CELL-WALL HEMICELLULOSES AS MOBILE CARBON STORES IN PLANTS

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CHAPTER 1

GENERAL INTRODUCTION

General introduction

Carbon storage

In view of the currently increasing atmospheric CO_2 concentrations (IPCC 2001) investigations on the carbon balance of plants have drawn considerable attention over the last decades. Plants take up carbon via photosynthesis and invest it into various C-sinks, like respiration, structural growth, export or mobile reserves (Körner 2003). Because carbon reserves accumulate at times of overabundant C-supply (C-sources > C-sinks) and decrease during periods of insufficient photo-assimilation, C-source and sink-balances are mirrored in the size of the plants' mobile carbon reserve pools. C-storage in plants can be achieved by three different processes: reserve formation, accumulation and recycling (Chapin *et al.* 1990). Reserve formation is an active formation of carbon reserve compounds, which therefore competes directly for carbon with other carbon sinks within a plant. Accumulation is not competing for photoassimilates with a plant's C-sinks (growth, respiration, etc.) but occurs when carbon assimilation exceeds the need of carbon for growth and maintenance. Recycling, finally, is the re-utilization of carbon from organic compounds, the immediate physiological function of which is not storage (e.g. organic acids). Storage can occur at different time scales such as daily storage (e.g. short term starch accumulation during the day for use at night) or long term storage (Chapin *et al.* 1990).

Carbon storage is an important component of plant fitness and increases the possibility of recovery after any kind of disturbance which might be crucial with respect to global change (higher frequency of extreme events and disturbances, higher temperatures, CO_2 increase and species competition). Increasing CO_2 concentrations are known to increase the mobile carbon reserve pools since in most ecosystems the observed increase in net-photosynthesis is not matched by higher rates of growth and other carbon sinks (e.g. Poorter *et al.* 1997; Penuelas and Estiarte 1998; Körner 2003).

Mobile carbon stores

The most important and widespread mobile carbon compound is starch which is stored in plastids as a direct product of photosynthesis and therefore is subject to daily fluctuations. Starch consists of exclusively α-1,6 and α-1,4 linked glucose units which build water insoluble granules. Furthermore low molecular weight carbohydrates (e.g. glucose, fructose, sucrose) and oligosaccharides of the raffinose-family are also considered to serve as C-sources but have a primary function as metabolites for many physiological processes. Even sugar alcohols can serve as reserve sugars like in the case of the acyclic sorbitol which substitutes sucrose as the main long-distance transport compound of carbon in Rosaceae. Besides starch, fructans are stored in the vacuole of different plant families (e. g. Asteraceae, Campanulaceae, Liliaceae and Poaceae) and play an important role as mobile carbohydrates (Meier and Reid 1981; Hendry 1987). Certain plant families store large amounts of lipids, which are considered to be long term storage pools since their production and remobilization is costly (e.g. Fischer and Höll 1991; Kozlowski and Pallardy 1991; Fischer and Höll 1992; Hoch and Körner 2003). In addition to all these well known carbon reserve compounds, cell wall hemicelluloses may also serve as potential mobile carbon reserves additionally to their structural function. Cell wall compounds that serve a specific cell-function (cell wall structure) are considered mobile, if they can be recycled and thus be used for other cell functions, such as growth or respiration. It is already known that cell wall polysaccharides are modified during expansion of primary cell walls, fruit softening and other developmental processes when cell wall loosening does occur. In such situations polysaccharide are modified by enzymes (e.g. Fry 1995; Cosgrove 2000). The dynamic nature of cell walls and re-mobilized cell wall polysaccharides might represent additional C-sources for growth or other physiological processes.

Hemicelluloses

Cell walls are by far the largest carbon pool of biomass and therefore play a key role in the carbon cycle and carbon budget of ecosystems. Cell walls consist of a complex mixture of polysaccharides and other polymers that are tightly interconnected. The proportion of the single cell components can vary strongly between tissues, species and functional groups (Fig. 1). Although hemicellulose concentrations are generally lower in leaves than in wood, hemicelluloses account for about one quarter of the total biomass (considering that tree stems account for 90% of the entire biomass (Körner 2003)) and are therefore the second most abundant polysaccharide on the planet. In contrast to cellulose, which consists exclusively of 1,4-linked β-D-glucose units, hemicelluloses occur in a large variety of structural types which are classified according to their main type of sugar: xylans, xyloglucans, mannans and mixed linkage β-glucans (for a detailed review see Ebringerova (2005)). The most common hemicelluloses are the xylans that comprise about three quarter of the entire hemicellulose pool in hardwood species, grasses, and which are present in lower amounts in all other plant functional types and tissues. Xylans consist of a backbone of 1,4-linked β-D-xylopyranose units linked together with different sugar and sugar acid residues such as arabinose, glucose, galactose and glucuronic acid and, in lower amounts, rhamnose and galacturonic acid (e.g. Puls 1993; Smith and Harris 1999; Saha 2003; Willför *et al.* 2005b). Xyloglucans are quantitatively a major component of primary cell walls of all higher plants and have a structure similar to cellulose, but with frequent branching of α-D-xylose residues that prevent them from

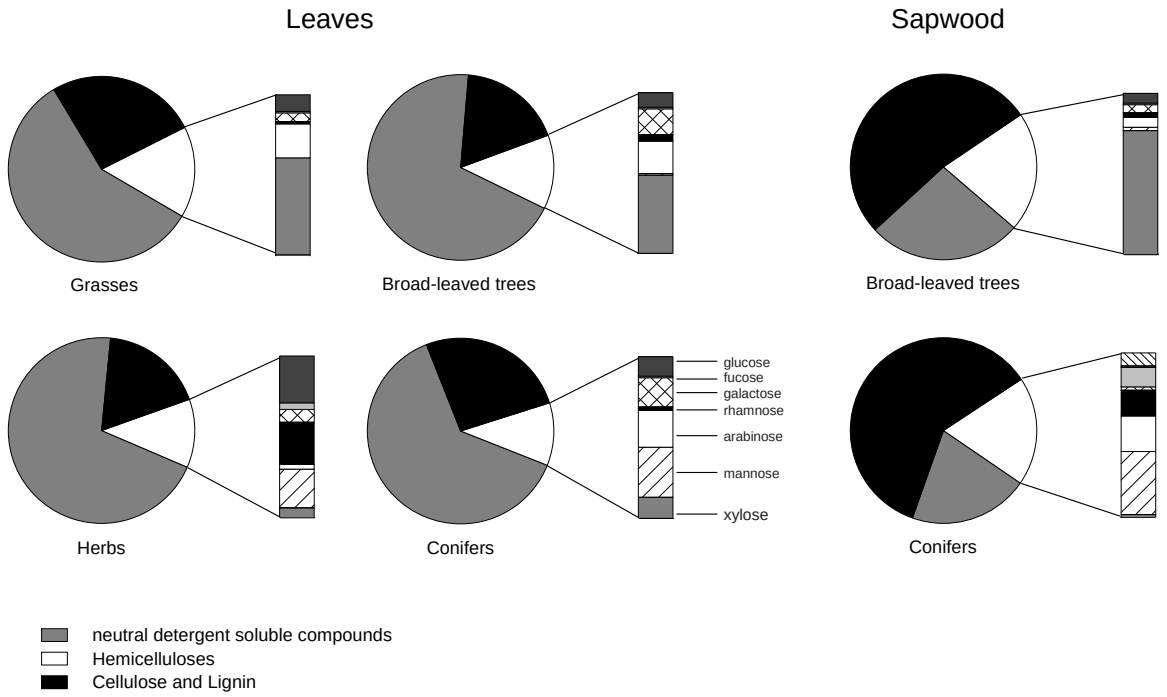

Figure 1: Composition of plant cells and hemicellulose composition for leaves of four different plant functional groups and sapwood of broad-leaved trees and conifers. Neutral detergent soluble compounds include non-structural carbohydrates, pectins, proteins, lipids and other water-soluble compounds. Data were collected within the current thesis and are mean values of 5-8 species.

forming microfibrils (e.g. Hayashi 1989; Buckeridge *et al.* 2000b; Cosgrove 2005). Mannan-type hemicelluloses are very common in gymnosperms and seeds of Leguminosae and consist of a 1,4-linked β-D-mannose backbone mixed with glucose and galactose residues (e.g. Buckeridge *et al.* 2000b; Willför *et al.* 2005a). The fourth group of hemicelluloses are the mixed linkage 1,3 and 1,4 linked β-D-glucans that essentially occur in Poales (e.g. Carpita 1996; Smith and Harris 1999; Buckeridge *et al.* 2004) and in *Equisetum* species (Fry *et al.* 2008; Sorensen *et al.* 2008).

Quantity and structure of hemicelluloses are well studied since hemicelluloses, as part of the lignocellulosic material, belong to the most abundant organic resources on earth that can be converted to industrial products or biofuels (e.g. Saha 2003; Gray *et al.* 2006; Somerville 2007; Pauly and Keegstra 2008). Hemicelluloses are well-known in the pulp and paper industry, where they are also discussed as wet-end additives to improve the mechanical properties of paper (e.g. Lima et al. 2003; Puls et al. 2006) but are not as important as cellulose, starch or pectins. In contrast to industrial research, surprisingly little is known about the ecological and physiological properties of hemicelluloses. Thus, the frequently discussed additional function of hemicelluloses as mobile carbon stores is still equivocal (Hoch 2007).

Mobility of hemicelluloses

Cell wall polysaccharides of the hemicellulose classes of mannans, xyloglucans and galactans are known to be mobile carbon reserves in endosperm reserves and cotyledons of seeds of many plant families such as Arecaceae, Compositae Convolvuceae, Leguminosae and Liliaceae (Meier and Reid 1981; Buckeridge *et al.* 2000a; Buckeridge *et al.* 2000b). These hemicellulose classes are proposed to be multifunctional molecules that became storage polysaccharides during evolution but still kept their structural function (Buckeridge *et al.* 2000b). These hemicellulose classes are actively mobilised during germination, which is accompanied by high activities of different enzymes responsible for the re-mobilization of hemicelluloses as well as the re-translocation of hemicellulose-derived sugar-residues from the cell wall into the cytoplasm (Buckeridge *et al.* 2000b).

As hemicelluloses from non-reproductive tissues contain the same hemicellulose classes as were found in seeds, the question arises, if and to which extend hemicelluloses in tissues other than seeds are mobile, as well. Several studies described temporal fluctuations of hemicelluloses to resemble that of non-structural carbohydrates and thus suggested that they might serve as additional C-sources in times of enhanced C-demand. One way to explore the flexibility of hemicelluloses in a carbon supply context is to manipulate the C-source-sink availability. For example, elevated CO_2 concentrations increased hemicellulose concentrations in *Castanea sativa* saplings (Couteaux *et al.* 1996), leaves of *Quercus myrtifolia* (Hall *et al.* 2006) and in leaf litter of *Betula pendula* (Oksanen *et al.* 2005). Both increasing and decreasing hemicellulose

concentrations in response to elevated CO_2 were found in leaves of Mediterranean shrub species (Penuelas *et al.* 2002), whereas no response to elevated CO_2 was found in wood of young *Pinus sylvestris* trees (Kilpeläinen *et al.* 2003). Poorter *et al.* (1997) investigated cell wall polysaccharides (no separation of cellulose and hemicelluloses) in leaves of different herbaceous species and found no response to elevated CO_2. High soil nutrient availability increases growth and therefore C-sink-activity, which in turn potentially reduces carbon reserves. Interestingly, soil fertilization reduced hemicellulose concentrations in conifer wood in several previous studies (Anttonen *et al.* 2002; Kilpeläinen *et al.* 2003; Kaakinen *et al.* 2004). Seedlings of barley (*Hordeum vulgare*) that were exposed to darkness exhibited increased concentrations of two mixed-linkage-glucan degrading enzymes which resulted in strongly decreased concentrations of cell wall (1,3 and 1,4)-β-D-glucans (Roulin *et al.* 2002). This decrease suggests a re-mobilization of the cell wall glucans in dark-incubated leaves and the additionally available glucose may serve as an energy source. Naturally occurring situations of stronger C-sink-activity than C-source-activity are to be expected during bud break. Investigations of hemicelluloses in previous seasons' needles and sapwood of evergreen trees revealed trends of decreasing hemicellulose concentrations after flushing, similar to what is known for non-structural carbohydrates (Glerum and Balatinecz 1980; Renault and Zwiazek 1997; Robakidze and Patov 2000; Robakidze and Bobkova 2003).

The carbon balance in plants is important in the ongoing debate about climate change mitigation since biomass carbon pools are sinks for atmospheric CO_2. If hemicelluloses are additional carbon reserves they would strongly increase the sink strength of carbon reserves under elevated CO_2 and thus change the whole carbon budget, especially as hemicelluloses account for one quarter of the total dry plant biomass. Should the hemicellulose concentrations increase with increasing CO_2 concentrations this would also be important for forage quality and litter decomposition as higher hemicellulose concentrations implicate higher foliar C/N ratio which would have major impacts on many ecosystem processes like herbivory.

The following chapters aim at clarifying the role hemicelluloses play as carbon reserves of plants. So far, many studies described the quantity and structure of hemicelluloses but only very few studies focused on hemicelluloses as carbon reserves. Since hemicelluloses are the second most abundant polysaccharide in plants that differ quantitatively and qualitatively among plant species and tissues, I aimed at investigating hemicelluloses in different tissues of several species from different plant functional types to arrive at a more general picture of the carbon reserve capacity of hemicelluloses.

Chapter 2: This chapter presents the first broad comparative analysis of hemicellulose concentration and composition over different tissues of several plant functional types within a single study. For this study, the fiber analysis after Van Soest (1963; 1967) was modified to enable the simultaneous quantitative and qualitative measurements of hemicelluloses and to assess the ratio of cellulose and lignin to hemicelluloses in small sample volumes. Subsequent HPLC analyses of the hydrolyzed monosaccharides were added to analyze the monosaccharide composition of hemicelluloses which varied greatly among plant functional types and tissues.

Chapter 3: A broad-scale experiment was conducted to determine the responsiveness of hemicelluloses to different CO_2 concentrations in 16 species of four different plant functional types (grasses, herbs, broad-leaved trees and conifers). Three concentration-levels of CO_2 (140, 280 and 560 ppm) were applied within separate growth chambers to induce situations of carbon under- or oversupply. The exceptionally low CO_2 concentrations of 140 ppm (which is half the pre-industrial atmospheric CO_2 concentration) induced carbon limiting situations for plants, which, besides reduced growth, led to very low concentrations or even a depletion of mobile carbon compounds. On the contrary, high CO_2 concentrations strongly increased growth rates under the given rich soil conditions but also induced accumulation of carbon reserves. Hemicelluloses were investigated quantitatively and qualitatively and the data were compared with the concentration of non-structural carbohydrates (which are well-known C-reserve compounds) to assess the responsiveness of hemicelluloses to varying carbon supply.

Chapter 4: This study was a quantitative assessment of the role of non-structural carbohydrates and cell wall hemicelluloses in young branches of mature forest trees during enhanced carbon demand at spring bud break. Especially in deciduous species, the initial phase of leaf and shoot growth drains heavily on stored C-reserves, hemicelluloses might be used as additional C-sources, besides non-structural compounds to satisfy the high C-demand at flushing. Terminal branches of two deciduous tree species (*Carpinus betulus* and *Fagus sylvatica*) and two evergreen tree species (*Picea abies* and *Pinus sylvestris*) were sampled in short intervals before, at and after bud break using a forest canopy crane in order to capture even short term fluctuations in the mobile carbon pools. Similar to the CO_2 manipulation experiment (chapter 3) hemicellulose fluctuations were compared with the fluctuations of non-structural carbohydrates (i.e. starch and low molecular weight sugars) to reveal the significance of hemicelluloses as mobile carbon stores.

Finally, **chapter 5** will present a short general summary synthesizing the main conclusions derived from the single studies of this thesis.

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CHAPTER 2

QUANTIFICATION AND MONOSACCHARIDE COMPOSITION OF HEMICELLULOSES FROM DIFFERENT PLANT FUNCTIONAL TYPES

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Research article

Quantification and monosaccharide composition of hemicelluloses from different plant functional types

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ABSTRACT

Hemicelluloses are the second most abundant polysaccharide in nature after cellulose. So far, the chemical heterogeneity of cell-wall hemicelluloses and the relatively large sample-volume required in existing methods represent major obstacles for large-scale, cross-species analyses of this important plant compounds. Here, we apply a new micro-extraction method to analyse hemicelluloses and the ratio of 'cellulose and lignin' to hemicelluloses in different tissues of 28 plant species comprising four plant functional types (broad-leaved trees, conifers, grasses and herbs). For this study, the fibre analysis after Van Soest was modified to enable the simultaneous quantitative and qualitative measurements of hemicelluloses in small sample volumes. Total hemicellulose concentrations differed markedly among functional types and tissues with highest concentration in sapwood of broad-leaved trees (31% d.m. in *Fraxinus excelsior*) and lowest concentration between 10-15% d.m. in leaves and bark of woody species as well as in roots of herbs. As for total hemicellulose concentrations, plant functional types and tissues exhibited characteristic ratios between the sum of cellulose plus lignin and hemicelluloses, with very high ratios (> 4) in bark of trees and low ratios (< 2) in all investigated leaves. Additional HPLC analyses of hydrolysed hemicelluloses showed xylose to be the dominant hemicellulose monosaccharide in tissues of broad-leaved trees, grasses and herbs while coniferous species showed higher amounts of arabinose, galactose and mannose. Overall, the micro-extraction method permitted for the simultaneous determination of hemicelluloses of various tissues and plant functional types which exhibited characteristic hemicellulose concentrations and monosaccharide patterns.

1. Introduction

Cell-walls are by far the largest pool of organic carbon in plants. They consist of a variety of different compounds, mainly structural polysaccharides, pectins, lignin, proteins and other minor components. Structural polysaccharides are cellulose and hemicelluloses with the ratio of cellulose:hemi-celluloses commonly varying between 2:1 to 1:1 [1]. Hemicelluloses are thus the second most abundant polysaccharide group in plants and, depending on the tissue, generally account for 10 to 30% of a tissue's dry biomass. They are defined as those cell-wall polysaccharides that are insoluble in water but can be extracted with aqueous alkali and hydrolysed into its component monosaccharides with diluted sulphuric acid [2]. Hence, hemicelluloses are rather defined methodically, than by structure and they comprise different classes of polysaccharides, of possibly different physiological and mechanical functions.

Despite their quantitative importance, hemicelluloses are often neglected in ecological studies, mainly due to analytical difficulties associated with their chemical heterogeneity. Therefore, our current knowledge about hemicelluloses derived largely from structural research, dietary fiber analyses and the paper industry, but only few studies investigated the ecophysiological significance of these important plant compounds [references cited in 1, 3].

Hemicelluloses are grouped into four classes according to their main types of sugar residues present: xyloglucans, xylans, mannans and mixed linkage β-glucans (for a detailed review on hemicellulose diversity see Ebringerova et al. [4]). Xyloglucans occur in primary cell-walls of all higher plants and seem to be tightly bound to cellulose fibrils by hydrogen bonds [5]. Hemicelluloses of the xylan type are the most abundant hemicellulose class in secondary cell-walls of hardwood species and herbaceous plants [6, 7]. Glucomannans and galactomannans are the main representatives of the mannan-type hemicelluloses, which are the predominant hemicelluloses in secondary cell-walls of conifers [8]

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and seeds of *Leguminosae* [9]. Finally, mixed-linkage (1→3, 1→4)-β-D- glucans occur exclusively in *Poales* [10] and some pteridophytes (*Equisetum*, [11]).

The great chemical diversity of hemicelluloses is the biggest challenge for quantitative and qualitative analyses of these cell-wall compounds. Many published methods isolate hemicelluloses by complex sequential extraction [12-14]. Using multiple extraction steps allows for a good separation of the cell-wall polysaccharides from low-molecular-weight water soluble compounds, proteins, lipids and lignin. Hemicelluloses are then usually extracted with aqueous alkaline solutions and further hydrolysed with acid into their monosaccharides which are subsequently measured by chromatographic methods [12]. However, these multi-step extractions are very labour-intensive preventing large-scale screenings over a multitude of samples. In contrast to these very elaborate methods one procedure, which was originally developed for quality analysis of forage in agriculture [15-17], has been established as the most promising avenue for large-scale determination of hemicelluloses. The basic principle of this method is a two step separation of bulk hemicelluloses from all non-structural cell compounds (the protoplasmatic content) and cell-wall pectins on one hand, and cellulose and lignin on the other hand. Since hemicelluloses are hydrolysed into their corresponding monosaccharides during the extraction process, the hemicellulose containing extract can be directly used for the qualitative determination of the hemicelluloses' monosaccharide composition (e.g. by HPLC). However, the standard procedure after Van Soest requires relatively large amounts of sample material which conflicts with the parallel analysis of many samples.

Here we report on a study in which total concentrations of hemicelluloses as well as their monosaccharide composition in different tissues from 28 plant species (comprising four different plant functional types) were analysed by a micro-extraction method. This method, which is based on the original Van Soest fiber analysis [15-17], decreases the required amount of plant material per sample (50 mg dry weight) and thus permits rapid screening for hemicelluloses in up to 80 samples within a single run. Particularly we substituted all filtering steps by sequential centrifugation steps, which enabled a drastic reduction of the required sample volume. Subsequent HPLC analyses of the hydrolysed monosaccharides permitted their identification. To our knowledge this is the first broad comparative analysis of hemicellulose concentrations and composition over different tissues of several plant functional types within a single study. The novel analytical procedure is a long awaited break through that will permit a better exploration of this important field of plant science. The results are compared with data from previous studies using different methods for the quantification and description of hemicelluloses. Finally, ecological implications of the functional type-specific differences in hemicellulose content and composition are discussed.

2. Results

2.1. Hemicelluloses in different plant functional types

The gravimetric analyses of total hemicelluloses showed characteristic differences among plant functional types and tissues with the highest concentrations in sapwood of broad-leaved trees and roots of grasses (both about 25% d.m. (dry matter)) followed by conifer sapwood (approximately 20% d.m., Fig. 1). Smaller hemicellulose concentrations of around 15% d.m. were present in leaves of grasses and herbs with high variation between species. Leaves and bark of broad-leaved trees as well as needles and bark of conifers and roots of herbs exhibited the lowest mean hemicellulose concentrations of 10-12% d.m.

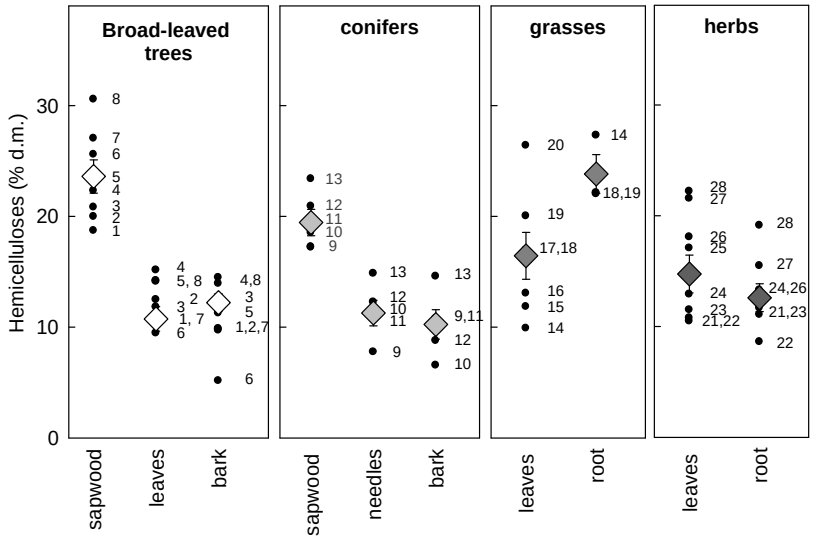

Figure 1: Total hemicellulose concentrations [gravimetrically calculated as total cell wall fraction – cellulose and lignin fraction] in % dry matter in different tissues of four functional plant groups. The black dots are values for single species; open diamonds are means for the respective tissue ± standard error. The numbers given beside the black dots indicate the species identity as listed in Table 1.

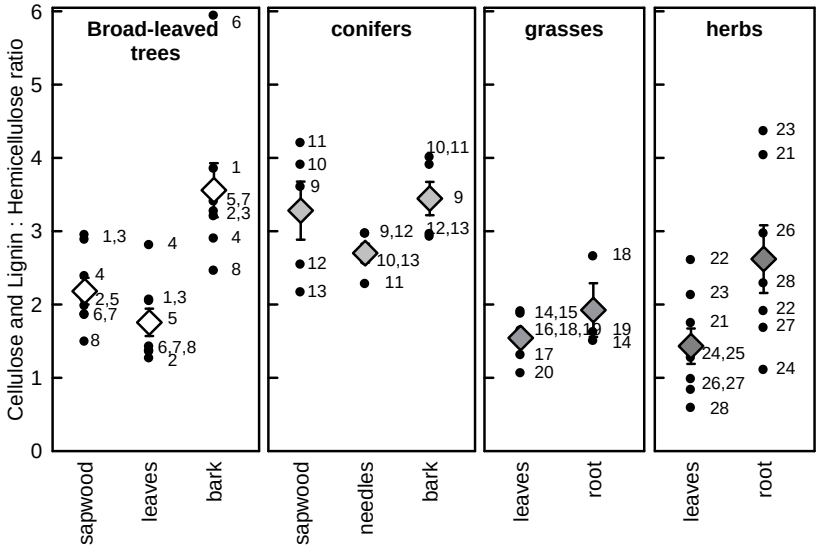

Figure 2: The ratio of the 'cellulose and lignin fraction' to hemicelluloses in different tissues of four functional plant groups. The black dots are values for single species; diamonds are means for the respective tissue ± standard error. The numbers given beside the black dots indicate the species identity as listed in Table 1.

Depending on plant functional type and tissue, the sum of cellulose plus lignin accounted for 14 to 70% of the dry biomass. Overall, the 'cellulose and lignin fraction' was 1.5 to 3.5 times greater than the gravimetric hemicellulose pool (Fig. 2). Among the analysed tissues, leaves of broad-leaved trees, grasses and herbs exhibited relatively smaller ratios of 'cellulose and lignin' to hemicelluloses, with leaves of two herbaceous species (*Silene flos-cuculi* and *Urtica dioica*) having even slightly more hemicelluloses than cellulose and lignin. In contrast, exceptionally high ratios were found in the bark of broad-leaved trees and conifers, which resulted rather from low hemicellulose than high cellulose and lignin concentrations.

2.2. Monosaccharide spectrum of hemicelluloses

Since the acidic extraction of cell wall hemi-celluloses hydrolysed the heteropolysaccharides into their corresponding monosaccharides, the de-termination of hemicellulose-derived monomers by HPLC could be performed directly within the acidic extract. The used separation column and water:NaOH eluent gradient provided sufficient peak separation among the monosaccharides for optimal detection of even small sugar concentrations (Fig. 3, detection limit at 0.05 mg g^{-1} d.m.).

The monosaccharide spectrum of hemicelluloses varied among plant functional types and, to a lesser extent, among tissues within a plant functional type (Fig. 4). Hemicelluloses in sapwood of broad-leaved trees revealed a very high proportion of xylose (about 80% of all hemicellulose-derived monosaccharides), which has been hydrolysed from xylans as the main hemicellulose type in this tissue. In contrast, hemicelluloses in bark and leaves of broad-leaved tree species had additionally significant amounts of galactose and arabinose (15% resp. 25%), likely deriving from arabino-galactans (Fig. 4). The monosaccharide composition of conifer hemicelluloses was more equally distributed between xylose, mannose, arabinose and galactose, indicating the presence of substantial pools such as arabinogalactans and galacto-glucomannans. Similar to wood of broad-leaved trees, leaf and root hemicelluloses of grasses showed high proportions of xylose (around 60%), almost no mannose, but considerable amounts of arabinose and galactose. The monosaccharide spectrum of hemicelluloses in herbaceous tissues was divers with on average 40% xylose, 30% arabinose and galactose, and 6% glucose in leaves. Hemicelluloses of herbaceous roots exhibited lower proportions of galactose (14%) but higher proportion of glucose (15%, Fig. 4). Fucose, rhamnose and glucuronic acid were present in small but constant proportions of 1-2% of the total hemicelluloses in all species and tissues.

2.3. Methodical aspect

The gravimetric determination of total hemicellulose concentration with the micro-extraction method proofed to be highly reproducible. Repeated gravimetric determinations of total hemicellulose concentrations in the same plant material revealed only very small differences among the single measurements. Thus, five independent analyses of the same material resulted in standard errors of less than 4% of the mean for gravimetric and HPLC analyses.

Initial HPLC-measurements of hemicellulose deriving monosaccharides in starch-rich material exhibited very high glucose proportions within the hemicellulose fraction, if samples were only extracted with the neutral detergent before the hemicellulose extraction. The use of heat-stable α-amylase (as was originally suggested by Van Soest et al. [17]) as an initial step, effectively removed starch from the pellet,

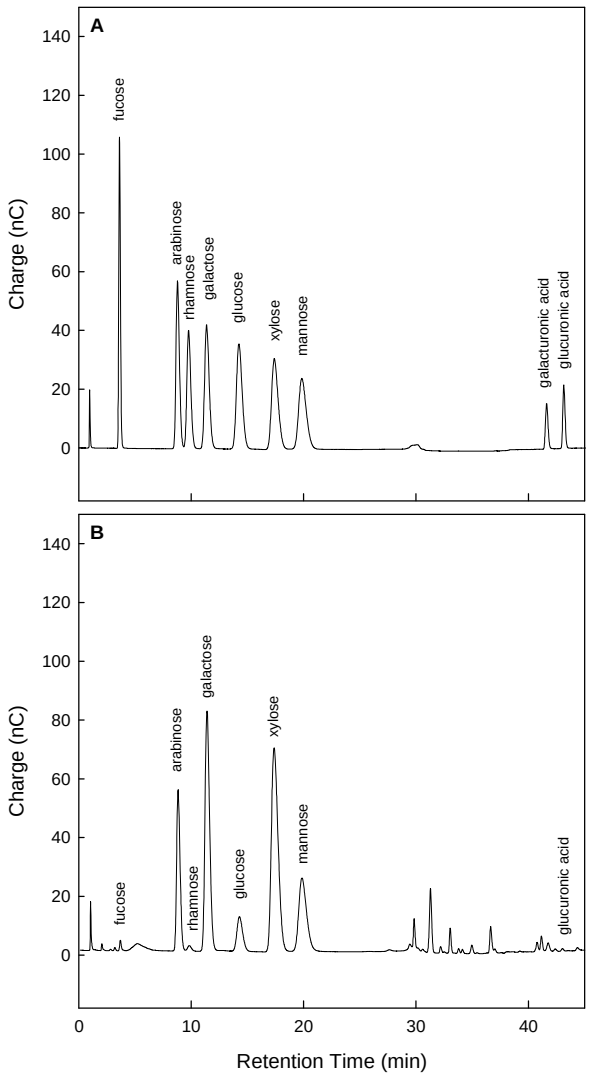

Figure 3: HPLC-chromatograms of the hemicellulose fraction of A) standard solution in water, B) *Picea* sapwood extracted with 1N H_2SO_4, dilution 1:100. See material and methods for details.

which resulted in significantly lower proportions of glucose within the hemicellulose-derived monosaccharides. Importantly, the use of α-amylase did not affect the concentration of any other hemicellulose monosaccharide (data not shown).

3. Discussion

The current study used a micro-extraction method for hemicelluloses, based on the well established method of Van Soest, which allowed for the simultaneous analysis of many samples from different plant tissues and species (28 species from 4 plant functional types). Hence, we were able to directly compare hemicellulose amount and composition among species, without any methodical bias that might occur when existing data from studies that use different methods for the determination of hemicelluloses are compared. The micro-extraction method therefore can be used to determine hemicellulose content and composition of any species in a given ecosystem with a passable amount of work, allowing for more precise estimates of the hemicellulose pool of a specific plant community or ecosystem.

3.1. Hemicelluloses in different plant functional groups

On average, the total hemicellulose concentrations in sapwood of broad-leaved trees in this study accounted for about 25% of the dry biomass, which matches well with previous reports [18-20]. The relatively low hemicellulose concentrations of 11% in leaves of broad-leaved trees agree with reported 8-15% d.m. in leaves of three Mediterranean shrub species as described by Peñuelas [21]. High variability of leaf hemicellulose concentrations was found within a survey across 45 tropical tree species where hemicellulose concentrations in green leaves ranged from 4-45% d.m., with a cross-species mean of 16% d.m. [22]. Cell wall polysaccharides in bark of woody species have rarely been studied, but become increasingly important as renewable sources of organic carbon [23]. In this study bark hemicelluloses of broad-leaved trees accounted for 12% d.m. Lower bark hemicellulose concentrations of 5.6% for *Quercus robur* and higher concentrations (17% d.m.) for *Fagus sylvatica* were reported by Dietrichs et al. [24].

Hemicellulose concentration of conifer branch sapwood in this study was around 20% d.m., which is slightly lower than most of the existing data. For example, Willför [25] measured a total non-cellulose cell wall carbohydrate concentration of 25% d.m. in heartwood and sapwood of *Picea abies* stems (vs. 18.5% d.m. branch sapwood in the current study). Although this difference might at least be partially caused by methodical differences, it may also indicate different hemicellulose concentrations in stem and branchwood. Like for sapwood, the measured hemicellulose concentrations in conifer needles were also lower in this study than described previously; on average 60% more hemicelluloses were found in needles of *Abies balsamea* and different *Picea* species [26, 27]. Bark hemicelluloses accounted for 7-15% d.m., which is also slightly less than the reported concentrations of *Pinus pinaster* bark (18% d.m.) analysed by Fradinho et al. [28].

Leaves of grasses showed a high variation in hemicellulose concentrations among species which might be due to the selected species as a literature comparison revealed strongly differing hemicellulose concentrations among different *Poaceae* species [e.g. 12, 18, 29]. Almost all published data on hemicelluloses in grasses derived from crop species, which are adapted to strong light conditions (with a higher leaf mass per area and a higher proportion of cell wall per volume), while the grasses investigated for this assessment were mainly wild species sampled at more shaded sites (with relatively lower leaf mass area [30]). To our knowledge, this study is the first to present hemicellulose concentrations in grass roots. Generally, grass roots exhibited significantly higher hemicellulose concentrations than grass leaves.

Like in grasses, tissues of herbaceous species also

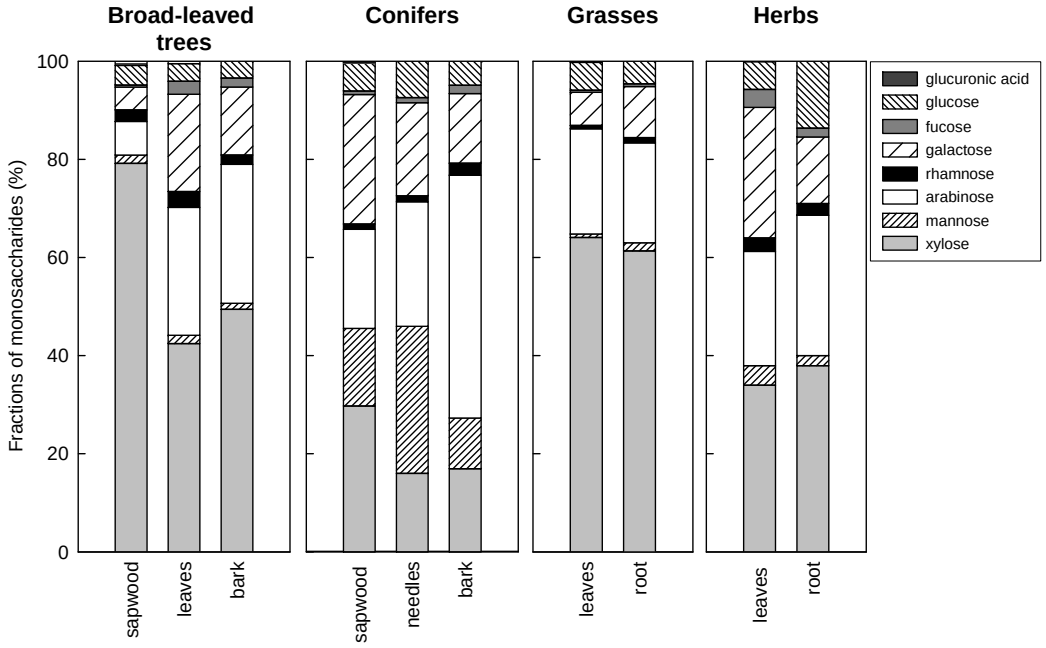

Figure 4: Fractions of hemicellulose-derived monosaccharides in different tissues of four functional plant types as measured by HPLC within the acid detergent (1 N H_2SO_4) soluble hemicelluloses. Values are means of at least three species (except grass roots, where n = 1).

revealed high variability of hemicellulose concentrations among herbaceous species as was described in a former study by Martens and Loeffelmann [31] who reported hemicellulose concentrations of 22% in alfalfa, 23% in flax and even 33% d.m. in leaves of soybeans.

Cellulose and lignin were not separated in this study, but in woody tissues cellulose generally accounts for 40-50% d.m. and lignin for 20-35% d.m. [32-34]. Accordingly, the 'cellulose and lignin' fraction measured in the current study accounted for 60-70% d.m. in branch sapwood of trees, resulting in a cellulose and lignin to hemicellulose ratio of 2-3. Similarly, the cellulose and lignin to hemicellulose ratio of 1.5-2.8 in foliar tissues of trees matches with previous studies that reported leave concentrations of 26– 60% d.m. for the sum of cellulose plus lignin in leaves [e.g. 21, 22, 35]. The low concentrations of 'cellulose and lignin' in leaves of grasses and herbs, and the resulting low cellulose and lignin to hemicellulose ratio reflects the low lignification rate of these tissues.

Despite of species-specific variations within the same tissues, the current study revealed characteristic differences in the total hemicellulose concentrations among plant functional types. Hemicellulose concentrations in sapwood of broad-leaved trees are twice as high as in leaves and roots of the same plant functional type. Hemicellulose concentrations of woody and grass species have received a lot of attention in different studies where hemicelluloses, together with cellulose, are discussed as the most abundant renewable resource for biofuels [e.g. 6, 36]. Although the hemicellulose concentration in leaf and root tissues is markedly less than one quarter of the total biomass, it's the woody stems of trees that account for close to 90% of the global biomass [37]. In wood, cell walls resemble more than 90% of the total biomass, and hemicelluloses account for about 35% of the cell wall fraction. Thus, on a global scale, about 25% of the biomass are hemicelluloses [38].

3.2. Monosaccharide spectrum of hemicelluloses

The HPLC analyses revealed xylans as the main hemicellulose type in hardwoods [e.g. 4, 8, 20], while larger amounts of mannose and galactose in tissues of softwoods indicated the presence of galacto-mannans in this functional group [e.g. 25, 39]. High concentrations of xylose and arabinose in grass species investigated in this study agree with previous findings reviewed in Ebringerova et al. [4] where arabinoxylans were found to occur in considerable concentrations in cereals such as wheat, rye, barley and oat. Although Pauly and Keegstra [36] described great variability of the degree of arabinosylation of hemicelluloses among grass species, we found very similar arabinose proportions within the total hemicellulose pools of the three grass species investigated via HPLC.

3.3. Methodical comparison

The most important modification of the micro-extraction method to the original Van Soest fiber analysis was a 20-fold reduction for the required sample size from 1 g as described in Van Soest and Wine [16] to 50 mg for the micro-extraction. Importantly, the sample size of 50 mg was still enough material for the accurate gravimetric determination of hemicelluloses, when a 0.01 mg scale was used for weighing (considering an original sample mass of 50 mg, a hemicellulose concentration of 20% d.m. gives a 10 mg mass-difference between the non-structural cell content and the cellulose and lignin fraction). The

Table 1: Sampled plant species ordered by functional plant type with indication of the analyzed tissue.

Functional plant type	Species	analyzed tissues	gravimetric/HPLC[a]
Broad-leaved trees			
	Acer campestre L. (1)	sapwood[b], leaves, bark[c]	+/-
	Carpinus betulus L. (7)	sapwood[b], leaves, bark[c]	+/-
	Fagus sylvatica L. (4)	sapwood[b], leaves, bark[c]	+/+
	Fraxinus excelsior L. (8)	sapwood[b], leaves, bark[c]	+/+
	Prunus avium L. (6)	sapwood[b], leaves, bark[c]	+/-
	Quercus petraea Matt. (Liebl.) (5)	sapwood[b], leaves, bark[c]	+/-
	Sambucus nigra L. (3)	sapwood[b], leaves, bark[c]	+/+
	Tilia platyphyllos Scop. (2)	sapwood[b], leaves, bark[c]	+/-
Conifers			
	Abies alba Miller (10)	sapwood[b], needles, bark[c]	+/-
	Larix deciduas Miller (9)	sapwood[b], needles, bark[c]	+/-
	Picea abies L. (11)	sapwood[b], needles, bark[c]	+/+
	Pinus nigra ARNOLD. (12)	sapwood[b], needles, bark[c]	+/+
	Pinus sylvestris L. (13)	sapwood[b], needles, bark[c]	+/+
Grasses			
	Alopecurus pratensis L. (19)	leaves, roots[d]	+/-
	Arrhenaterum elatius L. (15)	leaves	+/-
	Avena sativa L. (14)	leaves	+/-
	Bromus erectus HUDS. (18)	leaves, roots[d]	+/+
	Brachypodium sylvaticum HUDS. (20)	leaves	+/+
	Dactylis glomerata L. (17)	leaves	+/+
	Digitaria ischaemum SCHREB. (16)	leaves, roots[d]	+/-
Herbs			
	Geum urbanum L. (24)	leaves, roots[d]	+/+
	Leontodon hispidus L. (21)	leaves, roots[d]	+/+
	Medicago lupulina L. (26)	leaves, roots[d]	+/+
	Ranunculus acris L. (22)	leaves, roots[d]	+/+
	Salvia pratensis L. (23)	leaves, roots[d]	+/+
	Silene flos-cuculiL. (27)	leaves, roots[d]	+/-
	Trifolium pratensis L. (25)	leaves	+/+
	Urtica dioica L. (28)	leaves, roots[d]	+/+

Numbers beside species names correspond to the numbers given in Fig. 1 and Fig. 2.
[a] +/+ hemicelluloses measured gravimetrically and with HPLC, +/- hemicelluloses measured only gravimetrically
[b] sapwood of 3 to 5 year-old branches
[c] bark of 3 to 5 year-old branches
[d] roots are coarse and fine roots

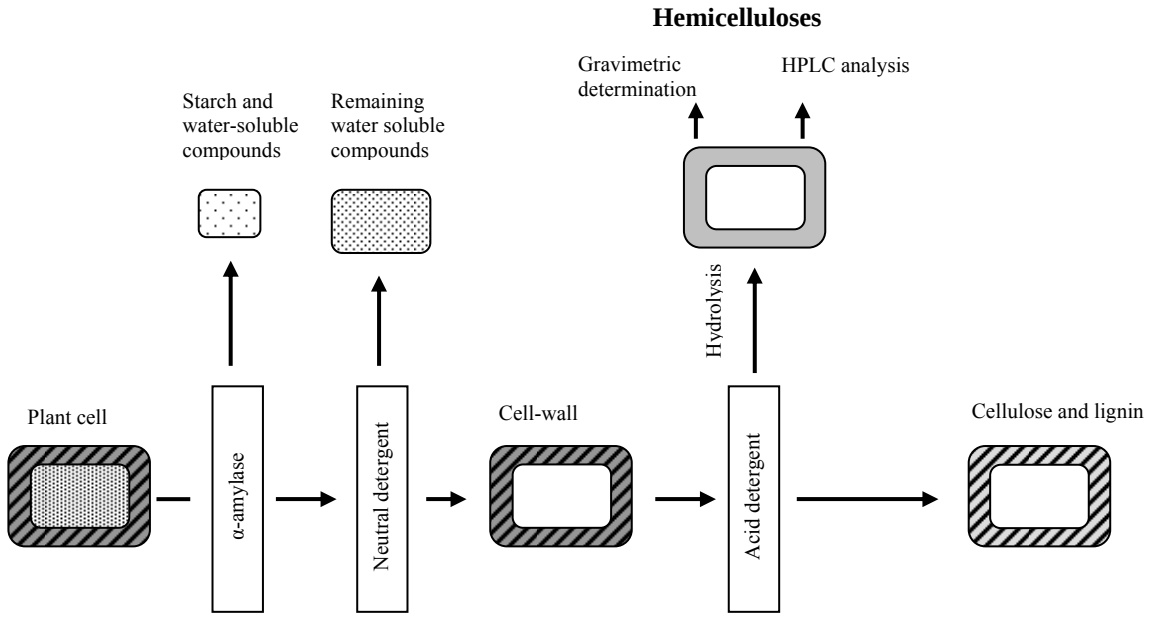

Figure 5: Schematic depiction of the micro-extraction method for hemicelluloses, modified after the Van Soest fiber analysis.

gravimetric determination is an easy and fast method for the quantitative measurement of bulk-hemicelluloses in different tissues and species, but so far, it was applied primarily for fibre-analyses of forage [40, 41]. Hemicelluloses are hydrolysed during the extraction step within the Van Soest method, which permits to directly identify monosaccharides by HPLC. Surprisingly, this convenient possibility to jointly analyse hemicelluloses quantitatively and qualitatively has not been used within the past.

Initially, we compared the new micro-extraction method with the established extraction method for hemicelluloses by Lawther et al. [12]. Both methods were applied to sapwood of *Acer campestre*, *Carpinus betulus* and *Fagus sylvatica* and hemicelluloses were analysed quantitatively (gravimetrically) and qualitatively (by HPLC). While there was a generally good match between the two methods, the total hemicellulose concentration measured with the method by Lawther [12] were constantly about 20% lower than the hemicellulose concentrations measured with the micro-extraction method. Because NMR screenings revealed no 'contamination' of the hemicellulose extract from the micro-extraction method by non-carbohydrate compounds, and its relatively low concentrations of glucose suggest no significant co-extraction of cellulose, an under-estimation of the hemicellulose content from the method by Lawther is more likely than an over-estimation by the micro-extraction method. The difference in absolute hemicellulose yield most likely reflects the higher number of extraction and washing steps for the method by Lawther, during which plant material might be lost.

Because every method using a hydrolytic step for the determination of hemicelluloses is a trade-off between maximum yield of hemicelluloses and minimum 'contamination' of the hemicellulose fraction with other cell wall compounds (e.g. cellulose), slightly different hemicellulose yields for the same plant material are inescapable effects, if different methods are used. This again emphasizes the value of the new micro-extraction method, which, for the first time, enables real broad-scale analyses of hemicelluloses in many different species, as they are required for studies of ecosystems that reach beyond the analysis of single model organisms.

4. Material and methods

4.1. Quantitative and qualitative screening of hemicelluloses in different tissues and species

28 plant species belonging to four different plant functional types (8 broad-leaved trees, 5 conifers, 7 grasses and 8 herbs) were sampled during summer 2005 in the surrounding area of the city of Basel, Switzerland (47°33' N, 7°35' E). The sampled species and the analysed tissues per species are given in Table 1. The different plant organs were separated in the field and immediately heated in a microwave oven at 600 W for 90 s after return to the laboratory in order to stop any enzymatic activity [42]. The samples were then oven dried at 75°C and ground to fine powder. All samples were analysed gravimetrically for their bulk hemicellulose concentrations. In addition, the hemicellulose-containing extracts of diverse tissues of 16 species were also analyzed for their specific hemicellulose monosaccharide composition by HPLC (Table 1).

4.2. Sequential hemicellulose extraction:

A schematic diagram of the sequential steps of the hemicellulose extraction after Van Soest [15, 16] is given in Fig. 5. The used method comprised three extraction steps: 1. an enzymatic hydrolysis of starch, 2. a neutral detergent extraction of non-structural compounds and water-soluble cell-wall constituents and 3. an acid detergent extraction of bulk-hemicelluloses.

50 mg of plant powder was extracted with heat stable α-amylase (EC: 3.2.1.1; 15 U mg^{-1} solid) from *Bazillus licheniformis* (Sigma, Buchs, Switzerland) to remove starch. After incubation in a water bath at 85°C for 30 min the samples were centrifuged (13.000 g, 10 min), the supernatants discarded and the pellet washed with deionised water. The starch-free residue was dissolved in a neutral detergent (1.5 mL per 50 mg dry weight) to extract remaining water-soluble compounds and water soluble cell-wall pectins. 1 L of the neutral detergent solution contained: 18 mmol sodium tetraborate decahydrate, 66 mmol EDTA (ethylendiaminetetraacetic acid) reagent grade, 10.4 mmol sodium dodecyl sulphate (SDS), 10 mL triethylene glycol, 32 mmol dibasic sodium phosphate and 990 mmL deionised water. Just prior to the neutral detergent extraction, sodium sulfite (0.5 mg g^{-1} solid) was added to each sample to facilitate the dissolving of proteins. The samples were extracted in a water bath with boiling water for 60 min while shaking. After centrifugation the pellet ('cell-wall fraction' containing hemicelluloses, cellulose and lignin) was washed (2x hot deionised water, 1x acetone and 1x deionised water) and dried over night in a vacuum concentrator at 60°C (Eppendorf 5301, Basel, Switzerland) and the dry mass was weighed on a 0.01 mg scale (Mettler Toledo, AT261 delta range).

Following the neutral detergent extraction, an acid detergent solution (containing 1 N H_2SO_4 and hexadecyl trimethylammonium bromide, 55 mmol L^{-1} deionised water) was added to the dried pellet and the samples were placed in a water-bath at 100°C for 1 hour to hydrolyse hemicelluloses and separate them from the remaining cell-wall components (cellulose and lignin). Preliminary ^{1}H NMR analyses of the supernatant containing the hemicelluloses revealed exclusively carbohydrates to be present in the hemicellulose fraction indicating no 'contamination' of the hemicellulose fraction with other compounds (e.g. cell-wall proteins, lignin). Following centrifugation the supernatant was removed and post-hydrolysed with 4 N H_2SO_4 to ensured highest possible polysaccharide hydrolysis. The pellet (cellulose and lignin fraction) was dried over night at 60° in a vacuum concentrator

and weighed. Total hemicellulose concentrations were calculated as the gravimetric difference between 'total cell-wall fraction' and 'cellulose and lignin fraction'.

4.3. Monosaccharide analysis by HPLC

In addition to the gravimetric determination of bulk hemicelluloses, hemicellulose derived monosaccharides were analysed by HPLC on a Dionex ICS-3000 ion-chromatography system equipped with a pulsed amperometric detector (Dionex, Austria) using a CarboPac^{TM} PA20-column (3 × 150 mm), modified after Obro et al. [43]. Monosaccharides were eluted with water (0-20 min), following a gradient to 0.8 N NaOH (20-45 min) and holding this concentration for 15 min at a flow rate of 0.5 mL min $^{-1}$ to elute D-Galacturonic acid and D-Glucuronic acid. The column and detector were thermostated at 20°C to enable good separation of the neighbouring peaks arabinose/ rhamnose and xylose/mannose. Post-column addition of 0.1 N NaOH assured a high base concentration for optimal detection.

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CHAPTER 3

HEMICELLULOSE CONCENTRATION AND COMPOSITION IN PLANT CELL WALLS UNDER EXTREME CARBON SOURCE-SINK IMBALANCES

Hemicellulose concentration and composition in plant cell walls under extreme carbon source-sink imbalances

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Abstract Hemicelluloses account for one quarter of the global dry plant biomass and therefore are the second most abundant biomass fraction after cellulose. Despite their quantitative significance, the responsiveness of hemicelluloses to atmospheric carbon oversupply is still largely unknown, although hemicelluloses could serve as carbon sinks with increasing CO_2 concentrations. This study aimed at clarifying the role hemicelluloses play as carbon sinks, analogous to non-structural carbohydrates, by experimentally manipulating the plants' carbon supply. Sixteen plant species from four different plant functional types (grasses, herbs, seedlings of broad-leaved trees and conifers) were grown for two months in greenhouses at either extremely low (140 ppm), medium (280 ppm) or high (560 ppm) atmospheric CO_2 concentrations, thus inducing situations of massive C-limitation or -oversupply. Above and belowground biomass as well as non-structural carbohydrates significantly increased in all species and tissues with increasing CO_2 concentrations. Increasing CO_2 concentrations had no significant effect on total hemicellulose concentrations in leaves and woody tissues in all species, except for two out of four grass species, where hemicellulose concentrations increased with atmospheric CO_2 supply. Despite the overall stable total hemicellulose concentrations, the monosaccharide spectrums of hemicelluloses showed a significant increase in glucose monomers in leaves of woody species as C-supply increased. In summary, total hemicellulose concentrations in *de-novo* built biomass seem to be largely unaffected by changed atmospheric CO_2 concentrations, while significant increases of hemicellulose-derived glucose with increasing CO_2 concentrations in leaves of broad-leaved and conifer tree seedlings showed differential responses among the different hemicellulose classes in response to varying CO_2 concentrations.

Keywords: CO_2, glucose, non-structural carbohydrates, plant functional types, polysaccharides, starch, monosaccharides

Introduction

Cell walls are by far the largest carbon pool of the plant's biomass and therefore play a key role in the carbon-cycle and carbon-budget of ecosystems. They consist of a complex mixture of polysaccharides and other polymers with high chemical and structural variability of the single cell wall compounds among tissues, species and plant functional groups (Rose 2003; Schädel et al. 2009a). Major compounds of cell walls from every plant tissue are hemicelluloses, which are mixed polysaccharides and account for up to one third of the total dry plant biomass. Although hemicellulose concentrations are generally lower in leaves than in wood, hemicelluloses account for one quarter of the total global biomass (considering that tree stems account for almost 90% of the entire biomass (Körner 2003a)) and are therefore the second most important biomass fraction. Structurally, hemicelluloses either interconnect the cellulose microfibrils through hydrogen bonds or regulate as 'scaffolds' the space among the cellulose fibrils (Cosgrove 2000; Thompson 2005). Chemically, hemicelluloses can be grouped into four classes: xylans, xyloglucans, mannans and mixed-linkage β-glucans (Ebringerova et al. 2005). Xylans are composed of a backbone of β-(1→4)-D-xylose units with side chains containing different sugars and sugar acid residues. These side chains comprise arabinose, glucose, galactose and in lower amounts rhamnose, glucuronic acid and galacturonic acid (Puls 1993; Saha 2003a). Xylans differ in their degree of branching and the chemical nature of the side chains. Xyloglucan is the main hemicellulose-type in primary cell walls of all higher plants, with a backbone similar to that of cellulose, consisting of β-(1→4)-linked D-glucopyranose units, but with frequent branching of α-D-xylose residues. Glucomannans and galactomannans are the principle hemicelluloses in tissues of conifers and consist of a branched backbone of β-(1→4)-linked D-mannose and D-glucose units (Timell and Syracuse 1967). A fourth type of hemicelluloses are mixed (1→3, 1→4) linked β-glucans which are restricted to grass species (Buckeridge et al. 2004) and some pteridophytes (*Equisetum*, (Sorensen et al. 2008)).

The heterogeneity of hemicelluloses within the cell wall matrix is also expressed in different binding strengths to cellulose fibrils among and within the different hemicellulose classes (Morrison 1980), which may correspond to different functions of hemicelluloses within the cell wall (Sun et al. 2004), with some hemicelluloses being more flexible than others (Bootten et al. 2004). Quantity and structure of hemicelluloses are well studied since hemicelluloses, as part of the lignocellulosic material, belong to the most abundant organic resources on earth that can be converted to industrial products or biofuels (e.g. Saha 2003b; Gray et al. 2006; Somerville 2007; Pauly and Keegstra 2008). In contrast, there is still considerably little knowledge about the physiological and ecological properties of hemicelluloses in living plants (Hoch 2007), especially regarding their responsiveness towards C-source-sink imbalances.

It is well established that the size of a plant's carbon reserve pool mirrors its carbon supply status, i.e. it depends on the balance of its source- (i.e. photosynthesis) and sink- activities (Chapin et al. 1990; Körner 2003a). With respect to atmospheric CO_2 concentrations, previous studies have already demonstrated that concentrations of non structural carbohydrates (starch and low molecular weight sugars) of plants increase at high CO_2 concentrations (e.g. Poorter et al. 1997; Würth et al. 1998; Körner 2003a) and decrease at low CO_2 concentrations (e.g. Agüera et al. 2006). In previous studies, the reaction of hemicelluloses to changing C-source-sink relations has been described to resemble that of non-structural carbohydrates and thus indicating cell wall hemicelluloses to likely function as an additional C-storage pool for surplus photoassimilates. For example, hemicellulose concentrations increased with elevated CO_2 concentrations (Couteaux et al. 1996) and decreased in plants when fertilization enhanced growth, i.e. increased C-source-sink activity (Anttonen et al. 2002; Kilpeläinen et al. 2003; Kaakinen et al. 2004a). Several studies found decreasing hemicellulose concentrations in mature tree tissues (sapwood and mature needles) during the period of high C demand at spring bud break (Robakidze and Patov 2000; Robakidze and Bobkova 2003; Schädel et al. 2009b).

Despite this evidences, we are currently lacking an extensive test for the responsiveness of hemicelluloses in plant tissues to carbon limitation or oversupply. All existing studies that indicated changing tissue concentration of hemicelluloses due to different carbon availabilities were restricted to one or a few species within one plant functional type. In view of this lack of knowledge a broad-scale experiment was conducted to determine the responsiveness of this significant biomass fraction to different CO_2 concentrations. Three concentrations of CO_2 (140, 280 and 560 ppm) were used to induce situations of massive carbon under- or oversupply. The reactions of hemicelluloses to the varying CO_2 concentrations were investigated quantitatively and qualitatively. To assess the degree of responsiveness of hemicelluloses, these data were compared with the concentration of non-structural carbohydrates (starch and soluble sugars).

The response of hemicelluloses, as one of the most abundant compounds in biomass, towards increased atmospheric CO_2 concentrations is of special importance for the ongoing debate if plants can serve as C-sinks that mitigate the continuous increase of CO_2 in the earth's atmosphere. Increasing hemicellulose concentrations at increased atmospheric CO_2 concentrations would consequently lead to higher C/N ratios of the plants' biomass, which would entail impacts on many ecosystem processes like herbivory and litter decomposition (e.g. Ceulemans and Mousseau 1994; Cotrufo et al. 1998; Körner 2003b). For the current study, the hypothesis was that CO_2 enriched plants show highest growth rates and highest concentrations of non structural polysaccharides and hemicelluloses, whereas exceptionally low CO_2 concentrations will induce carbon limiting situations for plants, which, besides reduced growth, will lead to very low concentrations or even a depletion of non structural carbohydrates and hemicelluloses.

Material and methods

Experimental setup:

Carbon source-sink relations were changed experimentally by treating six closed air conditioned daylight growth chambers with different CO_2 concentrations at the Institute of Botany, University of Basel, Switzerland. Each chamber had a volume of 0.86 m^3 (1.3 x 1.1 x 0.6m) and a metal grid separated the plants from the air conditioning system. Each chamber was connected to a CO_2 concentration controlling system checking CO_2 values every 7 seconds and maintaining the targeted CO_2 concentrations by either supplying concentrated CO_2 or by removing surplus CO_2 by pumping chamber-air through a box containing soda lime. The targeted CO_2 concentrations were 140 ppm (half pre-industrial), 280 ppm (pre-industrial) and 560 ppm (double pre-industrial) CO_2. Each CO_2 treatment was replicated twice in separate chambers. All chambers were additionally equipped with a vacuum cleaner attached to a box containing soda lime to rapidly decrease the chamber CO_2 concentrations below ambient concentrations after opening a chamber.

Chamber temperatures were set to 22°/14°C day/night, which were computer controlled every 6 seconds. If natural day-light was lower than 150 µmol photons s^{-1} m^{-2}, additional lamps on top of each chamber maintained favourable light conditions. The soil used was sieved forest soil (collected at a calcareous mixed forest near the city of Basel) mixed with quartz sand and universal potting soil (12 : 8 : 1, v:v:v). This mixture assured sufficient nutrient concentration for optimal plant growth, as was evidenced by no further biomass increase in additionally fertilized control plants (data not shown).

Table 1: List of study species according to their plant functional type

PFT[1]	Species	Family
Grasses		
	Alopecurus pratensis L.[3]	Poaceae
	Arrhenaterum elatius L.[3]	Poaceae
	Bromus erectus Huds.[2]	Poaceae
	Dactylis glomerata L.[2]	Poaceae
Herbs		
	Geum urbanum L.[3]	Rosaceae
	Leontodon hispidus L.[2]	Asteraceae
	Saliva pratensis L.[2]	Lamiaceae
	Silene flos-cuculi L.[3]	Caryophyllaceae
Broad-leaved trees		
	Acer campestre L.[3]	Aceraceae
	Fagus sylvatica L. [2]	Fagaceae
	Quercus petraea Matt. (Liebl.)[3]	Fagaceae
	Tilia platyphyllos Scop.[2]	Tiliaceae
Conifers		
	Abies alba Miller[2]	Pinaceae
	Larix decidua Miller[2]	Pinaceae
	Picea abies L.[3]	Pinaceae
	Pinus sylvestris L.[3]	Pinaceae

[1] Plant functional type

[2]Species exposed to CO_2 concentrations treatment from March 13th – May 12th

[3]Species exposed to CO_2 concentrations treatment from May 13th – July 7th

Grasses and herbs were planted into 1L pots, while pots for woody species had a volume of 2L.

Plant species and harvest

The plant species used in this study comprised four plant functional types (grasses, herbs, seedlings of broad-leaved trees and conifers) with each group represented by four species (Table 1). Seeds of grasses and herbs were obtained from a commercial agriculture supplier (Landi, Winterthur, Switzerland). For grasses, 20 seeds were sawn directly into each of the 1L pots. Seeds of the four herbaceous species were sawn and germinated in larger trays, from which four individuals were transplanted into each of the 1L pots a few days after germination. All seeds germinated at ambient CO_2 concentrations, but as soon as the first leaves after the cotyledons became visible, the pots were randomly assigned to one of the three CO_2 treatments and put into the respective chambers. For all tree species, two-year-old seedlings were obtained from a local nursery (WSL, Birmensdorf, Switzerland), where they were excavated at the end of winter (late February 2006) and kept in the dark at 4° C to avoid bud break before the start of the experiment. One tree seedling was planted per pot. Due to limited space in the chamber, the experiment had to be conducted in two separate experimental growth cycles; the first cycle took place from March 13 to May 12 2006, the second from May 13 to July 7 2006. For each cycle two species were used per plant functional group, with each species (pots) replicated four times per chamber. Randomisation was performed exclusively during the night to avoid enhanced photosynthetic activity in low CO_2 treated plants. All chambers and CO_2 treatments, as well as the positions of pots within each chamber, were randomised in intervals of 8-10 days.

Plants were harvested after 61 (first cycle) and 56 days (second cycle), respectively. All individuals were separated into the following tissues: leaves (or needles in the case of conifer seedlings), roots, sapwood and bark, which were immediately heated in a microwave oven at 600 W for 90 s to stop any enzymatic activity (Popp et al. 1996). The samples were dried to weight constancy in an oven at 75°C and biomass data was collected by weighing each tissue separately before grinding to fine powder. Chemical analyses were performed with leaves of all species and sapwood of all tree seedlings, as well as with roots of two species within each plant functional type.

Non-structural carbohydrates

NSC (the sum of the three quantitatively most important low molecular weight sugars, i.e. glucose, fructose and sucrose, plus starch) were analysed after Wong (1990) as described in detail in Hoch *et al.* (2002). About 10 mg of plant powder was extracted with 2 mL distilled water at 100°C for 30 min. An aliquot of the extract was used for the determination of low molecular carbohydrates after enzymatic conversion of fructose and sucrose to glucose. The concentration of free glucose was determined photometrically after enzymatic conversion of glucose to gluconate-6-phosphate on a 96-well multiplate reader. After the degradation of starch to glucose by a crude fungal amylase ('Clarase' from *Aspergillus oryzae*, Enzyme Solutions Pty Ltd., Crydon South, Victoria, Australia) at 40°C overnight, NSC were determined in a separate analysis. The concentration of starch was calculated as NSC minus the free low molecular carbohydrates. All NSC concentrations are given on a % dry matter basis.

Gravimetric hemicellulose determination

Bulk hemicelluloses were extracted following the standard method by Van Soest (1963; 1967), modified for the use of small sample volumes (< 100 mg) and starch rich material (Schädel et al. 2009a). In a first step starch was removed with heat stable α-amylase from *Bacillus licheniformis* (Sigma, Buchs, Switzerland). The starch-free pellet was dissolved in a

neutral detergent solution (sodium tetraborate decahydrate, EDTA, SDS, triethylene glycol, sodium phosphate and distilled water) and boiled for 60 min in a water bath to extract proteins, low molecular weight carbohydrates, lipids and pectins. Subsequent centrifugation separated these dissolved compounds from all remaining cell wall components (cellulose, lignin and hemicelluloses, e.g. 'total cell wall fraction'), which were washed with water and acetone, dried over night and weighed. The dry pellet was mixed with an acid detergent containing 1 N H_2SO_4 and hexadecyl trimethylammonium bromide and boiled for 60 min to dissolve and hydrolyze hemicelluloses. After centrifugation the remaining 'cellulose and lignin fraction' was washed and dried and hemicelluloses were determined gravimetrically as 'total cell wall fraction' minus 'cellulose and lignin fraction'. Hemicellulose concentrations were calculated on a % dry matter basis as well as on a % NSC-free dry matter basis, which allows accounting for possible 'dilution' effects of hemicelluloses in NSC-rich material.

Analytic hemicellulose monosaccharide determination

Post-hydrolysis of the hemicellulose containing supernatant with 4 N H_2SO_4 for 60 min while boiling ensured optimal hydrolysis of all polysaccharides. In addition to the gravimetric hemicellulose determination, hemicellulose derived monosaccharides were analyzed by pulsed amperometric HPLC (Dionex ICS-3000 ion-chromatography system) using a CarboPac^{TM} PA20-column (3 x 150 mm). Monosaccharides (L-Fucose, L-Arabinose, L-Rhamnose, L-Galactose, D-Glucose, D-Xylose, D-Mannose) were eluted with water (0-20 min), following a gradient to 0.8 N NaOH (20-45min) and holding this concentration for 15 min at a flow rate of 0.5 mL/min D-Galacturonic acid and D-Glucuronic acid were eluted (Obro et al. 2004). The column and detector were thermostated at 20°C to enable baseline separation of the neighbouring peaks arabinose/rhamnose and xylose/mannose and post-column addition of 0.1 N NaOH assured high base concentration for optimal detection.

Statistics

The experiment followed a split-plot design with chambers as blocks, CO_2 concentration as between subject factor and plant functional group as within subject factor. Chemical analyses were performed with individual samples but for data analyses individuals grown in the same chamber were averaged to avoid pseudoreplication resulting in robust mean values per chamber (3 CO_2 concentrations x 2 chambers). On the level of plant functional types means were calculated over all four species (n=4). Prior to statistical analyses biomass data were log-transformed and NSC and hemicellulose data were arcsine transformed to achieve normal distribution and equal variances. A linear mixed-effects model was used to calculate effects of CO_2 and functional type as well as their interaction. Effects were considered significant at $P<0.05$. Within CO_2 concentrations and plant functional type differences in biomass, NSC, hemicelluloses and hemicellulose monosaccharides were analyzed for significance by Tukey-Kramer honestly significant difference (HSD) test. All errors given are standard errors. Statistical analyses were carried out using R version 2.7.2 (R Development Core Team2004).

Results

CO_2 concentrations and biomass

Overall, the targeted CO_2 concentrations were reached very precisely for all three treatments. Across the whole experiment daytime medians of CO_2 concentrations were 143 ppm, 283 ppm and 561 ppm, respectively. For the low CO_2 treatment, 19% of the hourly mean values were 5% higher and 17% were 5% lower than the median concentration. Only 11% of the hourly mean values of the 280 ppm CO_2 treatment were more than 5% higher as the median and 0.5% were more than 5% lower. For the high CO_2 concentrations 2.8% of the hourly mean values were more than 5% higher as the median and 7.5% of the values were lower.

Above and belowground biomass of all plant functional groups responded with significant higher biomass to increasing CO_2 concentrations (Table 2, Fig. 1). Generally, grasses and herbs approximately doubled their biomass from 140 – 560 ppm with larger increments between 140 and 280 ppm CO_2 than between 280 and 560 ppm CO_2. Broad-leaved tree seedlings and conifer seedlings also exhibited increased biomass with increasing CO_2 concentrations (Fig. 1). While the mean leaf and needle biomass increased by about 90%, the biomass of woody tissues and roots increased by about 40% from 140 to 560 ppm CO_2.

Non-structural carbohydrates (NSC)

Significant increases of non-structural carbohydrate concentrations with increasing CO_2 concentrations were found in leaves of all species with concentrations varying from 5 to 25% dry matter (Fig. 2).

Table 2: ANOVA table of the fixed effects from the mixed-effects model analysis for the increase in total biomass for four different plant functional groups. Biomass includes all tissues of one plant. P-values smaller than 0.05 are in bold.

	Biomass		
	df	F	P
CO_2	2	27.4	< **0.04**
Functional group	3	372.6	< **0.001**
CO_2 x Funct. group	6	0.7	0.63

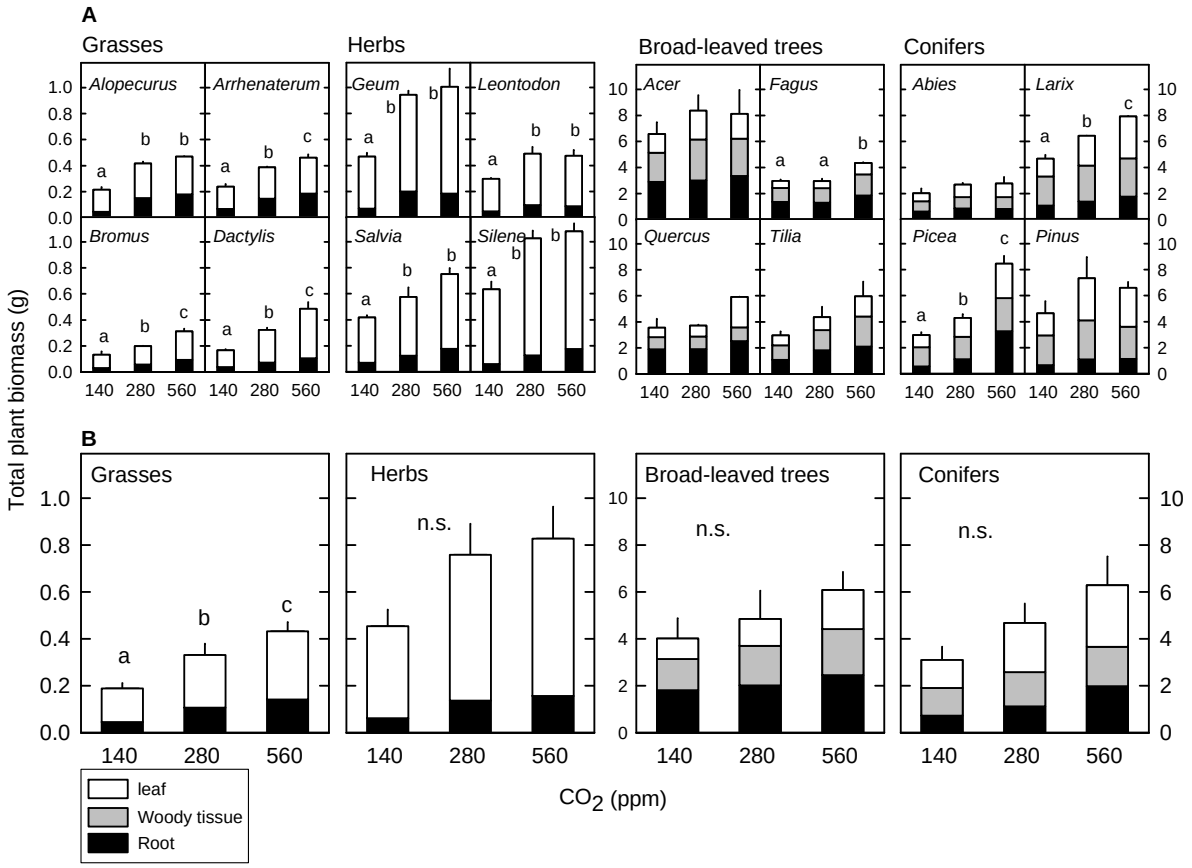

Figure 1: Total plant biomass (mg per individual) in response to CO_2 treatments for A) species and B) plant functional groups. Values are means of chamber means on species level and means of four species on plant functional level, + SE. High variability on species level induces high standard errors on plant functional groups and influences significances. Different letters indicate significant CO_2 effects by Tukey-Kramer HSD-test. Note the different scale for woody species. Note the different scale for woody species

Table 3: ANOVA table of the fixed effects from the mixed-effects model analysis for the increase in concentrations of NSC and hemicelluloses in leaves and sapwood of four different plant functional groups. Broad-leafed trees and conifers produced sapwood. P-values smaller than 0.05 are in bold.

	NSC			Hemicelluloses		
	df	F	P	df	F	P
leaves						
CO_2	2	38.7	**<0.03**	2	0.19	0.84
Funct. group	3	7.9	**<0.001**	3	11.13	**<0.001**
CO_2 x Funct. group	6	1.0	0.40	6	0.25	0.96
sapwood						
CO_2	2	2.6	0.28	2	0.02	0.98
Funct. group	1	514.6	**<0.001**	1	66.6	**<0.001**
CO_2 x Funct. group	2	0.3	0.76	2	0.78	0.46

The highest increase was found in *Silene* where leaves of plants grown at 560 ppm enhanced their NSC 7-fold compared to 140 ppm (Fig. 2). For all other species the increase in leaf NSC concentration from 140 ppm to 560 ppm was lower but still ranged from +40% to +250%. The proportion of soluble sugars within NSC was very high in leaves of grass species (70-80% of total NSC), whereas herbaceous species contained equal amounts of soluble sugars and starch and the proportion stayed constant among all CO_2 treatments. In contrast, soluble sugars in leaves of broad-leaved tree seedlings did not increase as much as starch with increasing CO_2 concentrations (8-fold higher starch values in *Acer* and *Quercus* at 560 ppm compared to 140 ppm CO_2, Fig. 2). Conifer seedlings showed a more diverse pattern in the composition of NSC with high starch concentration in *Abies* and *Picea* and lower concentrations in *Larix* and *Pinus*. A linear mixed-effects model revealed CO_2 concentrations and plant functional group to have a significant influence on the total NSC concentration with no interaction between CO_2 concentrations and plant functional group (Table 3).

Non-structural carbohydrates in sapwood of broad-leaved tree seedlings were not significantly affected by increasing CO_2 concentrations, but tended to increase between 140 ppm and 280 ppm CO_2 (Fig. 3). In contrast, the NSC concentrations in sapwood of conifer seedlings increased significantly from 140 ppm to 560 ppm by about 80% (Fig. 3).

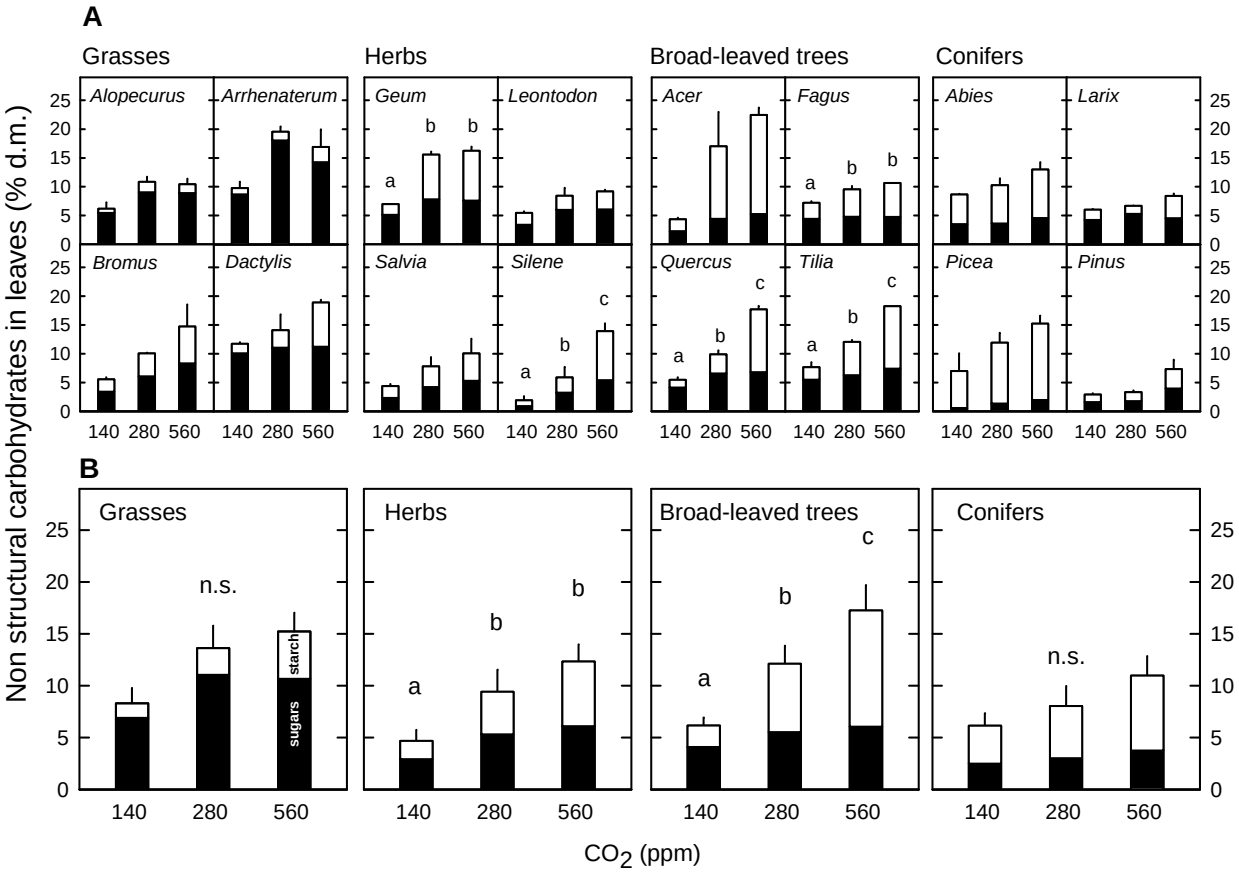

Figure 2: Increase in concentrations of non structural carbohydrates in response to 3 different CO_2 concentrations in leaves of A) species and B) plant functional groups. Black bars represent the soluble sugar fraction and white bars the starch fraction. Values are means of chamber means on species level and means of four species on plant functional level, + SE. Different letters indicate significant CO_2 effects at $P< 0.05$ by Tukey-Kramer HSD test.

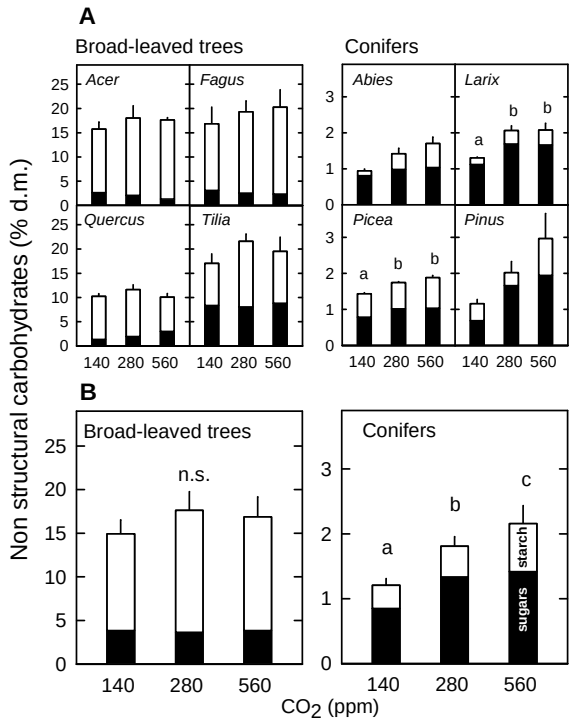

Figure 3: Increase in concentrations of non structural carbohydrates with increasing CO_2 concentrations in sapwood of broad-leaved trees and conifers of A) species and B) plant functional types. Note the different scales for broad-leaved trees and conifers. Black bars represent the soluble sugar fraction and white bars the starch fraction. Values are means of chamber means on species level and means of four species on plant functional type level, + SE. Different letters indicate significant CO_2 effects at $P< 0.05$ by Tukey-Kramer HSD test.

A linear mixed-effects model detected no significant influence of CO_2 concentrations on sapwood but a highly significant difference between the two plant functional groups (Table 3), since NSC concentrations in sapwood of broad-leaved tree seedlings were about ten times higher than in conifer seedlings. Both plant functional groups differed strongly in their composition of NSC; starch was the main component (80%) in broad-leaved tree seedlings, whereas in conifer seedlings the proportion of starch within NSC was only 33%.

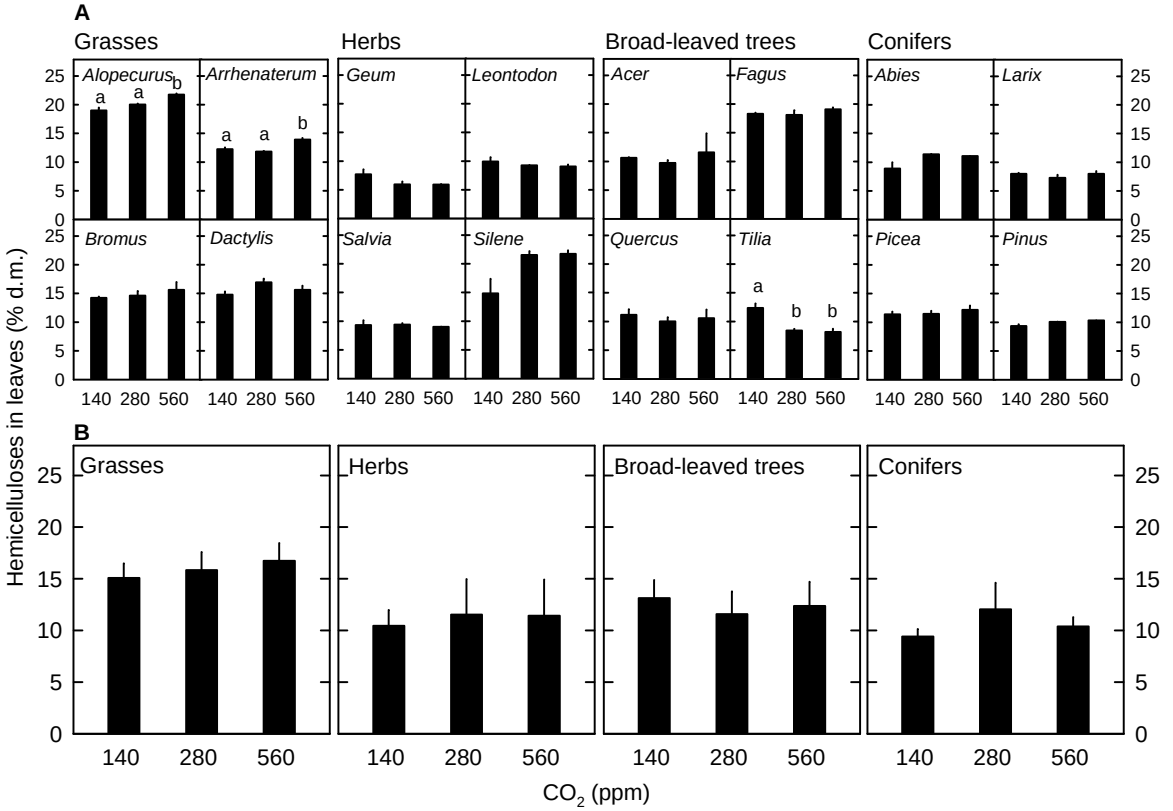

Figure 4: Bulk hemicellulose concentrations as determined gravimetrically in leaves of A) species, B) plant functional types in response to 3 different CO_2 concentrations. Values are means of A) chamber means and B) means of four species. Different letters indicate significant differences ($p < 0.05$) among CO_2 concentrations levels.

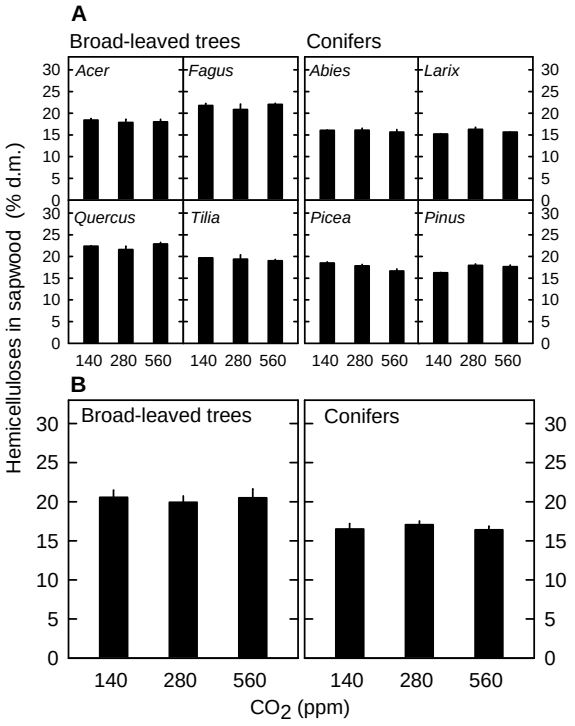

Figure 5: Bulk hemicellulose concentrations as determined gravimetrically in sapwood of broad-leaved trees and conifers of A) species, B) plant functional types in response to 3 different CO_2 concentrations. Values are means of A) chamber means and B) means of four species.

Total hemicelluloses

Total gravimetric hemicellulose concentrations (on a % total dry matter basis) in leaves varied from 6-22% dry matter among the sixteen investigated species (Fig. 4). Higher concentrations were found in all four grasses and in *Silene* and *Fagus*, while the lowest hemicellulose concentrations were measured in leaves of *Geum*, *Leontodon*, *Salvia* and *Larix* with less than 10% d.m. (Fig. 4A). In contrast to NSC, the CO_2 treatments had no significant effect on hemicellulose concentrations in leaves except in two out of four grass species (*Alopecurus* and *Arrhenaterum*) in which increasing CO_2 concentration significantly increased total hemicellulose concentration by about 14% (Fig. 4A). Although *Silene* showed a 46% increase of hemicellulose concentrations from 140 to 560 ppm CO_2, this increase was not significant due to the high variability between individuals. Hemicellulose concentrations in seedlings of broad-leaved trees and conifers were constant over all CO_2 treatments except in *Tilia* where 34% more hemicelluloses were measured at 140 ppm than at the two higher CO_2 concentrations. Averaged over all species of one plant functional type, no CO_2 effect was found for the total hemicellulose concentrations (Fig. 4B, Table 3).

Hemicellulose concentrations in sapwood were similar for seedlings of broad-leaved trees and

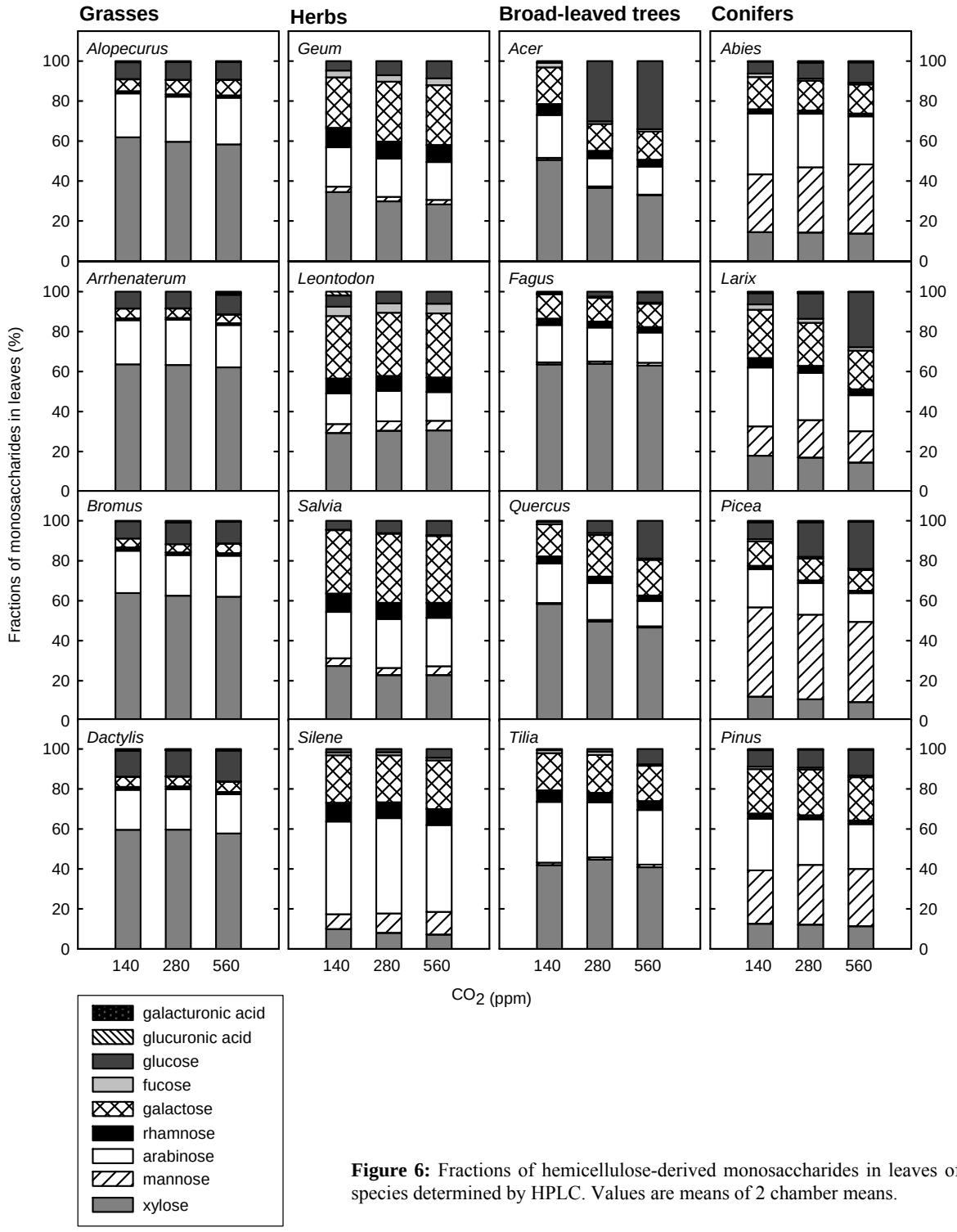

Figure 6: Fractions of hemicellulose-derived monosaccharides in leaves of all species determined by HPLC. Values are means of 2 chamber means.

conifers and ranged from 15-23% with slightly higher values in broad-leaved tree seedlings (Fig. 5). There was no significant effect of CO_2 on sapwood hemicellulose concentrations in any of the eight investigated tree species. Likewise, hemicellulose concentrations in roots did not show any CO_2 effect (data not shown).

Because the strong increase of NSC with CO_2 concentrations in most species might mask ('dilute') more moderate increases of other cell compounds, hemicelluloses were additionally calculated not only relative to the total dry weight but also on a NSC-free basis. The general pattern of hemicellulose concentrations on a NSC-free basis in leaves and sapwood in response to increasing CO_2 concentrations did not differ from that on a % dry matter basis, but became more pronounced, especially with an average 20% (not significant) increase in the hemicellulose concentration in grass leaves and a 17% (not significant) increase in needles of conifer seedlings from 140 to 560 ppm CO_2 (data not shown).

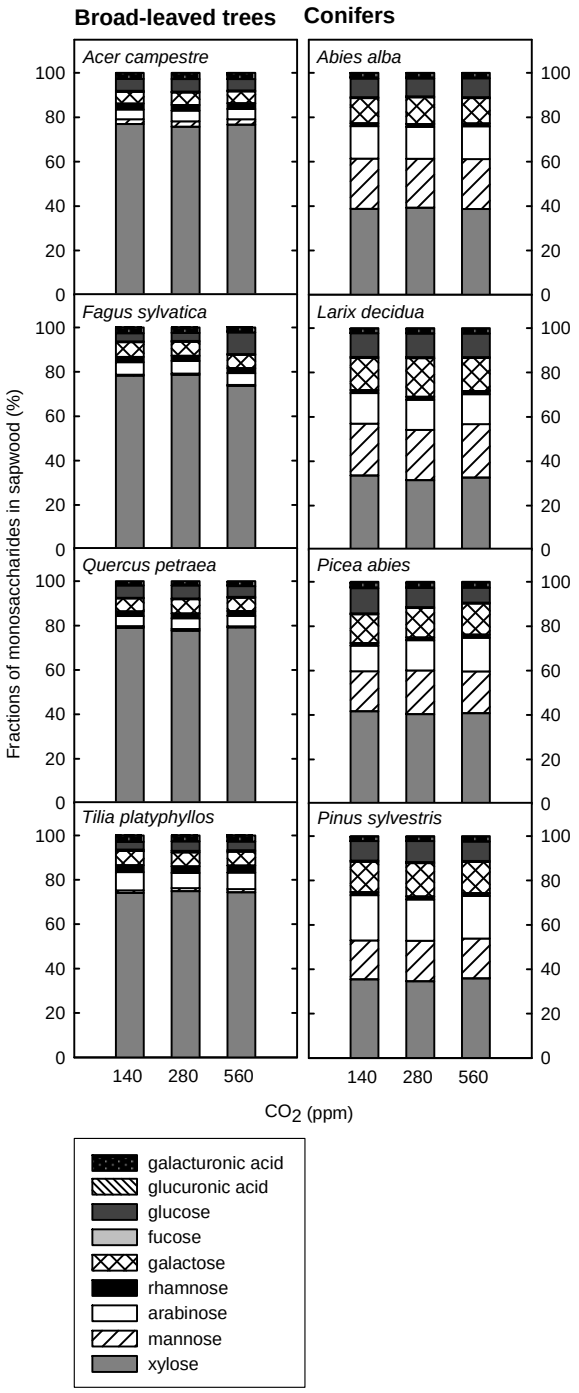

Figure 7: Fractions of hemicellulose-derived monosaccharides in sapwood of all species from broad-leaved trees and conifers determined by HPLC. Values are means of 2 chamber means.

Monosaccharide spectrum of hemicelluloses

The analysis of the monosaccharide spectra of hemicelluloses by HPLC revealed specific patterns for each plant functional group (Fig. 6 and 7). Hemicelluloses of grass leaves contained over 60% xylose, c. 20% arabinose and mainly galactose, glucose and fucose within the remaining 20%. Xylose, arabinose and galactose were the major sugars in leaves of herbaceous species with smaller (8 - 10%) proportions of rhamnose and glucose. Leaves of broad-leaved tree seedlings revealed a high proportion of xylose, likely deriving from xylans, while mannose was the major monosaccharide in needles of conifer seedlings, indicating high proportions of mannans with also arabinose, galactose and glucose as sugar residues (Fig. 6). In leaves of broad-leaved tree seedlings and conifer seedlings significantly ($p < 0.04$ by linear mixed-effects model) higher glucose concentrations were measured with increasing CO_2 concentrations (Fig. 6). Although present in leaves of all tree species, the increase in glucose content was especially pronounced in *Acer*, *Quercus*, *Larix* and *Picea*. However, because of the relatively small proportion of glucose within the total hemicelluloses, this increase in glucose concentration had no significant effect on the total amount of hemicelluloses (Fig. 4) and no other monosaccharide changed significantly with changing CO_2 concentrations.

Unlike leaves hemicelluloses in seedling sapwood of both broad-leaved trees and conifers revealed no change in the monosaccharide spectrum with increasing CO_2 concentrations (Fig. 7). Xylose accounted between 70 – 80% of all monosaccharides in broad-leaved tree seedlings with minor fractions of arabinose, rhamnose, galactose and glucose. The monosaccharide pattern in sapwood of conifers exhibited lower xylose concentration with proportions of 40% and significant proportions of mannose, arabinose and galactose (10-20% each).

Discussion

Biomass and NSC responses to elevated CO_2 concentrations

The successful manipulation of the plants' carbon supply was evident by the significant changes in growth and NSC concentrations in response to increasing atmospheric CO_2 in all plant functional types. All species investigated in this study revealed high sensitivity of growth to different CO_2 concentrations with pronounced biomass increases from 140 ppm to 560 ppm CO_2. A relative biomass increase of around 30% from pre-industrial (280 ppm) to twice pre-industrial CO_2 (560 ppm) as it was found for most species in the current study matches reports for other plant species in previous studies that were grown under optimal (mostly horticultural) growth conditions (e.g. Kimball and Idso 1983; Poorter 1993; Wullschleger et al. 1997; Makino and Mae 1999; Körner 2000).

The generally very strong growth reductions all species at 140 ppm CO_2 evidenced severe carbon limitation in the lowest CO_2 treatment. However, with the exception of *Pinus* (where in few individuals mature needles died off during the first days at low CO_2 concentrations), all species still revealed measurable growth even at the exceptionally low 140

ppm CO_2 treatment. Campbell *et al.* (2005) calculated a whole plant CO_2 compensation point of 65 ppm CO_2 for young tobacco plants, which reached robust sizes and even produced viable seeds at 100 ppm CO_2.

Across all species, NSC concentrations increased markedly with CO_2 concentrations. The accumulation of photoassimilates at high atmospheric CO_2 concentrations was identified as one of the most consistent reactions of plants to elevated CO_2 concentrations in numerous previous studies irrespective of growth conditions (e.g. Körner and Miglietta 1994; Poorter et al. 1997; Kaakinen et al. 2004b; Sholtis et al. 2004; Körner et al. 2005; Kostiainen et al. 2006). Further, non-structural carbohydrates of plants have been described as sensitive to other situation of changed carbon source-sink-balances like defoliation or branch girdling of trees (Jordan and Habib 1996; Iglesias et al. 2002; Hoch 2005). These studies, as well as the current experiment, clearly demonstrated that non-structural carbohydrates represent mobile carbon compounds that mirror carbon shortage or surplus situations and therefore, the qualitative analysis of non-structural carbohydrates is a good approximation for a plant's net carbon balance.

Total hemicelluloses

Unlike NSC, the overall response of total hemicellulose concentrations to changes in the carbon supply was very weak for all tissues and species in the current study. Only two out of four grass species (*Alopecurus* and *Arrhenaterum*) responded significantly to elevated CO_2 concentrations with 14% higher hemicellulose concentration in leaves at 560 ppm compared to 140 ppm CO_2. All other species revealed constant hemicellulose concentrations with increasing CO_2 concentrations, except *Tilia* which was the only species that unexpectedly showed significantly higher hemicellulose concentrations in leaves developed at 140 ppm compared to higher CO_2 concentrations; a phenomenon which needs further explanation. The overall picture of stable hemicellulose concentrations prevailed, even if the 'diluting' effect of the marked changes in NSC was considered and hemicellulose concentrations were expressed on a % NSC-free d.m. basis.

Hemicelluloses have rarely been studied in relation to increasing CO_2 concentrations. Most of those studies were restricted to woody species and the reported effects were rather inconsistent. In addition, the focus of these studies had not been on the reaction of hemicelluloses, and the quality and precision of the different methods used to determine hemicelluloses varied greatly among the studies. Peñuelas *et al.*(2002) found increasing hemicellulose concentrations with increasing CO_2 concentrations in leaves of the Mediterranean shrub *Myrtis communis*, whereas two other shrub species (*Erica arborea* and *Juniperus communis*) growing at the same site showed no change in hemicellulose concentration to elevated CO_2. The authors explained this inconsistency with different carbon investment strategies among different plant species. Within another study, leaf litter of *Castanea sativa* saplings grown at 700 ppm CO_2 revealed 23% higher hemicellulose concentrations than at ambient conditions (Couteaux et al. 1996). Similarly, Hall *et al.* (2006) found increased hemicellulose concentrations with elevated CO_2 concentrations in leaf litter of *Quercus myrtifolia*. Leaves of *Betula pendula* revealed significantly higher hemicellulose concentrations under elevated CO_2 but this effect disappeared again when leaves were exposed to ozone (Oksanen et al. 2005). No change in hemicellulose concentration at increased CO_2 concentrations was found in wood of 15-year old *Pinus sylvestris* trees, while elevated temperatures led to slightly decreased hemicellulose concentrations in the same study (Kilpeläinen et al. 2003). Poorter *et al.* (1997) found no changes in structural carbohydrate concentrations under elevated CO_2 concentrations in leaves of crop and wild herbaceous species as well as in leaves of woody species. However, Poorter *et al.* (1997) did not differentiate between hemicelluloses and cellulose within the polysaccharide fraction of the cell wall.

Because of the high abundance of hemicelluloses in all plant tissues in most tissues, already small concentration changes of hemicelluloses might have substantial influence on a plant's carbon relations. Thus in addition to the relative variation of hemicellulose concentrations, it is also important to consider the absolute amount of C that is sequestered or released by changing tissue concentrations of hemicelluloses. In the current study, hemicelluloses in leaves of grasses accounted on average for 15% of the total dry biomass, which increased by about 11% between 140 and 560 ppm CO_2. An increase in hemicellulose concentration of 11% corresponds to 7.3 mg of carbon per g dry mass (assuming a carbon proportion of 44% in polysaccharides). In comparison, the 85% increase in NSC in grasses between the 140 and the 560 ppm CO_2 treatment corresponds to a difference of 32 mg carbon per g dry mass. Considering that within the other investigated plant groups, hemicellulose concentrations were even more stabile, the overall responsiveness of hemicelluloses in newly synthesized cells towards changed CO_2 supply seems very limited in most plants and tissues. This unresponsiveness of total hemicellulose concentrations in tissues formed at different carbon availabilities is in contrast to several studies, which described decreasing hemicellulose concentrations in mature tissues at situations of high carbon demand, like bud break (Zwiazek 1991; Renault and Zwiazek 1997; Robakidze and Bobkova 2003). In Schädel *et al.* (2009b) a decline of 10% in the hemicellulose concentration in branch sapwood of the deciduous tree *Carpinus betulus* before bud break accounted for two-thirds of the carbon that was derived from the strong decrease of starch in the same tissue. This study showed that in mature tissues, like branch sapwood and previous season's coniferous needles, hemicelluloses can serve as additional mobile

carbon reserves besides their primarily structural function during times of extremely high carbon demand.

Monosaccharide spectrum of hemicelluloses

Independent of the CO_2 treatment, characteristic monosaccharide spectra for each plant functional type were found in this study, which are largely consistent with previous findings (Timell and Syracuse 1967; Ebringerova et al. 2005; Willför et al. 2005a; Willför et al. 2005b). High proportions of xylose indicated xylans to be the most abundant hemicellulose class in hardwoods while larger amounts of mannose and galactose in tissues of conifers mirrored the high abundance of galacto-mannans in this functional type. Measuring only the quantitative reaction of bulk-hemicelluloses towards changed CO_2 concentrations might mask some more subtle changes within the monosaccharide composition of hemicelluloses. Especially, since the different chemical and spatial structure of hemicelluloses result in different binding-strengths to cellulose fibrils (Morrison 1980; Sun et al. 2004), a varying degree of flexibility among the hemicellulose classes seems plausible. For example, Lima *et al.* (2004) found the binding strength of xyloglucans to cellulose fibrils to depend on the length of the polysaccharide backbone as well as the chemical nature of their side-chains. To our knowledge, the effect of CO_2 concentrations on the monosaccharide spectrum of hemicelluloses has not been investigated so far. A significant change of the monosaccharide composition of hemicelluloses, as well as a pronounced change of hemicellulose concentrations in plant tissue, could ultimately affect ecosystem processes like herbivory or litter decomposition (Couteaux et al. 1996). Within this study, glucose was the only monosaccharide that showed significantly higher proportions with increasing CO_2 concentrations in most of the investigated tissues, which led to significantly higher proportions of glucose in the total hemicellulose pools of tree leaves. The strong responsiveness of glucose towards changed CO_2 supplies can be most likely explained by the fact that glucose is a key metabolite in the sugar metabolism (Fujikawa et al. 2002) and surplus carbon would be stored within hemicelluloses as glucose units rather than other monosaccharides, because it can promptly re-enter the metabolism, while other hemicellulose monosaccharides require energy demanding conversions. Similarly, Schädel *et al.* (2009b) also found that observed changes of hemicellulose concentrations in mature needles of evergreen conifers during bud break could be entirely explained by changing proportions of glucose-units within the needles hemicellulose pools. On the other hand, in grasses, which was the plant functional group exhibiting the most distinct increase of hemicellulose concentrations with increasing CO_2 concentrations, the proportion of hemicellulose-derived glucose did not increase significantly at high CO_2, indicating that in grasses the higher hemicellulose concentrations derived from an approximately even increase among all hemicellulose monosaccharides.

Conclusion

This study is the first broad-scale investigation on cell wall hemicelluloses in response to varying CO_2 concentrations in different plant functional types. Only leaves of two grass species exhibited a significant increase in hemicellulose concentration with increasing CO_2 concentrations, while all other plant functional types did not show significant changes in the hemicellulose concentration with C-source-sink imbalances. Thus, hemicellulose concentrations in *de-novo* built biomass seem to be largely unaffected by changed atmospheric CO_2 concentrations. In contrast, significant increases of hemicellulose-derived glucose with increasing CO_2 concentrations in leaves of broad-leaved and conifer tree seedlings showed that hemicellulose compositions change in response to varying CO_2 concentrations. Overall, this experiment showed that the structural functions of hemicelluloses do generally not allow for pronounced variations of the total hemicellulose content of tissue built under situations of different carbon availabilities. In conclusion, a ubiquitous quantitative increase of cell wall hemicelluloses in plants at future high atmospheric CO_2 concentrations is not expected.

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CHAPTER 4

SHORT-TERM DYNAMICS OF NON-STRUCTURAL CARBOHYDRATES AND HEMICELLULOSES IN YOUNG BRANCHES OF TEMPERATE FOREST TREES DURING BUD BREAK

Tree Physiology 29, 901-911

Short-term dynamics of non-structural carbohydrates and hemicelluloses in young branches of temperate forest trees during bud break

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Summary Non-structural carbohydrates (NSC) are the most important C-reserves in tissues of deciduous and evergreen tree species. Beside NSC, cell-wall hemicelluloses as the second most abundant polysaccharides in plants have often been discussed to serve as additional mobile C-reserves during periods of enhanced carbon-sink activities. In order to assess the significance of hemicelluloses as mobile carbon reserves, branches of two deciduous (*Carpinus betulus* L., *Fagus sylvatica* L.) and two evergreen (*Picea abies* L., *Pinus sylvestris* L.) tree species were sampled in a mature mixed forest stand in short intervals before and during bud break to assess NSC and hemicellulose concentrations in response to the increased carbon demand during bud break. Starch concentrations in branch sapwood of deciduous trees strongly decreased immediately before bud break and increased after bud break. In both evergreen species, only small changes of NSC were found in branch sapwood. However, one-year old needles exhibited a significant increase in starch concentration shortly before bud break which declined again after flushing. Hemicellulose concentrations (on a NSC-free dry matter basis) in branch sapwood of *Carpinus* decreased significantly shortly before bud break but increased again after bud break. Contrarily, in *Fagus* branch sapwood hemicellulose concentrations remained constant during bud break. Moderate increases of total hemicellulose concentrations prior to bud break were found in one-year old needles of both conifers, which could be explained by an accumulation of glucose-units within the hemicellulose fraction. Overall, cell-wall hemicelluloses appeared to respond species specific to the enhanced carbon demand during bud break. Hemicelluloses in branch sapwood of *Carpinus* and in one-year old needles of conifers likely act as additional carbon reserves similar to starch.

Keywords: carbon reserves, deciduous trees, evergreen trees, glucose, starch.

Introduction

Carbon storage is an essential process of plants to cope with situations in which carbon demand exceeds carbon assimilation. Mobile carbon reserves are defined as organic carbon compounds that accumulate in plant tissues at times of surplus carbon supply, and which can be re-mobilized to support carbon sink activities (e.g. growth, respiration, etc.) at periods of negative carbon source-sink-balances (Chapin et al. 1990). The most important and abundant C-storage compounds are starch and various low molecular carbohydrates. Further carbon storage compounds such as fructans (Meier and Reid 1981) or neutral lipids (Kozlowski and Pallardy 1991) are restricted to certain plant families. Beside these known mobile carbon reserve pools, cell-wall hemicelluloses have also been discussed to be mobile carbon reserves, in addition to their primarily structural function (e.g. Glerum and Balatinecz 1980; Chapin et al. 1986; Renault and Zwiazek 1997). Cell-wall hemicelluloses and pectins in seeds of several plant families have been identified to be re-mobilized as carbon reserves during germination (Buckeridge et al. 2000). However, up to date, it is still unclear, if cell-wall hemicelluloses indeed function as carbon reserves in non-reproductive tissues as well (Hoch 2007).

Hemicelluloses occur in primary and secondary cell walls of all plants and are the second most abundant polysaccharide after cellulose. They are water insoluble, mixed polysaccharides and can be grouped into four classes according to their main type of sugar: xylans, xyloglucans, mannans and mixed-linkage β-glucans (e.g. Ebringerova et al. 2005). Xylans have a backbone of exclusively 1,4-β linked D-xylose units with different sugar and sugar acid residues and are the most abundant hemicellulose class in all higher plants (Puls 1993; Saha 2003). Xyloglucans are mainly represented in primary cell walls of higher plants and seem to be tightly bound to cellulose fibrils by hydrogen bonds (Pauly *et al.* 1999). Hemicelluloses of the mannan-type are

mainly glucomannans and galactomannans and are found in secondary cell-walls of conifers (Timell and Syracuse 1967) and in seeds of Leguminosae (Buckeridge *et al.* 2000). Finally, mixed-linkage (1→3, 1→4)-β-D- glucans occur exclusively in Poales (Buckeridge *et al.* 2004) and some pteridophytes (*Equisetum*, Fry et al. 2008; Sorensen et al. 2008).

Although quantity and structure of hemicelluloses in cell walls are well-known, comparatively little is known about their eco-physiological relevance (Hoch 2007). Obviously, hemicelluloses are not completely resistant to mobilization, particularly those hemicellulose classes which are less tightly bound to cellulose fibrils (Morrison 1980). Sun et al. (2004) suggested this variable binding strength with cellulose to be responsible for different functions of hemicelluloses within cell walls. During the last decades poly-saccharide-modifying enzymes were described in cell-walls of living tissues (Fry 1995), and there is increasing evidence for considerable turnover of structural polysaccharides in mature cell-walls (Gibeaut and Carpita 1991; Kaczkowski 2003).

It is well-established that non-structural carbohydrates (NSCs) in perennial tissues of trees (wood and evergreen needles or leaves) serve as carbon sources to meet the carbon demand for flushing of new leaves and shoots in spring (e.g. Fischer and Höll 1991; Terziev et al. 1997; Landhäusser and Lieffers 2003). Obviously, the lack of photosynthetic active tissues at bud break makes broad-leaved trees dependent on last season's carbon reserves for flushing (Kozlovsky and Pallardy 1997). Nevertheless the high carbon demand during bud break may exceed carbon supply by current photosynthesis in evergreen species as well. So far, several studies indicated that hemicelluloses of previous seasons' needles and sapwood of trees reacted similar to non-structural carbon reserves, especially starch, at situations of high C-demand for growth (Glerum and Balatinecz 1980; Chapin et al. 1986; Kozlowski 1992; Renault and Zwiazek 1997). Furthermore hemicellulose concentrations tended to decrease in stem wood of conifers following the enhanced growth (increased carbon-sink activity) after fertilization (Anttonen et al. 2002; Kilpeläinen et al. 2003; Kaakinen et al. 2004).

This study aimed at a detailed record and quantification of the fluctuations of carbon reserves in branch sapwood and previous season's needles in terminal branches of mature temperate forest trees during the time of bud break. The sampling of two deciduous broad-leaved and two evergreen conifer species enabled a comparison between this two functional tree types. To clarify the role of hemicelluloses as possible mobile carbon reserves, total hemicellulose concentrations were compared with concentrations of free sugars and starch. In addition, a screening of the sugar composition of hemicelluloses allowed assessing, if observed changes within the hemicellulose pools are restricted to specific hemicellulose classes. We hypothesized that for woody species hemicelluloses are an additional C-source in times of increased C-demand during bud break and that concentration changes similar to non-structural carbohydrate reserves in terminal branches of mature forest trees would be observable.

Material and Methods

Study site, plant material and sampling

The study site was located near the village of Hofstetten (44°33'N, 7°36'E, 500 m a.s.l.), close to the city of Basel, Switzerland in a mature (about 100 years old), mixed forest stand with a canopy height of 30-35 m. The infrastructure provided by the Swiss Canopy Crane (Pepin and Körner 2002) was used to reach the upper canopy of the mature trees by a gondola. The mixed forest stocks on rendzina type soil over calcareous bedrock, with ground cover dominated by herbaceous plants (*Helleborus foetidus*, *Mercurialis perennis* and *Paris quatrifolia*) and shrubs (e.g. *Daphne mezereum* and *Lonicera xylosteum*). The climate is oceanic with mean January and July temperatures of 2° and 19° C, and a mean annual precipitation of close to 1000 mm.

Young branches of sun-exposed parts within the canopies of two deciduous broad-leaved tree species (*Carpinus betulus* L., *Fagus sylvatica* L.) and two evergreen conifer species (*Picea abies* L., *Pinus sylvestris* L.) were sampled between March and July 2007 at several dates before, during and after bud break (Table 1). In the following, the species will be referred to by their genus only. Different phases of bud break were identified following a modified classification after Richardson(2006) as shown in Table 1.

Xylogenesis in the sampled branches was tracked by microscopic inspection of microtome-cuts after safranin-staining. Five trees per species were selected

Table 1: Sampling dates for the four investigated species. The numbers indicate the different phases of bud break, which are defined as: 0 = unexpanded buds, 1 = buds swollen, 2 = green leaves/needles emerging, 3 = leaves/ needles 50 % of final size, 4 = leaves/needles fully expanded, + and – indicate transitional stages, f = start of flowering. The days at which samples were collected for a specific species are given in bold.

Sampling date	Deciduous trees		Evergreen trees	
	Carpinus[1]	*Fagus*[1]	*Picea*[2]	*Pinus*[2]
2 March	**0**	**0**	**0**	**0**
29 March	**1, f**	**1**	**0**	**0**
12 April	**2**	1+	0	0
16 April	2+	**2**	1-, f	1-
26 April	**3-**	**3-, f**	**2**	**2, f**
3 May	3	3	**3-**	**3-**
16 May	**3+**	**3+**	**3**	**3**
7 July	**4**	**4**	**4**	**4**

[a] branch sapwood
[b] branch sapwood and previous seasons' needles

with the exception of *Carpinus*, where only three replicates were available within the reach of the crane. Young (c. last 4-5 years) branches and, in the case of evergreen trees, needles from previous seasons were sampled at each sampling date (Table 1).Bark and phloem were removed from the sapwood (xylem) by knife and all tissues were immediately heated in a microwave oven at 600 W for 90 s to stop any enzymatic activity (Popp *et al.* 1996). All samples were dried to weight constancy and ground to fine powder before analysis.

Chemical analyses

Non-structural carbohydrates

The pool of non-structural carbohydrates (NSCs) analysed in this study comprised starch and the quantitatively most important low molecular weight carbohydrates (glucose, fructose and sucrose). The NSC analyses followed the method by Wong (1990), modified as described in Hoch et al. (2002). 10 mg of the dried plant powder were extracted with 2 mL deionised water at 100° C for 30 min. An aliquot of each sample extract was taken for the determination of low molecular weight carbohydrates using invertase (from baker's yeast, Sigma Aldrich) to break sucrose into glucose and fructose. Glucose and fructose were converted to gluconate-6-phosphate with glucose-hexokinase (Sigma Diagnostics, St. Louis, MO, USA) and phosphoglucose-isomerase (from baker's yeast, Sigma-Aldrich). The total amount of formed gluconate-6-phosphate was determined photometrically as the increase in $NADH+H^+$ (Photometer: HR 7000; Hamilton, Reno, NE, USA). For NSC determination the remaining extract was incubated at 40°C for 15 h with dialyzed clarase (an amylase from *Aspergillus oryzae*, Enzyme solutions Pty Ltd, Crydon South Victoria, Australia) to break starch into glucose. NSC was determined as the total amount of glucose as described above. Starch content was calculated as total NSC minus free sugars. All concentrations were calculated on a % dry matter basis. Previous comparisons of the NSC method used for the current study with gas chromatographic based analyses for low molecular weight carbohydrates and starch of two external labs (A. Richter, University of Vienna and M. Raessler, Max Planck Institute, Jena), revealed high quantitative matches among the methods. An earlier study at the Swiss Canopy Crane site (Hoch et al. 2003) showed that the used NSC analysis accounts for more than 80% of the entire non-structural carbohydrate pool of all investigated tree species.

Bulk hemicelluloses

Bulk hemicelluloses were sequentially extracted following the method by Van Soest (1963; 1967) modified for the use of small sample volumes and starch-rich material. Dried plant powder (50 mg) was extracted with a heat stable α-amylase (from *Bacillus licheniformis*, Sigma, Buchs, Switzerland) for 30 min at 85°C to previously extract even high amounts of starch. The starch-free pellet was dissolved in a neutral detergent (sodium tetraborate decahydrate, EDTA, SDS, triethylene glycol, sodium phosphate and distilled water) and boiled for 60 min in a water bath. The detergent extracted proteins, low molecular weight carbohydrates, lipids and pectins, which were separated and removed from the pellet by centrifugation. The pellet containing the cell wall fraction (cellulose, hemicelluloses and lignin) was washed (2x hot deionised water, 1x acetone, 1x deionised water) and dried over night before weighing. The dried pellet was then dissolved with an acid detergent containing 1 N H_2SO_4 and cetyltrimethylammonium bromide and boiled for 60 min to extract and hydrolyze hemicelluloses. The remaining cell wall fraction (cellulose and lignin) was washed with deionised water and acetone before drying and weighing. Hemicelluloses were determined gravimetrically as 'total cell wall fraction' minus 'cellulose and lignin' fraction and expressed on a % dry matter basis as well as on a % NSC-free dry matter basis, which allows to account for possible 'dilution' effects of hemicelluloses in NSC-rich material.

Qualitative hemicellulose analyses

Post-hydrolysis of the hemicellulose containing supernatant with 4 N H_2SO_4 ensured highest polysaccharide hydrolysis of all hemicelluloses and enabled additional analyses of the hemicellulose derived monosaccharides by pulsed amperometric HPLC (Dionex ICS-3000 ion-chromatography system) using a CarboPac^{TM} PA20-column (3 x 150 mm). Monosaccharides (L-Fucose, L-Arabinose, L-Rhamnose, L-Galactose, D-Glucose, D-Xylose, D-Mannose) were eluted with water (0-20 min) at a flow rate of 0.5 mL/min, following a gradient to 0.8 N NaOH (20-45 min). Holding the NaOH concentration at 0.8 N for 15 min resulted further in an elution of D-Galacturonic acid and D-Glucuronic acid (Obro *et al.* 2004). The column and detector were thermostated at 20°C to enable baseline separation of the neighboring peaks arabinose/rhamnose and xylose/mannose and post-column addition of 0.1N NaOH assured high base concentration for optimal detection.

Statistics

Chemical analyses were performed per sampling date, individual tree and tissue. NSC and hemicellulose data were arcsine transformed to achieve normal distribution and equal variances. A linear mixed-effects model was used to calculate repeated measures analyses of NSC, total hemicelluloses and hemicellulose monosaccharides with sampling date and functional group as fixed factors and tree as random factor. Effects were considered significant at $P<0.05$ and for each species significant differences in NSC, hemicelluloses and hemicellulose mono-

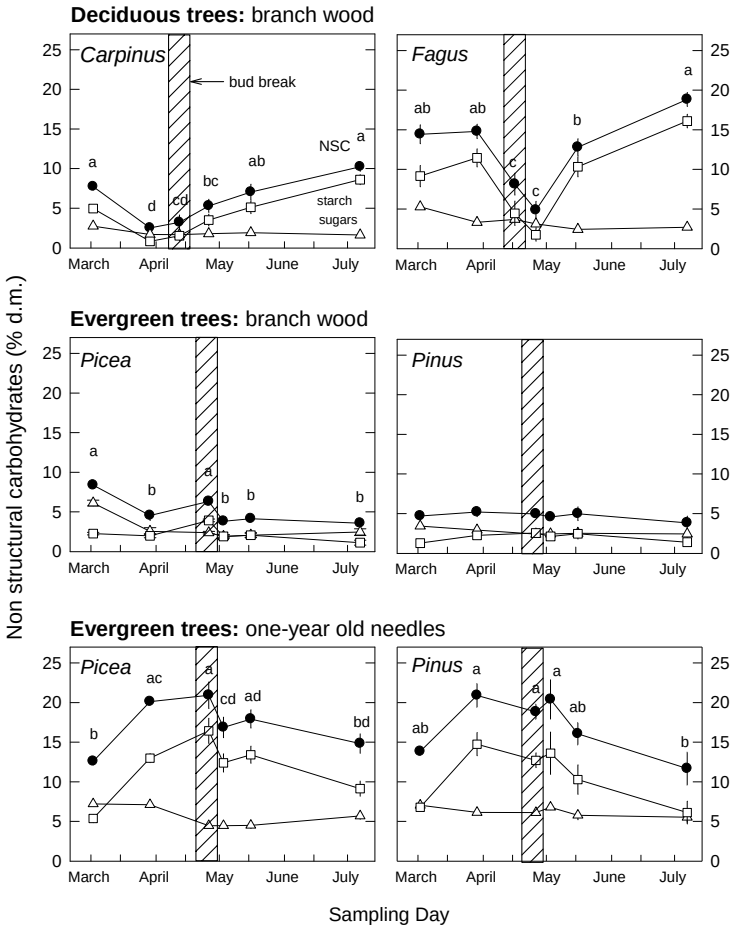

Figure 1: Non-structural carbohydrates (starch and sugars) in branch sapwood and one-year old needles of two deciduous and two evergreen tree species during bud break. For each species the time of bud break is indicated by a hatched bar. Values are means of five trees per species, except *Carpinus* where *n*=3, ± SE. Different letters indicate significant differences in NSC con-centrations among sampling dates by Tukey-Kramer HSD test. No letters indicate no significant differences found.

saccharide concentrations among the sampling dates were analyzed by Tukey-Kramer honestly significant difference test. Statistical analyses were carried out using R version 2.7.2 (R Development Core Team, 2004).

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Results

The buds of all trees were still dormant at the first sampling date in early March (Table 1). Flower buds of *Carpinus* started expanding at the end of March and fully flowered 5-10 days before leaf flushing, whereas *Fagus* flowered approximately ten days after leaf bud break. Flowering and needle bud break of both evergreen species started around two weeks later than for deciduous species by the end of April (Table 1). Microscopic inspections of sapwood-cross-sections revealed no new xylem growth at the time of bud break in all four species. Xylogenesis of the new growth rings in terminal branches was near to completion by mid July in deciduous and conifer species.

Non-structural carbohydrates (NSC)

Non-structural carbohydrates in branch sapwood of the two deciduous tree species, *Carpinus* and *Fagus,* accounted for 9% d.m. and 15% d.m., respectively, on the first sampling date in early March (Fig. 1). Both deciduous trees showed a strong decrease in NSC just before and at the beginning of flushing. While in *Carpinus* NSC reached its minimum concentration shortly before leaf bud break, in *Fagus* the lowest concentration of NSC was measured ten days after bud break. In both species NSC concentrations increased again after bud break (during most extensive shoot growth) and reached the same concentrations as prior to bud break within about 7 weeks. For both species the variation of NSC concentrations was mainly due to changes in starch, while the concentrations of low molecular weight sugars remained almost constant withonly a slight decrease from March to July (Fig. 1). A repeated measures analysis showed significant effects of time and species, as well as for their interaction on NSC (Table 2).

In comparison to the deciduous species, NSC concentrations in branch sapwood of both evergreen conifers were significantly lower at all sampling dates and were always below 10% d.m.. In both conifers, the ratio of low molecular weight carbohydrates to starch was about 1:1, except for the first sampling date in early spring, where free sugar concentrations were found to be more than twice as high as those of starch (Fig. 1). In branch sapwood of *Picea* a slight decrease of low molecular weight sugar con-centrations was evident between April and July, whereas starch approximately doubled its con-centration immediately before bud break (end of March to end of April) and declined again during

Table 2: Repeated measures analysis for time, species and their interaction on seasonal dynamics of NSC and bulk hemicelluloses in branch sapwood of deciduous and evergreen trees and in one-year old needles of evergreen trees.

	NSCs (% d.m.)			Hemicelluloses (% d.m.)			Hemicelluloses (%) NSC- free d.m.		
	df	F	P	df	F	P	df	F	P
Branch sapwood									
Deciduous trees									
Time	5	23.5	**<0.001**	5	7.5	**<0.001**	5	3.3	**<0.016**
Species	1	40.5	**<0.001**	1	87.9	**<0.001**	1	17.3	**<0.001**
Time x species	5	3.2	**0.020**	5	12.3	**<0.001**	5	2.7	**0.040**
Evergreen trees									
Time	5	11.4	**<0.001**	5	7.8	**<0.001**	5	8.0	**<0.001**
Species	1	13.8	**<0.005**	1	10.0	**0.013**	1	5.9	**0.018**
Time x species	5	9.2	**<0.001**	5	4.6	**0.02**	5	5.6	**<0.001**
One-year old needles									
Evergreen trees									
Time	5	16.4	**<0.001**	5	3.04	**0.02**	5	3.3	**0.013**
Species	1	0.08	0.79	1	0.7	0.42	1	0.7	0.413
Time x species	5	5.3	**0.001**	5	0.75	0.59	5	0.7	0.589

P-values < than 0.05 are in bold.

flushing. NSC dynamics in branchwood of *Pinus* were similar to *Picea*, but were less pronounced and not significant (Fig. 1). A repeated measures analysis revealed highly significant effects of time and species on NSC concentrations of branch sapwood of each functional plant type (Table 2) and highly significant time x species effect, which can be explained by the different dynamics of NSC.

In previous season's needles of *Picea* and *Pinus*, NSC concentrations increased strongly up to 20% d.m. prior to bud break and then declined constantly until July (Fig. 1). The relative increase of previous season's needle NSC from March to May was about 70% in *Picea* and 50% in *Pinus*. Like in branch sapwood of the deciduous species, the short term increase before bud break was mainly due to changes in starch concentrations, while low molecular weight sugars showed a moderate decline from March to July (Fig. 1). In needles of both conifers, the fraction of starch within NSC was about 40% in early March and increased to 80% until bud break. Time significantly affected total NSC concentrations in previous season's needles but there was no significant difference between the two conifer species (Table 2). The older needle cohorts (two- to five-year-old needles in *Picea* and two- to three-year-old needles in *Pinus*) showed the same seasonal NSC dynamics as the previous season's needles, but the overall NSC concentrations and the seasonal amplitude of the starch fluctuations were smaller the older the needles (data not shown).

Total hemicelluloses

On average, hemicelluloses in branch sapwood of deciduous trees accounted for 27% d.m. in *Carpinus* and 23% d.m. in *Fagus* with relatively small concentration changes among sampling dates on a dry matter basis (Fig. 2). Because the differences of hemicellulose concentrations among the sampled individuals of a species turned out to be exceptional small in branch sapwood of deciduous trees (standard errors of less than 2% of the mean, Fig. 2) concentration changes of about 10% in *Carpinus* and 15% in *Fagus* resulted in highly significant effects of sampling date within the repeated measures analysis (Table 2). Since the strong variation of NSC concentrations might influence the hemicellulose concentrations on a dry matter basis, we additionally calculated hemicelluloses on a percent NSC-free biomass for all investigated tissues (Fig. 2). While in *Fagus*, the expression of total hemicelluloses on a NSC-free dry mass basis resulted in very constant concentrations (no significant changes over the entire sampling period), a moderate, but significant decrease of hemicellulose concentrations at bud break was evident for *Carpinus* branch sapwood, which was re-filled rapidly after flushing (Fig. 2).

Total hemicellulose concentrations in branch sapwood of evergreen trees accounted for 20% d.m. in *Picea* and 18% d.m. in *Pinus*. From March to April hemicellulos concentrations in branch sapwood of *Picea* increased significantly by 30%,

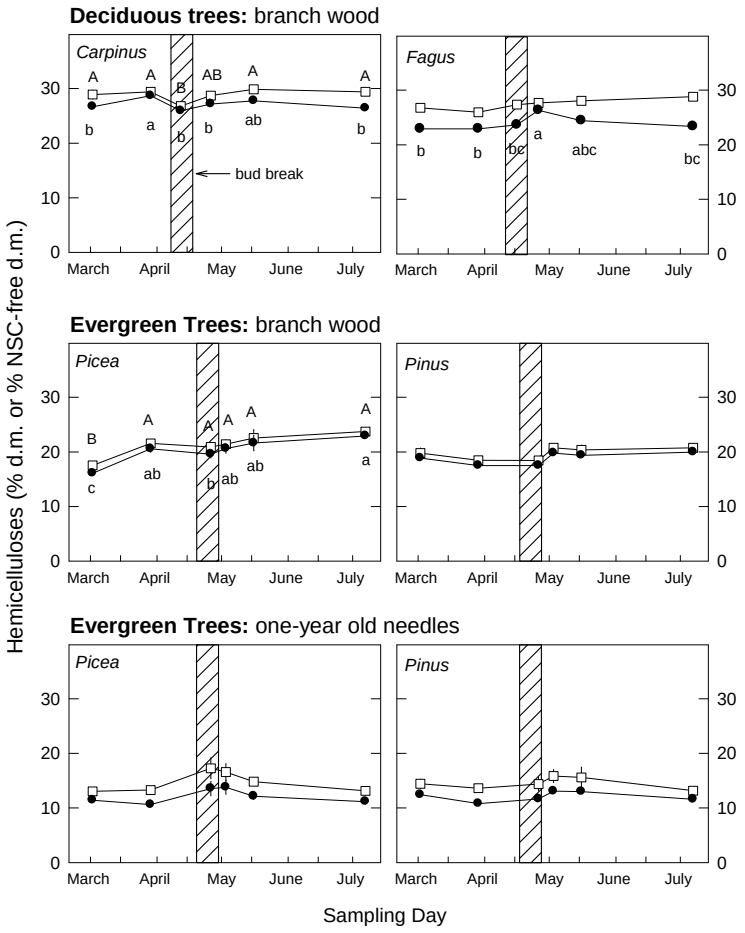

Figure 2: Bulk hemicellulose concentrations calculated as % d.m. (black circles) and % NSC-free d.m. (white squares) in branch sapwood and one-year old needles of two deciduous and two evergreen tree species during bud break. For each species the time of bud break is indicated by a hatched bar. Values are means of five trees per species, except *Carpinus* where n=3, ± SE. Different letters indicate significant differences in NSC concentrations among sampling dates by Tukey-Kramer HSD test. No letters indicate no significant differences found.

while no concentration changes occurred in *Pinus* (Fig. 2). Simultaneously to the increase in hemicelluloses the structural cell wall compounds cellulose and lignin also increased in branch sapwood of *Picea* but not in the other tissues and species. As for deciduous trees, the repeated measurement analysis revealed significant effects of time and species as well as a significant interaction between the two effects (Table 2).

Previous season's needles exhibited hemicellulose concentrations of 10-15% d.m. with increased concentrations during bud break (not significant for the individual species) and declining concentrations thereafter (Fig. 2). The increase in hemicellulose concentration was slightly delayed and less pronounced compared to the increase in starch in both conifers (Fig. 1 and 2). Total hemicelluloses in previous season's needles increased by 30% in *Picea* and 20% in *Pinus* during bud break, whereas NSC increased by 65% and 50% in the respective species during the same period. Within the repeated measures analysis, the uniform response pattern of total hemicellulose concentrations in needles resulted in a significant effect of time, but no significant differences between the two species or time x species interaction. The calculation of hemicellulose concentrations on a NSC-free dry mass basis did not change the observed seasonal trends of hemicelluloses in conifer sapwood and needles (Fig. 2).

Monosaccharide composition of hemicelluloses

Deciduous and evergreen tree species exhibited a distinct pattern of hemicellulose-derived monosaccharides (Fig.3). In branch sapwood of *Carpinus* and *Fagus* xylose was found to be the dominant mo osaccharide with 80% of all hemicellulose sugars and minor amounts of arabinose, galactose, glucose and fucose. These high xylose proportions indicated xylans as the dominant hemicellulose class in broad-leaved tree species. In the two conifer species xylose, mannose, arabinose and galactose accounted for similar proportions of 15-30% of the total, hemicellulose pool, pointing to substantial amounts of arabinogalactans and glucomannans (Fig. 3). In branch sapwood of deciduous tree species, glucose and fucose varied significantly with time (Table 3), whereas all other monosaccharides were not significantly affected by time. The monosaccharide spectrum in one-year old needles of evergreen species resembled that of conifer branch sapwood, although mannose concentrations were higher and galactose concentrations slightly lower. In contrast to branch sapwood, all hemicellulose derived mono-saccharides, except rhamnose, in one-year old needles changed significantly with time (Table 3). Only glucose concentrations, however, varied substantially, with a significant increase at the beginning of flushing

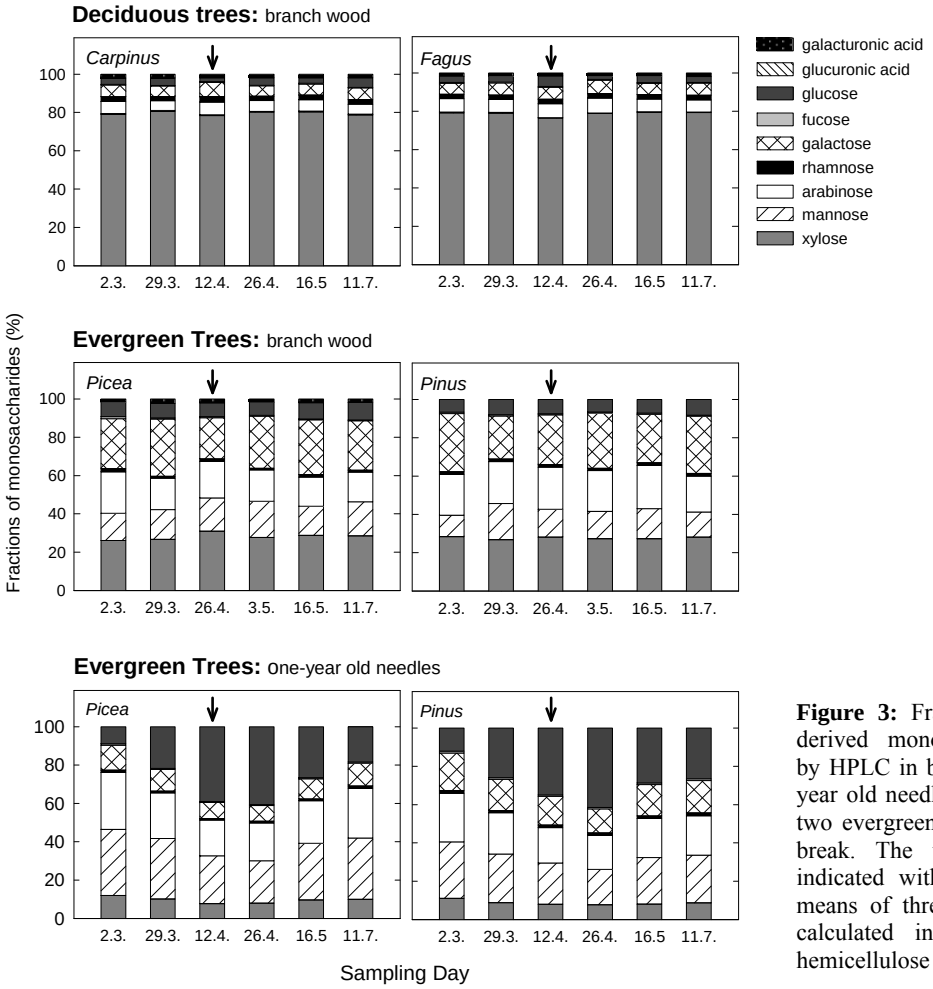

Figure 3: Fractions of hemicellulose derived monosaccharides determined by HPLC in branch sapwood and one-year old needles of two deciduous and two evergreen tree species during bud break. The time of bud break is indicated with an arrow. Values are means of three trees per species and calculated in percent of the total hemicellulose fraction.

Table 3: Summary of *P*-values from the repeated measures analysis of the monosaccharide fractions[1] (mg g^{-1} d.m.) in sapwood of deciduous and evergreen trees and in one-year old needles of evergreen trees.

	Monosaccharides						
	Xylose	Mannose	Arabinose	Rhamnose	Galactose	Glucose	Fucose
Branch sapwood							
Deciduous trees	0.399	0.392	0.095	0.930	0.700	**0.019**	**0.002**
Evergreen trees	0.180	0.115	0.098	0.052	0.415	**0.033**	**< 0.001**
One-year old needles							
Evergreen trees	**0.002**	**0.003**	**0.028**	0.076	**<0.001**	**< 0.001**	**< 0.001**

P-values smaller than 0.05 are in bold.

in one-year old needles (5.5-fold increase in *Picea*, 3-fold increase in *Pinus*) and a constant decrease following bud break. Fluctuations in glucose concentration were also found in older needle cohorts although less pronounced than in the one-year-old needles (data not shown). The temporal dynamics of hemicellulose-derived glucose in one-year-old needles correlated significantly with that of starch in both conifers (for *Picea*: r=0.71, p<0.001; for *Pinus*: r=0.51, p=0.03).

Discussion

NSC dynamics

The pronounced changes in the concentration of starch during bud break demonstrated the important role of this non-structural carbohydrate as C-reserve in branch sapwood of deciduous woody species (e.g. Landhäusser and Lieffers 2003; Wong et al. 2003; Palacio et al. 2008; Spann et al. 2008). Interestingly, the lowest NSC concentrations in *Carpinus* were

found a few days before leaf bud break which can be explained by the high need of C-reserves for flowering which took place 10 days before leaf bud break. In contrast, *Fagus* branches reached their lowest NSC concentrations 20 days after leaf bud break, also coinciding with flowering in this species. Because NSC concentrations increased very rapidly after leaf bud break (most of the variation of starch occurred within 50 days) it can only be captured by sampling in short intervals. Previous studies, which used longer sampling intervals tended to underestimate the decrease of NSC in branchwood during flushing. For example, a previous survey at the same site as the current study found a general increase of NSC concentrations over the course of the entire growing season in young branch sapwood, but since sampling was performed monthly this study did not capture the dramatic change of NSC in young branches around bud break (Hoch et al. 2003). However, in mature trees, the sharp decreases of NSC concentrations in sapwood during flushing seems to be largely restricted to terminal branches and does not translate to the stem or roots of a tree (Newell et al. 2002). Apparently, the C- reserves stored within the terminal parts of a tree crown are sufficient to meet the carbon needs for bud break in mature trees, confirming the general conception of a high degree of carbon autonomy of branches in mature trees (Sprugel et al. 1991; Hoch 2005). The fast refilling of NSC pools in branches immediately after flushing indicates that very young shoots and developing leaves already exhibit a positive carbon balance, with net carbon export even during the time of strongest growth. This is supported by earlier models that predicted a transition from carbon sinks to sources for developing leaves of deciduous trees before they reached 50% of their final size (Turgeon 1989; Marchi et al. 2005).

In contrast to deciduous trees, branch sapwood of the evergreen conifers exhibited less variation during bud break, since they are able to utilize newly fixed carbon from current year photosynthesis in previous seasons' needles before flushing in spring (e.g. Hansen and Beck 1990; Hansen and Beck 1994). Like for deciduous species, mainly starch, rather than low molecular weight sugars are used in mature needles as short-term carbon sources for flushing, resulting in the well-known late-winter increase of starch in conifer needles (e.g. Fischer and Höll 1991; Schaberg et al. 2000).

Total hemicelluloses

Cell-wall hemicelluloses in seeds of species from different plant families are known to be carbon sources for germination (Reid 1985). Those storage hemicelluloses in seeds were identified as mannans, xyloglucans and β-glucans (Buckeridge *et al.* 2000), which also occur in non-reproductive tissues and were therefore suggested to serve as mobile C-reserves in non-reproductive tissues as well (Hoch 2007). While the present study found evidence for a reserve function of hemicelluloses in branches of mature trees, the roles of hemicelluloses during bud break appear to be species and tissue specific. Hemicellulose concentrations in branch sapwood of *Carpinus* decreased significantly when non-structural carbon pools were at their minimum, whereas in *Fagus* no change in total hemicellulose concentrations was found (after the effect of changing NSC concentrations was accounted for). Because xylogenesis in young branches did not occur until several days after bud break (as verified in microscopic cross-section), the observed hemicellulose decrease in *Carpinus* branch sapwood at bud break cannot be explained by the formation of new (immature) xylem. The different dynamics of hemicellulose concentrations between the two deciduous species might be explained by the fact that flowering of *Carpinus* took place before flushing, which likely increased the demand for stored carbon reserves. Further, the absolute starch concentrations in *Carpinus* branches are considerably smaller than in Fagus, which might necessitate the usage of hemicelluloses as additional carbon sources in *Carpinus* when starch reserves were almost depleted during flowering.

A previous study that investigated seasonal fluctuations of total hemicellulose concentrations in different tundra plants reported total hemicellulose concentrations to change similar to non-structural carbohydrates across the growing season in two deciduous shrubs, *Salix pulchra* and *Betula nana* ((Chapin et al. 1986). Hemicellulose concentrations were found to be lowest during mid-season, when growth was strongest and increased by almost 100% towards the end of the growing season. However possible short-term fluctuations of hemicellulose concentrations during bud break were not reported. Because of the high total hemicellulose concentrations in sapwood of close to 30% d.m., relatively small concentration changes within the hemicellulose pool might resemble a substantial carbon source. Hence, the moderate (10%) decline of hemicelluloses in branch sapwood of *Carpinus* prior to bud break accounted for approximately 12 mg of carbon per gram dry mass, assuming a carbon proportion in polysaccharides of 44%), which is comparable with the 18mg carbon per g dry mass derived from the strong decrease of starch by about 80% in the same tissue. The different dynamics of hemicellulose concentrations between the two deciduous species might be explained by the fact that flowering of *Carpinus* trees took place before flushing, which likely increased the demand on stored carbon reserves. Further, the starch concentration in *Carpinus* branches is considerably smaller than in *Fagus*, which might necessitate the usage of hemicelluloses as additional carbon sources in *Carpinus* when NSC concentrations were used up, but not in *Fagus*.

Hemicellulose concentrations in branch sapwood of both evergreen conifers remained relatively constant during bud break, but increased significantly in *Picea* sapwood from March to April. However, this

increase likely resulted from an overall (anatomical) change within the structural cell wall constituents of *Picea* wood, since it was accompanied by a simultaneous increase of the cellulose-lignin fraction, and therefore does not indicate a specific reserve function of hemicelluloses. In contrast to conifer sapwood, total hemicellulose concentrations (but not the cellulose-lignin fraction) of previous season's needles slightly increased at bud break in *Picea* and *Pinus*. This indicates a limited reserve function of hemicelluloses, although, the total amount of carbon derived from hemicelluloses was small compared to that from starch. We calculated the absolute amount of carbon mobilized from the hemicellulose pool of previous season's needles to be 14 mg g^{-1} d.m. in *Picea* and 10 mg g^{-1} d.m. in *Pinus*, while in both species the amount of carbon supplied from starch pools was 48 mg g^{-1} d.m and 35 mg g^{-1} d.m., respectively. A reserve function of hemicelluloses in conifers had previously been supported by Glerum and Balatinecz (1980), who traced the fate of carbon assimilated in autumn with ^{14}C-labeling in *Pinus banksiana* seedlings. The isotopic signal in structural compounds of mature needles was found to strongly decrease after sprouting and, although the cell wall compounds were not separated in that study, the authors suggested that mobilization of hemicelluloses might be the best explanation for these findings. In a more recent study, Renault and Zwiazek (1997) described hemicelluloses of last season's needles from *Picea glauca* seedlings to decrease during flushing. The same result was found in mature needles of *Picea obovata* where hemicelluloses tended to accumulate prior to the most active growth period and serve as mobile carbon reserves in addition to water-soluble polysaccharides (Robakidze and Patov 2000; Robakidze and Bobkova 2003). Those studies explicitly pointed at the possibility of a dual function of hemicelluloses in coniferous needles as structural polysaccharides on the one hand and carbon reserves on the other hand.

Monosaccharide spectrum of hemicelluloses

The monosaccharide spectrum of hemicelluloses found in the current study agreed well with existing literature data for both functional plant types (Timell and Syracuse 1967; Ebringerova et al. 2005; Willför et al. 2005a; Willför et al. 2005b). High amounts of xylans were indicated by a c. 80 % xylose fraction in branch sapwood of broad-leaved trees, while higher proportions of arabinose and mannose mirrored the presence of substantial amounts of arabinogalactans and glucomannans in wood and needles of evergreen species. The detailed analyses of hemicellulose-derived monosaccharides enabled the assessment of quantitative variations within the hemicellulose pools and possible more subtle changes within the monosaccharide fraction, which would not show up measuring only total hemicelluloses. Hence, although the changes of total hemicellulose concentrations in one-year-old needles were not significant at the $p<0.05$ level, we found a highly significant increase of hemicellulose derived glucose concentrations in mature needles before and at bud break. The increase in glucose concentration entirely explained the fluctuation in total hemicellulose concentration in one-year old needles of both coniferous species. Previous studies on qualitative changes of hemicelluloses in woody species during the growing season are rare. Similar to our study, Renault and Zwiazek (1997) found that hemicellulose derived arabinose decreased during bud break, whereas glucose from the hemicellulose fraction increased significantly in *Picea glauca* seedlings. In contrast to the situation in evergreen needles, decreasing glucose and xylose concentrations during sprouting were reported by Decassia et al. (1992) within the hemicellulose fraction of xylopodium of *Ocimum nudicaule* (Basil). Furthermore, increased hemicellulase activity was found in the xylopodium of *O. nudicaule* at the end of the dormant season whereas other glucosidases were more active in the following sprouting phase (Figueiredo-Ribeiro and Dietrich 1983).

The observed qualitative changes in needle hemicelluloses during bud break mirrored either an active carbon reserve mobilization, or a physiological modification of cell wall rigidness at the beginning of the growing season. There are hints that both may occur. For example, Gibeaut and Carpita (1991) reported modifications of the chemical structure of arabinoxylans and xyloglucans during elongation and maturation phases of grass coleoptiles. In hemicelluloses of evergreen needles, however, only glucose, as a key metabolite in sugar metabolism (Fujikawa et al. 2002) changed substantially during bud break within the current study, and the observed increase of total hemicelluloses by 30% in *Picea* needles during bud break can be completely explained by the increase of hemicellulose derived glucose during that time. Hence, we assume that in addition to starch, glucose from current photosynthesis prior to bud break can also be stored within the hemicellulose pool, which is subsequently re-mobilized after flushing.

Conclusion

This study showed that deciduous trees and to a lesser extent also evergreen trees rely strongly on mobile carbon reserves during bud break, although new shoots of deciduous trees rapidly refilled their carbon reserve stores, indicating a fast switch of new shoots from carbon sinks to net carbon sources. The fluctuations within the NSC pool therefore were only transient and strongly driven by changing starch concentrations, pointing to the important role of starch in terminal branches during the initial stage of new shoot formation.

Although hemicellulose concentrations varied much less over time, they nevertheless might contribute as carbon reserves, since due to their

overall high tissue concentrations even small concentration changes correspond to substantial amounts of mobilized carbon. Overall, this study showed that cell wall hemicelluloses in young branches are to great extent structural carbohydrates, but can serve as additional mobile carbon sources in some species during periods of exceptionally strong carbon demand.

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CHAPTER 5

GENERAL SUMMARY

General summary

In this thesis I investigated the responsiveness of hemicelluloses to varying carbon supply and the potential role of hemicelluloses as mobile carbon reserves in different tissues, species and plant functional groups. Mobile carbon reserves are important carbon sources in times when carbon demand for growth and metabolism exceeds carbon input via photosynthesis. Hemicelluloses make up one quarter of the total biomass and are therefore the second most abundant polysaccharide in plants. A mobile carbon reserve function of these polysaccharides would thus significantly increase the carbon storage capacity of plants even if, due to the structural function of hemicelluloses, only fractions of the total hemicellulose pool could be mobilized. The ubiquitous occurrence and the high tissue concentrations of hemicelluloses imply a considerable carbon sink function under future elevated atmospheric CO_2 concentrations.

Despite the quantitative importance of hemicelluloses they have drawn little attention in ecological studies so far mainly because of the analytical difficulties associated with their chemical heterogeneity. The method for the analysis of hemicelluloses used in this thesis followed a modified procedure (developed during the first year of this thesis) based on the Van Soest fiber analysis (Van Soest 1963; Van Soest and Wine 1967)[1]. Within a first step, this fiber analysis separates structural cell-wall compounds (cellulose, hemicelluloses and lignin) from all non-structural cell compounds as well as cell-wall pectins and proteins. In a second step hemicelluloses are hydrolyzed and extracted from the remaining cellulose and lignin fraction with an acid detergent. The main modifications to the original Van Soest procedure were a significant reduction of the required small sample volumes (a 20-fold reduction of the original sample volume still allowed accurate gravimetric determination of hemicelluloses) and the additional extraction of starch with heat-stable α-amylase allowed for the gravimetric analysis of hemicelluloses in starch rich material. Most importantly, the miniaturization of the sample volume, which was possible by replacing all filtering steps with centrifugation steps, allowed for the simultaneous analysis of many samples (up to 150 within a single run). Finally, the addition of HPLC-analyses of the hydrolyzed hemicelluloses enabled a qualitative assessment of the chemical composition (i.e. the monosaccharide identities and proportions) of the extracted bulk hemicelluloses.

Quantity and quality of hemicelluloses from different plant functional types (chapter 2)

By using the refined hemicellulose extraction method we compared hemicellulose concentrations and compositions of different tissues from broad-leaved trees, conifers, grasses and herbs within a single study (**chapter 2**). Overall, hemicellulose concentrations were highest in sapwood of broad-leaved trees with a maximum concentration of 31% d.m. in *Fraxinus excelsior*, while foliar and bark tissues of woody species exhibited smallest concentrations with 10-15% d.m.. Beside the quantitative diversity of hemicelluloses this study revealed characteristic spectra of monosaccharides for each tissue and functional plant group. The most prevalent monosaccharide was xylose (derived mainly from xylans) that accounted for 80% in sapwood of broad-leaved trees and was also abound in the other tissues and plant functional groups. Coniferous samples had larger concentrations of mannose, arabinose and galactose that clearly assigned different mannan-type hemicelluloses to be common in conifers. Hemicelluloses of grasses mainly consisted of xylose and arabinose whereas herbaceous tissues exhibited a broader mixture of xylose, arabinose, galactose and glucose. In conclusion, the modified Van Soest method proofed to be a reliable and fast way to measure cell-wall hemicelluloses quantitatively and qualitatively in any plant tissue.

The influence of changed C-source-sink activities on hemicelluloses (chapter 3 and 4)

While **chapter 2** of this thesis introduced the new micro-extraction method for hemicelluloses and documented the quantity and structural composition of hemicelluloses within a screening over several plant functional types, **chapter 3** and **4** investigated the ecophysiological importance of hemicelluloses.

Experimental manipulation of the C-supply

Within the first of these studies (**chapter 3**) 16 plant species of four different plant functional types (grasses, perennial herbs, broad-leaved trees and conifers) were grown under either extremely low (140 ppm), medium (280 ppm) or high (560 ppm) atmospheric CO_2 concentrations in order to induce situations of massive C-under or C-oversupply. The successful manipulation of the carbon supply for the plants evidenced the significant increments of biomass and NSC concentrations with increasing atmospheric CO_2 concentrations in all species. On average, species doubled their biomass from 140 ppm to 560 ppm CO_2 and NSC concentrations were up to 7-fold increased in plants growing under 560 ppm

[1] Van Soest PJ (1963) Use of detergents in analysis of fibrous feeds .2. A rapid method for determination of fiber and lignin. *Journal of the Association of Official Agricultural Chemists* 46: 829-&.
Van Soest PJ and RH Wine (1967) Use of detergents in analysis of fibrous feeds .4. Determination of plant cell-wall constituents. *Journal of the Association of Official Analytical Chemists* 50: 50-&.

CO_2 compared to the low CO_2 treatment. Surprisingly, although growth was strongly restricted in plants growing under extremely low CO_2 concentrations, the non-structural carbohydrate pools were never completely depleted in any of the investigated tissues. In contrast to the strong reaction of NSC towards changed atmospheric CO_2 concentrations, hemicellulose concentrations remained very stable in most of the investigated species. Only leaves of two out of four grass species exhibited a significant increase (14%) of total hemicellulose concentrations with increasing CO_2 availability. All other species and tissues (leaves and sapwood of woody species) showed no changes in the hemicellulose concentration.

Although the CO_2 treatments did not affect total hemicellulose concentrations, qualitative changes within the monosaccharide spectrum of hemicelluloses were found in leaves and needles of woody species. Hemicellulose-bound glucose increased significantly with increasing CO_2, yet, due to the relatively small proportion of glucose (about 16%) within the hemicellulose pool, this increase did not lead to a substantial (significant) increase of the total hemicellulose concentrations within the respective tissues. This result is of special interest since a pronounced change of the monosaccharide composition of hemicelluloses at elevated CO_2 concentrations could ultimately affect ecosystem processes like herbivory or litter decomposition.

Naturally occurring fluctuations in C-sink activity

In contrast to the experimental approach in **chapter 3**, the second study investigated the potential C-reserve function of cell-wall hemicelluloses (**chapter 4**) by using the naturally occurring fluctuation in C-sink activity during bud break in mature forest trees. Two deciduous and two evergreen species (*Carpinus betulus*, *Fagus sylvatica* and *Picea abies*, *Pinus sylvestris*) were sampled at the Swiss Canopy Crane facility in Hofstetten, near the city of Basel. Young branch wood, and in the case of conifers also needles from previous seasons, were sampled in short intervals before, at and after bud break to track the fluctuations of NSC and hemicellulose concentrations. With the onset of new leaf and shoot growth in early spring woody species, especially deciduous trees which are lacking perennial green leaves, rely on the mobilization of stored carbon reserves to supply the initial phase of bud break.

In sapwood of deciduous tree species a strong decline in the NSC concentration (mainly starch) was found shortly before bud break. Fast refilling of the non-structural carbon pools indicated that newly formed leaves very soon change from C-sinks to C-sources. Sapwood of conifers exhibited no change in NSC concentration during bud break but old needles revealed a significant short-term increase of starch shortly before flushing which declined again after the formation of new needles and shoots had started. This result showed that evergreen trees rely rather on recent assimilates than on reserves from previous seasons.

Hemicellulose concentrations significantly decreased in sapwood of *Carpinus* by about 10% prior to bud break, but with a time lag of two weeks compared to the decrease in starch. Changes of hemicelluloses, calculated as % NSC-free d.m. resulted in a net carbon decrease of 12 mg C g^{-1} d.m., which was two-thirds the decrease of carbon due to decreasing starch concentrations (18 mg g^{-1} d.m.), indicating a considerable C-reserve potential of hemicelluloses in *Carpinus* sapwood. In contrast to *Carpinus*, *Fagus* showed no change in the hemicellulose concentration when calculated on a % NSC-free d.m. basis. This discrepancy could be explained by a much stronger C-sink activity in *Carpinus*, since flowering starts several days before leaf bud break in *Carpinus*, additionally increasing the strain on carbon reserves in this species. In contrast, flowering in *Fagus* occurs 5-10 days after leaf bud break as was also reflected by the lowest NSC concentration few days after bud break in *Fagus* branch wood. In contrast to *Carpinus* no change in hemicellulose concentrations was found in *Fagus* branch wood.

Branch wood of *Picea* exhibited a 30% increase in hemicellulose concentration from March to July when calculated as % NSC-free dry matter, which rather mirrors developmental changes in connection with wood formation than a reserve function of hemicelluloses in this tissue. Unlike sapwood of *Picea*, one-year old needles of both coniferous species (*Picea* and *Pinus*) showed increasing hemicellulose concentration at and shortly after bud break that declined again towards summer (July). This variation in hemicellulose concentration in needles correlated with that of NSC in the same tissue, supporting the hypothesis that hemicelluloses in mature needles indeed can be re-mobilized to serve as an additional C-source during the period of strongest shoot growth. Although starch was identified as the major C-source in needles, the observed concentration changes of hemicelluloses during bud break accounted for at least 30% of the carbon derived from NSC.

HPLC measurements of the extracted hemicelluloses identified characteristic monosaccharide spectra for tissues of deciduous trees and conifers. The most significant change in the monosaccharide spectrum was found in one-year old needles of both conifers, in which glucose concentrations significantly increased before bud break, coinciding with the increase in total hemicellulose concentration prior to bud break. Most importantly, the increase in glucose concentration entirely explained the fluctuation in total hemicellulose concentration in both coniferous species, indicating that the glucose increase is not only a physiological change in the hemicellulose monosaccharide spectrum but rather constitutes a mobile reserve function.

Main conclusions

1) The developed micro-extraction method for hemicelluloses, which is based on the well-established Van Soest fibre analysis, proofed to be a reliable and fast procedure for the simultaneous analysis of cell-wall hemicelluloses in a great number of samples and different plant tissues. A screening over 28 plant species revealed the highest hemicellulose concentrations in wood of deciduous trees (c. 25% d.m.) and conifers (c. 20% d.m.), which also account for the major biomass fraction globally, confirming hemicelluloses to be the second most abundant compound class in biomass on a world-wide scale. Furthermore, the novel introduction of a HPLC analysis for hemicellulose-derived monosaccharides directly within the hemicellulose extract enabled the fast and accurate assessment of the monosaccharide composition of the total hemicellulose pool.

2) In all tissues and species NSC concentrations strongly mirrored the carbon balance of all investigated plants. On the one hand, high carbon availability within the CO_2 manipulation experiment induced significant increments of NSC concentrations in all tissues and species, and on the other hand, high carbon demand during bud break induced significant declines of NSC concentrations in terminal branches of mature deciduous trees. In most cases the variation of NSC in response to changing C-source-sink activities was mainly driven by variations of starch concentrations and not osmotically active low molecular weight sugars.

3) In contrast to NSC, cell-wall hemicelluloses remained unchanged in most species within the CO_2 manipulation experiment. Since hemicelluloses did not accumulate at high CO_2 supply I conclude that hemicelluloses in newly formed biomass are mainly structural bound and have a very limited potential as additional C-sinks under future elevated atmospheric CO_2 concentrations. The monosaccharide spectrum of hemicelluloses in leaves of woody species, however, qualitatively changed with increasing CO_2 concentrations, which potentially implicates consequences for many ecological processes (i.e. herbivory, litter decomposition).

4) The analysis of hemicelluloses in mature tissues during spring bud break revealed short-term fluctuations of hemicelluloses in branchwood of *Carpinus* and mature needles of both investigated conifers (*Picea* and *Pinus*). As these variations correlated with those of starch I suggest a mobile reserve function of hemicelluloses during the time of high carbon demand at bud break. The observed variations in hemicellulose concentrations in mature needles were solely due to variations in hemicellulose-bound glucose and the amounts of carbon liberated accounted for one-third of the contribution from starch.

In conclusion, this thesis showed that during the 'de-novo' synthesis of cell-walls, hemicellulose concentrations do not vary strongly with changing carbon supply. They thus likely will not play a significant role as additional carbon sinks at future elevated atmospheric CO_2 concentrations on an ecosystem level. On the other hand, hemicelluloses in mature tissues, like branch wood of deciduous trees and previous seasons' coniferous needles, can serve as additional mobile carbon reserves besides their primarily structural function during times of exceptionally high carbon demand, like at bud break.

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