate sampling dates: a, 23.05; b, 03.06; c, 11.06; d, 21.06; e, 01.07; f, 14.07; g, 02.08; h, 07.09; i, 02.10; j, 12.10; k, 18.10; cross-bars, ± SD on average; dashed lines, control; Fru = fructose; Glc = glucose; Suc = sucrose.

DISCUSSION

Almost equal deviations in the Glc and Fru content from control may indicate that in leaves the flux of carbon through the reaction catalysed by invertases was reduced in response to the treatment of soil with cement dust. Slower cleavage of sucrose through invertase was further supported by the lower ratio of total hexoses to sucrose in the treatment trees (Zrenner et al., 1996). The main reasons could be that the kinetic properties of invertase were affected (higher concentration of the substrate was required for the same reaction velocity), the amount of enzyme was smaller, or less sucrose was available to invertase. The levels of Fru and Glc were also correlated during development. The correlation between Fru and Glc is explainable by the action of invertases and hexose kinases: invertases yield Fru and Glc in equal amounts, and the expression of glucokinase (EC 2.7.1.1) and fruktokinase is increased by their substrates (Fox et al., 1998; Kanayama et al., 1998). A time interval occurred when the Fru/Glc ratio was not different from control at substantially lower levels of Fru and Glc in the treatment. Since the pool of hexoses is dynamic, it is plausible that the Fru/Glc ratio had some regulatory meaning for the leaf metabolism and the ratio was protected from the influence of cement dust by an intrinsic determination of the species.

Accumulation of starch in sink leaves can facilitate the sink–source transition. On the one hand, if a cell cleaves and synthesizes sucrose at a time, increased futile cycling of sucrose may lower the efficiency of metabolism (Trethewey et al., 1999). Branching of activated sugars to glucolysis or futile cycling is statistical in nature – some molecules are glucolysed and some cycled. On the other hand, rapid resynthesis of sucrose can be under control, and the ability of plants to adjust the efficiency of sugar metabolism may have adaptive value (Black et al., 1987; Büssis et al., 1997). Wasting in favourable conditions when metabolites could actually be used more efficiently and stopping the wasting under stress can be built up on regulation which is more useful than finding energy resources for adaptation at the time of stress.

Starch is generally known as a storage compound and starch reserves could be used in stress conditions (when photosynthesis is low or consumption of C-skeletons is high). Reduced growth of trees indicated that cement dust caused stress of trees. However, in the current experiment the starch content of leaves was not affected. Possibly the strain on the carbon flow was not a driving force for the reduction in tree growth or reserves from stems or roots may have been used for the adaptation. At least in spring (5 May), the starch content of treated trees was lower in bark (26.2 and 29.8 mg g^{-1} DW; treatment and control, respectively) and roots (12.9 and 14.7) but higher in leaves (14.2 and 13.0) and wood (18.2 and 11.6) than control.

Deciduous and coniferous trees show bimodal root growth in temperate climates. Roots grow in spring and winter. During the intensive growth of shoots, a small amount of sugar is allocated to roots (Hansen & Beck, 1994). In the current study, roots were immediately influenced by the cement dust and possibly

required extra carbon and energy compared to control roots. So, in summer, the allocation for growth could conflict with the allocation for adaptation. However, the summer had unusually dry periods and it is quite possible that drying increased the alkalinity of the treated soil. Comparison of scatterplots with (Glc+Fru)/sucrose versus Glc/Fru in Fig. 5 suggests that the internal regulation was superimposed by the fluctuating environmental conditions and these fluctuations were higher in the treatment trees.

The shoot/root ratio did not change significantly. The content of available manganese in soil was substantially reduced in our experiment and, as shown by Pearson & Rengel (1997), Mn deficiency has no effect on biomass partitioning between shoots and roots of wheat. Alkaline soil causes root damage in some species (Tang et al., 1993; Lehto, 1994). Possibly, trees did increase the availability of Mn and Fe, since Chl levels were seldom substantially reduced. It is likely that more sugars were consumed by the roots to replace or repair damaged roots and to increase the availability of Fe, Mn, and P with the help of root exudates.

The increased Chl*a* to Chl*b* ratio of Fe-deficient trees is associated with the reduced efficiency of photosynthesis (Morales et al., 2000). An increased Chl*a* to Chl*b* ratio was also observed in mutants where the last step in the pathway of Chl synthesis was inhibited (Falbel & Staehelin, 1994). Differences in the Fru/Glc and Chl*a*/Chl*b* ratio were qualitatively similar. Higher Fru/Glc ratios in treated trees than in controls were caused by the equal decrease in Fru and Glc. When the Fru/Glc ratio did not differ from control then the Chl*a*/Chl*b* ratio did not differ either. Most likely, the trees adapted to the treatment with the reduced growth and slower consumption of assimilates. However, it could be also speculated that on the plot treated with cement dust, fast growth or drought lowered the supply of mineral elements and the efficiency of photosynthesis fell, and the leaves adapted to the reduced photosynthesis by down-regulating the futile cycling of sucrose.

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KLINKRITOLMUGA TÖÖDELDUD MULLA MÕJU SÜSIVESIKUTE SESOONSELE DÜNAAMIKALE PAJULEHTEDES

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Pistokstena istutatud paju (*Salix dasyclados* Wimm.) kasvatati tsemenditolmuga töödeldud mullal (2 kg m^{-2} esimesel aastal + 1 kg m^{-2} teisel aastal). Mulla happesus tõusis väärtusele 7,6 (töötlemata mullal 6,5). Määrati lehtede lahustuvate süsivesikute, tärklise ja klorofüllide sisaldus teise töötlusaasta vegetatsiooniperioodi jooksul. Töötlusvariandis oli glükoosi ja fruktoosi kontrollist madalam sisaldus ligikaudu tasakaalus sahharoosi kõrgema sisaldusega. Maltodekstriinide ja tärklise hulgale töötlus ei mõjunud, selle toime klorofüllide sisaldusele oli tühine. Taimede kogu biomass oli tsemenditolmu tõttu kontrollist oluliselt väiksem, kuid biomassi jaotumine erinevate taimeorganite vahel ei erinenud kontrolli omast.