Calcium nutrition has a significant influence on wood formation in poplar

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Summary

• To test the effects of calcium on wood formation, *Populus tremula* × *Populus tremuloides* clones were supplied with Hoagland solution modified in its calcium contents. Energy-dispersive X-ray analysis (EDXA) revealed an increase in calcium in the phloem, the cambium and the xylem elongation zone with increasing Ca^{2+} supply in the nutrient solution.

• Using light and electron microscopy, a strong impact was shown on the cambial and the elongation zones under calcium starvation.

• Using Fourier transform infrared (FTIR) spectroscopy on wood and bark cells formed under calcium starvation, we detected a reduction of some absorptions, such as carbonyl and methoxy groups from S-lignin. Also, a significant reduction in fiber length was detected with decreasing calcium supply in the nutrient solution. High-performance liquid chromatography (HPLC) analysis revealed a large increase in sugar concentrations in the leaves, but reduced concentrations in the bark under Ca^{2+} deficiency.

• In conclusion, our results show a significant influence of calcium on the structure, chemistry and physiology of wood formation. Thus, efficient Ca^{2+} supply has to be considered a decisive factor in wood formation.

Key words: calcium, fiber length, Fourier-transform-infrared (FTIR), poplar, sugar analysis, wood formation.

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Introduction

Mineral nutrition has both specific and essential functions in plants. Amongst others, calcium is one of the so-called macronutrients, which are essential for plant metabolism. It binds only weakly with water, uptake happens passively via the fine root apoplast and its acropetal oriented transport takes place almost exclusively via the transpiration stream (Marschner, 1995). Therefore, this cation is regarded as phloem-immobile. In contrast to most macronutrients, calcium is mainly found in the apoplast. This is partly because of the multiple binding sites in the cell wall, especially the carboxyl groups of pectins in the middle lamella. In trees, this binding competence of calcium in the middle lamella as well as in the flexible primary cell wall therefore effects strengthening of the developing xylem tissue during xylem differentiation (Brett & Waldron, 1996; Guglielmino *et al.*, 1997). Under salt stress, cell-wall-bound Ca^{2+} was shown to play an important role in mediating long-term salt adaptation of *P. euphratica* (Ottow *et al.*, 2005). Also, Dünisch & Bauch (1994) found that the positive influence of K^+, Ca^{2+} and Mg^{2+} fertilization on wood formation in coniferous trees was stronger during periods of low precipitation than during periods of high soil water content. However, once bound in the cell wall compartments, calcium remains mostly unavailable for further plant metabolism. Another reason the apoplast is main cell compartment to localize calcium is the restricted calcium capacity of the protoplast. In the cytoplasm, free calcium concentrations range between 0.1 and 0.2 μM (Felle, 1988; Evans *et al.*, 1991; Hirschi, 2004), and hence enable this ion

to work as an effective signal transducer upon small changes in its concentration. Those signals can be induced by multiple biotic or abiotic stimuli (Marschner, 1995; Sanders *et al.*, 1999; Knight, 2000; Anil & Rao, 2001; Knight & Knight, 2001; Rudd & Franklin-Tong, 2001). Apart from acting as a messenger ion, intracellular calcium also functions as a membrane stabilizer, and thus acts as a protector against passive ion influx (White & Broadley, 2003), and is often associated with enzyme-activating processes (Plieth, 2005). In trees, excess calcium ions precipitate as calcium oxalate crystals in cell vacuoles (angiosperm trees; Borchert, 1990) or in intercellular spaces (gymnosperm trees; Fink, 1991a,b,c). In poplar bark, calcium oxalate crystals were found to fill the whole lumen of fibers ('Kristallkammerfasern') located close to phloem fibers (Trockenbrodt, 1995). Using secondary ion mass spectrometry, Follet-Gueye *et al.* (1998) observed a strong temporary increase in calcium concentration in the cambium and in the phloem of beech during the period of reactivation. This calcium increase may be involved in the regulation of cambial reactivation in spring, but its biochemical mechanism of action is still not fully understood.

Research into the impact of calcium on plant biology revealed multiple and inhomogeneous functions of this ion in many different aspects of plant life. With regard to wood formation, Du & Yamamoto (2003) obtained results which suggested an involvement of calcium in the gravistimulated compression wood formation. On the lower side of tilted decapitated *Taxodium distichum* seedlings treated with EGTA or $LaCl_3$, the tracheid walls were thinner than those of non-treated, tilted decapitated seedlings. No characteristics of compression wood developed, indicating a particular role of Ca^{2+} in compression wood formation (Du & Yamamoto, 2003). According to Westermark (1982) and Lohrasebi *et al.* (1999), calcium deficiency might reduce lignification during tracheid differentiation, suggesting that Ca^{2+} plays an important role in the lignification of compression wood. Also, in hypocotyls of *Picea abies* it has been observed that wall deposition was reduced at low Ca^{2+} concentrations, mainly because of the inhibition of lignin and noncellulosic polysaccharide deposition (Eklund & Eliasson, 1990; Eklund, 1991). Interestingly, IAA could partially overcome this Ca^{2+} deficiency (Eklund, 1991).

In this context, the present work focuses on the effect of calcium nutrition on the xylogenesis and phloem development of young poplar trees. The influence of calcium nutrition on xylem structure as well as ion concentration of the cambial zone and its derivatives was studied by microscopic analysis and X-ray microanalysis. Since earlier research on willow showed that assimilate transport within the phloem is reliant upon calcium nutrition of the trees (Schulte-Baukloh & Fromm, 1993), we further investigated the changes in sugar concentrations by high-performance liquid chromatography (HPLC) analysis in trees grown in different calcium regimes.

Materials and Methods

Plant material

Populus tremula L. × *Populus tremuloides* Michx. clones T89 (kindly provided by Dr R. Hedrich, University of Würzburg, Germany) were grown for 6 wk in hydroponic culture under long-day conditions (16 h light, 22°C : 8 h darkness, 18°C). At the beginning of the experimental procedure, all plants were 5–6 cm tall, with a stem diameter of 2–3 mm and four to six leaves. They were provided with macro- and micronutrients in a modified Hoagland solution (Hoagland & Arnon, 1950) containing either minimized Ca^{2+} (0 mM or 0.1 mM), reduced Ca^{2+} (1 mM), full-strength Ca^{2+} (5 mM) or increased Ca^{2+} (10 mM) concentration. To guarantee constant oxygen supply, hydroponics were aerated with pumps (Tetra*tec*®AP50; Tetra Werke, Melle, Germany).

X-ray microanalysis

Stem segments 2–4 mm long were frozen in precooled liquid isopentane immediately after being cut. After freeze-drying, the samples were coated with chromium and examined under a scanning electron microscope (AMR 1200, Leitz) equipped with an energy-dispersive X-ray (EDX) microanalysis system (KEVEX 4000). Element-specific X-ray spectra were obtained from cross-sections of phloem and cambial tissue as well as from developing xylem tissue using a reduced scan raster area at ×1000 magnification. Relative calcium concentrations were expressed as peak : background ratios from five recorded spectra.

Light microscopy

Stem tissue was fixed in a solution containing 1% (w/v) formaldehyde, 1 mM EGTA, 50 mM cacodylat buffer and 5% glutaraldehyde for 2 h, washed in cacodylat buffer and dehydrated in a graded ethanol series. After embedding in LR-white acrylic resin, semithin sections (2 μm thick) were cut with a diamond knife and stained with toluidine blue for light microscopy. Images were taken with a digital camera (Axiocam, Zeiss) and image analysis was done using a Zeiss Axio Vision system (Axio Vision 3.1).

For fiber maceration, small tangential slices of xylem were cut off the stem with a razor blade and put in a maceration solution, containing 10% HNO_3 and 10% CrO_3 in a 1 : 1 ratio. After 50 min in an incubation chamber (Memmert U30, Schwabach, Germany) at 60°C, the libriform fibers were decanted and rinsed off. Images were taken and analysis performed as already described.

Transmission electron microscopy (TEM)

Small sections of stem tissue were cut with a razor blade and immediately immersed in fixation medium (as already described).

After fixation for 2 h, the tissue was postfixed with 2% (w/v) osmium tetroxide overnight at room temperature, stained with 3% (w/v) uranyl acetate in 20% ethanol for 1 h, dehydrated in a graded series of ethanol and embedded in Spurr's epoxy resin (Spurr, 1969). Ultrathin sections were cut with a diamond knife on an ultramicrotome (Ultrotome Nova, LKB, Bromma, Sweden), transferred onto Formvar-coated copper grids and stained with lead citrate. Sections were examined in a Zeiss EM 10c transmission electron microscope at 80 kV.

Fourier transform infrared (FTIR) spectroscopy

The infrared spectra were measured using the potassium bromide (KBr) method on a spectrometer (Biorad FTS 40, Munich, Germany). After grinding the xylem and the bark samples by ball milling (Perkin Elmer, Boston, MA, USA) and sieving, pellets were pressed consisting of 300 mg potassium bromide and 1 mg ground tissue. As an internal standard we used potassium thiocyanate (KSCN), which was dried carefully under vacuum (Wiberley *et al.*, 1957; Fraser, 1959; Nakano & Miyazaki, 2003).

For each nutritional variation, five KBr pellets were produced and measured between 4000 cm^{-1} and 450 cm^{-1} with a resolution of 4 cm^{-1} and 16 scans. The FTIR spectra were baseline-corrected and normalized on the absorption band of the internal standard (2050 cm^{-1}).

Sugar analysis

Poplar plants were separated into leaf, root, bark and xylem tissues and each was homogenized in liquid nitrogen with mortar and pestle. For the analysis of monosaccharides 50 mg of homogenized material was mixed with 1.5 ml water and 50 mg washed PVPP (Sigma, Munich, Germany). Soluble sugars were extracted while shaking for 30 min at 4°C. Samples were incubated for 5 min at 95°C. In order to separate cell debris from soluble sugar solution, samples were centrifuged twice for 10 min at 13 000 ***g*** in a table-top centrifuge. Samples were analyzed as described (Heizmann *et al.*, 2001) by injecting 100 µl samples into a HPLC system (Konex DX 500, Dionex, Idstein, Germany). The precolumns NG1 (Dionex) and CarboPac Guard Column (Dionex) purified samples from phenoles and other organic contaminants. Separation of sugars was carried out on a CarboPac 1 column (250 × 4.1 mm, Dionex) with 40 mM NaOH as eluent at a flow rate of 1 ml min^{-1}. Carbohydrates were measured by means of a pulsed amperometric detector equipped with an Au working electrode (Dionex DX 500). Individual carbohydrates were identified and quantified with external standards.

Starch was analysed by quantifying its glucose units on the photospectrometer with the starch detection kit (Roche, Mannheim, Germany) according to the manufacturer's protocol.

Results

Populus tremula × *Populus tremuloides* saplings grown hydropoincally for 6 wk with different Ca^{2+} supplies revealed clear differences in shoot growth. Variants grown with a Ca^{2+} supply of 5 mM in the nutrient solution showed optimal leaf and shoot development, whereas those grown with a Ca^{2+} supply of 1 mM showed slightly reduced shoot development. However, poplars grown under excessive Ca^{2+} supply (10 mM) showed no significantly enhanced biomass production compared with the full-strength supply of 5 mM Ca^{2+} (Fig. 1b). Shoot growth was strongly inhibited with a concentration of 0.1 mM Ca^{2+} in the nutrient solution (Fig. 1a). In addition, with this

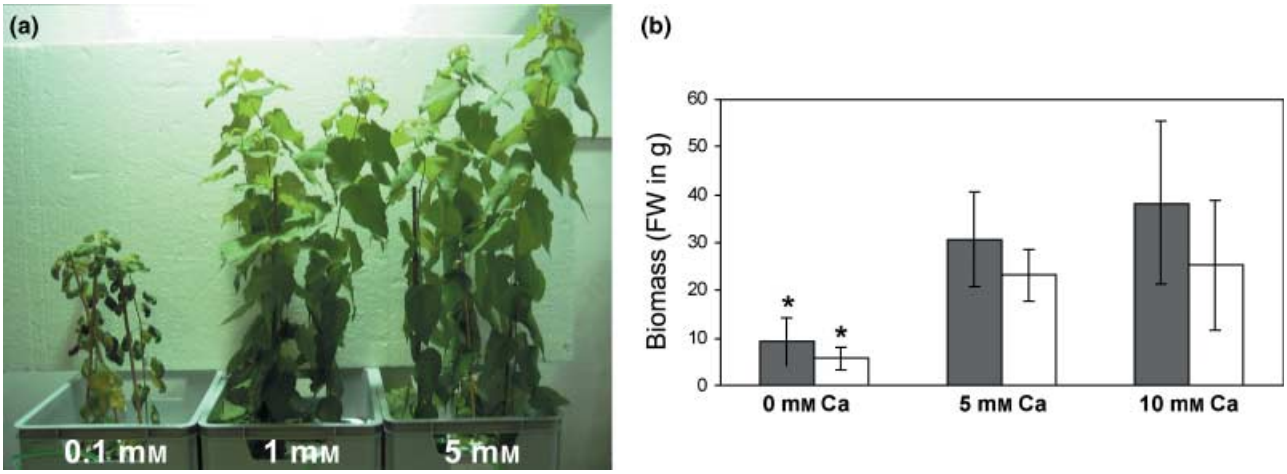


Fig. 1 (a) Poplars (*Populus tremula* × *Populus tremuloides* clones) grown for 6 wk in hydroponics under minimized Ca^{2+} supply (0.1 mM, left), reduced Ca^{2+} supply (1 mM, center) and optimal Ca^{2+} supply (5 mM, right). Under strong calcium stress, poplars show reduced shoot growth and aberrant leaf growth. (b) Biomass production (fresh weight, FW) of shoots and roots was significantly reduced under Ca^{2+} deficiency, but not enhanced under excessive Ca^{2+} supply. Data are means ± SD, $n = 3$. Asterisks represent statistically significant differences (*t*-test) compared with poplars with the highest (10 mM) Ca^{2+} supply: *, $P < 0.05$.

concentration, the relative leaf area of the poplar clones was greatly reduced and the leaves showed bends in the margins and constrictions in the leaf tissue (Fig. 1a, right). Similar symptoms were shown to be typical effects of calcium deficiency in strawberry leaves (Kochian, 2000).

Not only was poplar shoot growth affected by calcium stress, but root growth was also strongly inhibited. Under calcium starvation, root biomass production was significantly reduced compared with full strength and excessive Ca^{2+} supply (Fig. 1b).

Effect of Ca^{2+} supply on ion concentrations in stem tissues

Energy-dispersive X-ray analysis of cross-sections of various stem tissues (cambium, phloem and elongation zone) revealed a statistically significant increase in the relative calcium concentration with increasing calcium supply, in a dose-dependent manner ($n = 5$; Fig. 2). Thus, the Ca^{2+} concentrations were found to be in similar concentrations for each nutrient variation throughout the analyzed compartments of the stem cross-section. We only detected an aberrant increase in calcium in the phloem tissue when plants were grown with excessive Ca^{2+} (10 mM). However, since calcium is known to be almost phloem-immobile, this effect may be the result of Ca^{2+} oxalate formation caused by an apoplastic Ca^{2+} spread.

Microscopic analysis

Apart from a general reduction of wood increment under calcium starvation, light microscopic analysis of the stem cross-sections also exhibited Ca^{2+}-dependent changes in vessel size (Fig. 3). Measuring the vessel lumen area, we found a decrease

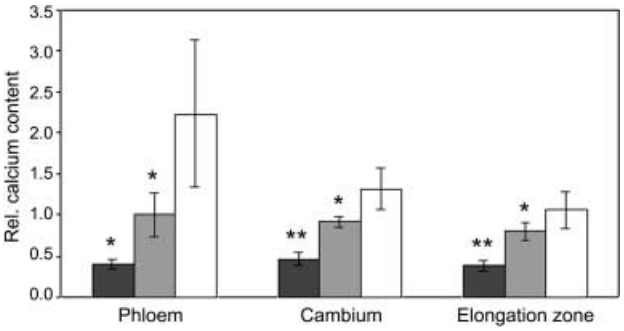


Fig. 2 Energy-dispersive X-ray (EDX) analysis of poplar (*Populus tremula* × *Populus tremuloides* clones) stem tissue. Relative calcium concentrations of phloem, cambium and developing xylem under different calcium supplies via the nutrient solution, expressed as peak : background values from five recorded spectra (closed bars, 0 mM; gray bars, 5 mM; open bars, 10 mM). A significant reduction in relative Ca^{2+} concentration along with reducing Ca^{2+} supply was detected in all samples measured ($P = 0.5$). Data are means ± SD, $n = 5$. Asterisks represent statistically significant differences (*t*-test) compared with poplars with the highest (10 mM) Ca^{2+} supply: *, $P < 0.05$; **, $P < 0.001$.

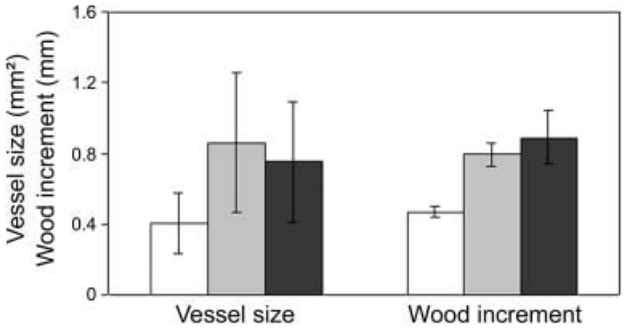


Fig. 3 Effect of different calcium supplies (open bars, 0 mM; gray bars, 5 mM; closed bars, 10 mM) on vessel size and wood increment of poplar (*Populus tremula* × *Populus tremuloides* clones). Both vessel size ($n = 170$) and wood growth ($n = 30$) decreased under calcium starvation. Data are mean values ± SD.

in the average area from 800 μm² under optimal Ca^{2+} supply (5 mM) to 400 μm² under Ca^{2+} deficiency (0 mM) in the nutrient solution. Light microscopy also revealed strong differences in the formation of the cambial zone and the xylem differentiating zone of poplars grown under different calcium supplies. After growing for 6 wk under optimal calcium supply, poplars developed a cambial zone of about seven to nine cambial cells in the radial direction, and secondary cell wall formation did not start until seven to 10 cell layers of differentiation after the cambial zone in the radial direction (Fig. 4a). Using TEM the cambial cells of optimally grown variants were shown to possess one large vacuole, with cytoplasm being confined to a narrow peripheral layer along the thin primary cell wall (Fig. 4b). Those characteristics are known to be typical for active vascular cambium cells of poplar wood (Arend & Fromm, 2003). In contrast, poplars grown under calcium starvation (0.1 mM) formed a limited cambial zone of only three to five cells in the radial direction and were found to have a very restricted cell elongation zone. Secondary cell wall formation started only at a distance of two cell layers in the radial direction of the cambial zone (Fig. 4c). Also, ultrastructural studies revealed a very different cytoplasmic appearance in contrast to the plants grown under optimal Ca^{2+} supply; under calcium starvation, instead of having one large central vacuole, the cytoplasm was very dense with numerous small vacuoles (Fig. 4d). Neither the cambial zone nor its adjacent tissues in poplars grown under excessive Ca^{2+} supply (10 mM) showed any differences compared with the full-strength variation (5 mM Ca^{2+} data not shown).

Fiber length

In measuring the fiber length of macerated libriform fibers of the young poplar wood, a distinct correlation with the calcium supply was observed. Fiber length decreased from 0.52 mm under optimal calcium supply (5 mM) to 0.47 mm under reduced calcium supply (1 mM), and a further decrease to only 0.38 mm was detected with a minimized calcium supply

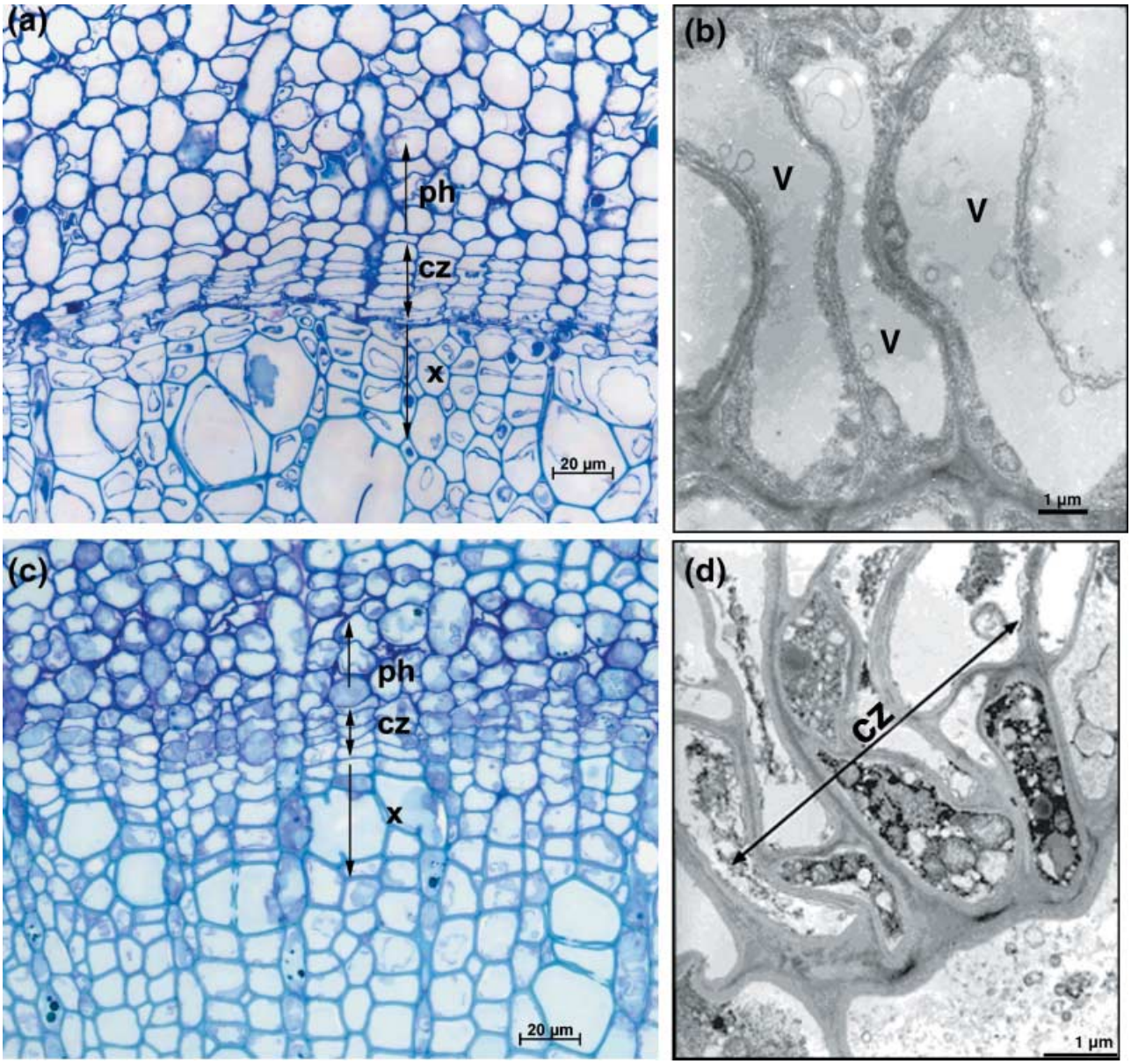


Fig. 4 Effect of different Ca^{2+} supplies on cambial cells and expanding xylem cells. Light microscopy of stem cross-section of poplar (*Populus tremula* × *Populus tremuloides* clones) grown under full-strength Ca^{2+} supply (5 mM, a) in comparison with reduced Ca^{2+} supply (0.1 mM, c) revealed that under Ca^{2+}-limiting conditions, cambial and xylem differentiation zones lack two to three cell layers in the radial direction. Secondary cell wall formation starts closer to the cambial zone. Transmission electron microscopy (TEM) analysis on cross-sections showed the cambial cells to be filled with a dense cytoplasm under calcium starvation (d), whereas cambial initials under full-strength Ca^{2+} supply showed a large vacuole (b), as is typical for active vascular cambium. cz, cambial zone; ph, phloem; x, xylem differentiation zone; v, vacuole.

(0.1 mM; Fig. 5). The mean values of the 120 measurements per nutrient variation were significantly different ($P < 0.001$).

FTIR spectroscopy

To compare FTIR spectra of poplar wood grown under different Ca^{2+} supplies, we chose the fingerprint region in the wavenumber range between 1800 and 800 cm^{-1}. In this close-up, the reduction of the methoxy groups at wavenumber 1325 cm^{-1} is shown in comparison to the aromatic skeletal vibrations of lignin at wavenumber 1505 cm^{-1} (Fig. 6a). Wavenumber 1325 cm^{-1} is that representing the C-O vibration of aryl-alkyl-ether, namely methoxy groups of S-lignin, for which a reduction in the wood cell walls of poplars grown under Ca^{2+} deficiency was detected. Moreover, other absorption bands also showed a lower intensity, for example typical bands of acetyl groups, such as wavenumbers 1740 cm^{-1} (C = O-stretch), 1240 cm^{-1} (C–O–stretch) and 1380 cm^{-1} (CH-deformations).

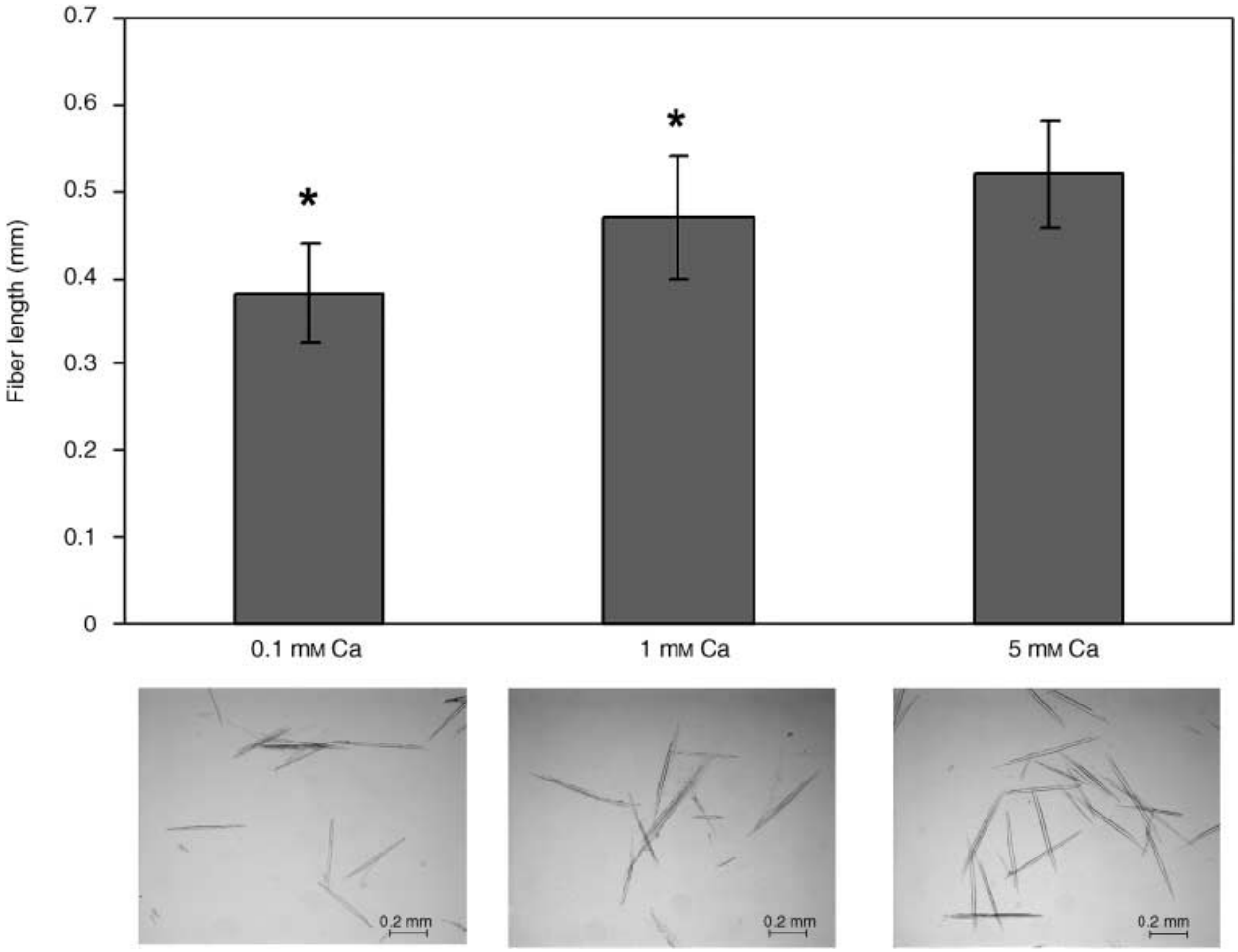


Fig. 5 Histometric measurements of fiber length of poplar (*Populus tremula* × *Populus tremuloides* clones). Along with a decrease in Ca^{2+} in the nutrient solution, the length of young libriform fibers is decreasing; mean values differ significantly. Data are mean values ± SD, n † 120. Asterisks represent statistically significant differences (t-test) compared with poplars with optimal (5 mM) Ca^{2+} supply: *, $P < 0.001$.

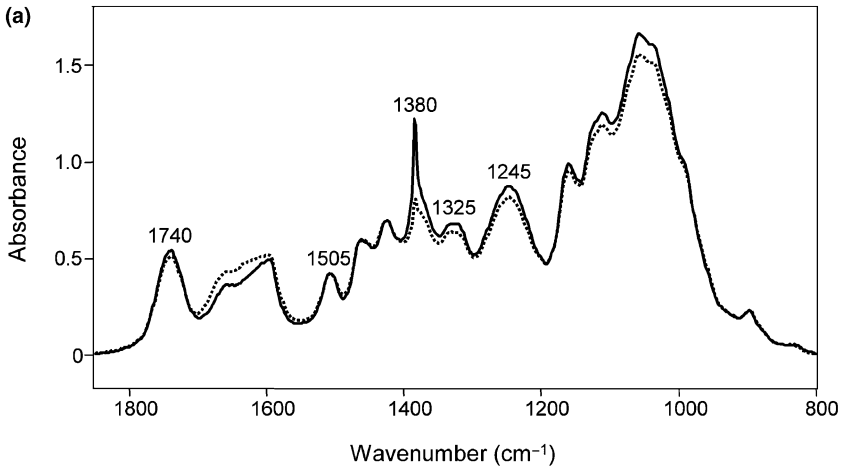
We observed Ca^{2+}-dependent changes not only in the cell walls of the wood, but also in those of the bark, with a distinct reduction in the absorption rate under Ca^{2+} starvation. Remarkably, a significant reduction of wavenumber 1640 cm^{-1} occurred under Ca^{2+} deficiency in comparison to the control plants (Fig. 6b). For lignin this absorption band represents the conjugated C = O stretch vibrations (e.g. in aryl ketones).

Sugar analysis

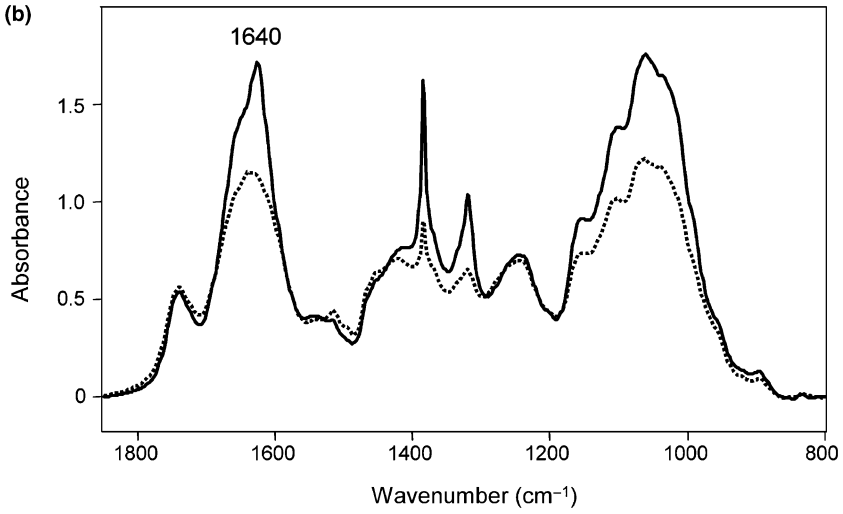
High-performance liquid chromatography analysis of sugars in the plants under different growth conditions showed that physiological processes in the leaves, bark, roots and wood are also affected by calcium. Under Ca^{2+} deficiency (0.1 mM) the concentration of sugars (Glu, Fru, Suc) increased greatly in the leaves, while a reduction occurred in the bark in comparison to both reduced (1 mM) and optimum (5 mM) Ca^{2+} supplies (Fig. 7a). These results confirm earlier findings of Schulte-Baukloh & Fromm (1993), who showed a Ca^{2+} dependency of assimilate translocation by autoradiography in poplar. Since there was also a large increase in the starch concentration of the leaves under minimized Ca^{2+} supply (0.1 mM, Fig. 7b), the loading process of sucrose into sieve elements seems to be partly inhibited. However, since starch also increased in the bark under Ca^{2+} deficiency (Fig. 7b), a direct effect of calcium on the biosynthesis of starch cannot be excluded. Analyzing individual concentrations of glucose, fructose and sucrose in the different tissues, we found much higher amounts of sucrose than of glucose and fructose in the bark and in the leaves of all variants (Fig. 8). Similarly, under Ca^{2+} deficiency, sucrose appears to be the main sugar in all tissues (Fig. 8).

Discussion

The aim of our study was to analyze the effect of calcium nutrition on the chemical and anatomical aspects of wood formation. Using light and electron microscopy as well as FTIR spectroscopy, EDXA and HPLC, we found changes in both



	Absorption height 1245 cm^{-1}	Absorption height 1330 cm^{-1}	Absorption height 1380 cm^{-1}	Absorption height 1740 cm^{-1}
0 mM	0.815 ± 0.021	0.649 ± 0.018	0.812 ± 0.047	0.511 ± 0.018
5 mM	0.875 ± 0.028	0.680 ± 0.025	1.222 ± 0.059	0.540 ± 0.020



	Absorption height 1245 cm^{-1}	Absorption height 1330 cm^{-1}	Absorption height 1380 cm^{-1}	Absorption height 1640 cm^{-1}
0 mM	0.698 ± 0.014	0.653 ± 0.010	0.903 ± 0.016	1.155 ± 0.009
5 mM	0.729 ± 0.017	1.039 ± 0.018	1.621 ± 0.072	1.713 ± 0.030

Fig. 6 Fourier transform infrared (FTIR) spectra of poplar (*Populus tremula* × *Populus tremuloides* clones) wood cells (a) and bark cells (b) grown under different Ca^{2+} nutrition conditions (dotted line, 0 mM; solid line, 5 mM calcium; $n = 5$). Under calcium starvation, a reduction of methoxy groups of S-lignin (wavenumber 1325 cm^{-1}) and typical bands of acetyl groups (wavenumbers 1740, 1240 and 1380 cm^{-1}) became obvious in xylem cell walls. Under the same nutrient conditions, in the bark tissue, a large decrease of conjugated C = O stretch vibrations and aldehyde was observed under Ca^{2+} stress compared with full-strength Ca^{2+} supply.

the anatomical and chemical structure of stem tissue cells. FTIR spectroscopy revealed a reduction of syringyl-units (S-units) of lignin polymers under calcium starvation. Since the ratio of S- and G-units is known to be rather stable in poplar (Hu *et al.*, 1999) this result suggests a general reduction of lignin concentration in poplars grown under calcium deficiency compared with optimal calcium supply. Even though its exact effects on the pathway of lignin biosynthesis are still not known, our results confirm earlier work of Eklund & Eliasson (1990) and Westermark (1982), who found a dependency of lignin biosynthesis on calcium supply in trees. One way of interpreting these findings is based on apoplasic peroxidases.

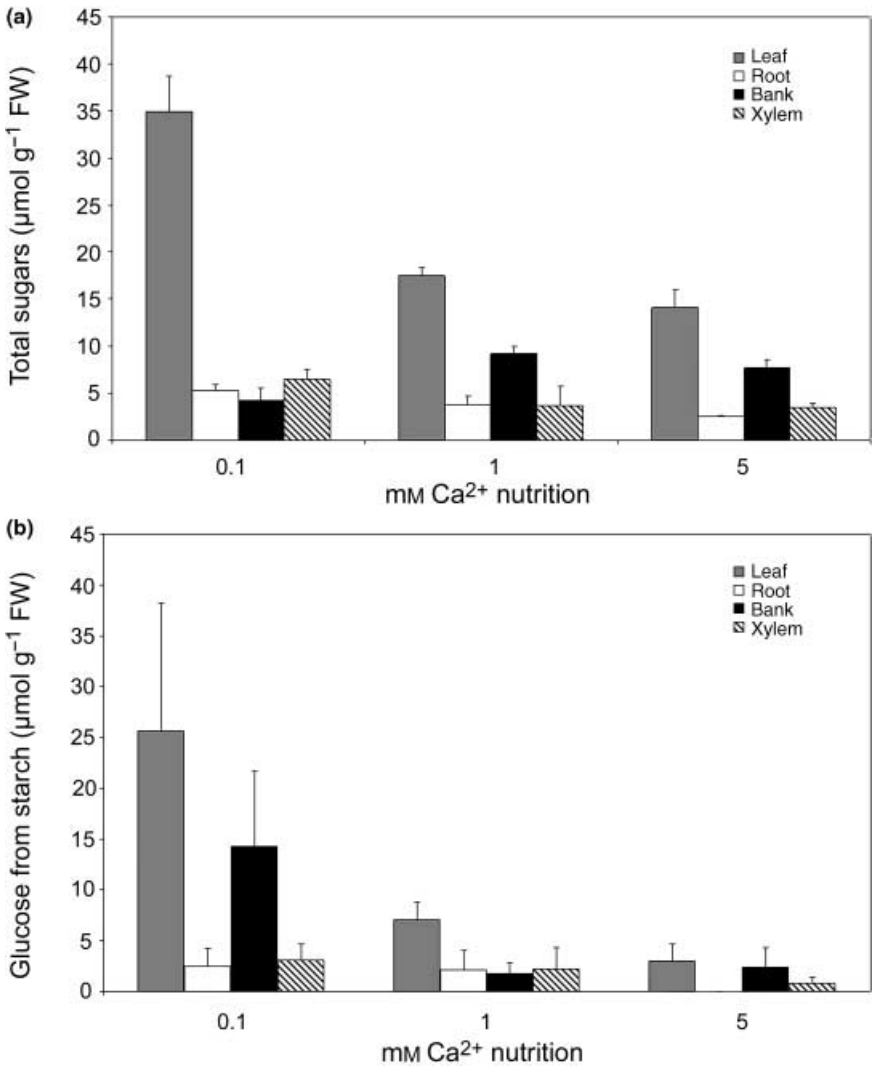


Fig. 7 Tissue-specific analyses of mono- and disaccharides (a) and starch (b) of poplar (*Populus tremula* × *Populus tremuloides* clones) grown under different Ca^{2+} nutrition conditions. Data are means ±SD, $n = 5$. (a) Mono- and disaccharides detected via HPLC analysis include glucose, fructose and sucrose. (b) Starch was hydrolyzed and resulting glucose units were detected in the photometer.

Several apoplastic peroxidases from zucchini and horseradish are known to bind pectin in their Ca^{2+}-induced conformation (Penel & Greppin, 1996). Since the middle lamella and the cell corners are rich in Ca^{2+} pectate (Carpita & Gibeaut, 1993) and are the first sites to be lignified, Ca^{2+}-pectate-bound peroxidases may play a role in the spatial control of lignin deposition, and changes in Ca^{2+} concentration may modulate the location of these peroxidases (Carpin *et al.*, 2001; Boerjan *et al.*, 2003) and therefore lignin biosynthesis.

FTIR spectroscopy conducted on xylem cell walls revealed changes in functional groups other than the lignin polymer, namely carbohydrates. In comparison to growth under full-strength calcium supply, the absorption band of wavenumbers 1300–900 cm^{-1}, representing functional groups of carbohydrates (Williams & Fleming, 1980), is clearly reduced under calcium starvation. FTIR analysis on carrot cell suspensions showed that cell wall sections of elongated cells contain significantly higher amounts of carbohydrates than before elongation (McCann *et al.*, 1993). However, these cells measured arise from isodiametric cells, unlike cambial cells in poplar, and therefore undergo a much more extended elongation.

In trees, differentiating xylem fibers develop from fusiform initials and undergo intrusive fiber tip growth with a species-dependent intensity. In poplar, xylem fiber elongation of about 0.4 mm has been reported (Larson, 1994). However, intrusive tip growth of cambial cells also depends on season and age. It is generally accepted that the younger the tree, the shorter the fibers. Therefore, short fiber lengths measured in this work by image analysis are the result of the (young) age of the poplars and this is the case for all the nutrition treatments. Measuring 120 fibers per variation we detected a significant reduction in fiber length in correlation with a reduction of calcium supply in the nutrient solution. This indicates a significant influence of calcium on xylem fiber elongation. The pectin matrix of growing cell walls may affect cell growth via controlling wall porosity, which depends on the availability of calcium (Carpita & McCann, 2000). Thus, a reduction of Ca^{2+} in the cell wall can affect cell elongation. We

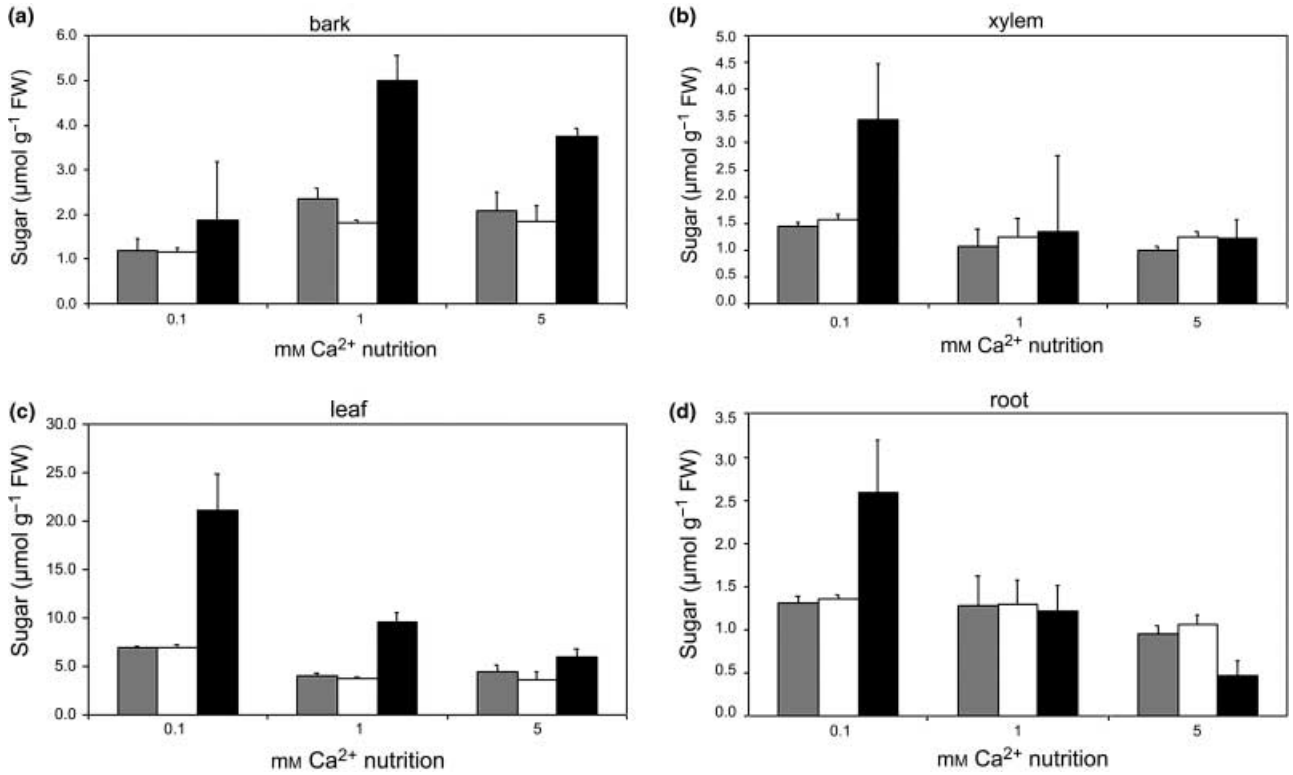


Fig. 8 Tissue-specific analyses of glucose, fructose and sucrose of poplar (*Populus tremula* × *Populus tremuloides* clones) grown under different Ca^{2+} nutrition conditions. (a) Bark, (b) xylem, (c) leaf, (d) root. Data are means + SD, $n = 5$. Gray bars, glucose; open bars, fructose; closed bars, sucrose. FW, fresh weight.

also detected a reduction of vessel diameter, which was another impact of calcium deficiency on wood formation. Vessel size appears to be the most important aspect of water transport, since transport efficiency improves with rising diameter, but the risk of damage from cavitation of the water column increases at the same time (Aloni, 1987). In the present study, the observed reduction in vessel diameter might either be caused by a reduced leaf area when plants were grown under calcium starvation and hence reduced transpiration rate, or generally reflect a stress situation caused by lack of Ca^{2+} nutrition. The latter hypothesis would be consistent with our observation of a narrowed cambial division zone under limited calcium supply. Similar results were detected in poplar when grown with a limited supply of potassium (Wind *et al.*, 2004). In that case, the authors observed a reduction in the xylem differentiation zone along with secondary cell wall formation close to the cambial zone under potassium limitation. In addition, in the present study TEM analysis revealed a dense cytoplasm under calcium-limiting conditions, compared with the highly vacuolated cambial initials of the poplars grown under optimal calcium supply, and hence points to greatly reduced cambial activity. Similar patterns can be found in the ultrastructure of dormant cambial cells (Krabel, 2000; Arend & Fromm, 2003). Since calcium stress seems to have a similar influence on the ultrastructure of the cambium, a reduction of the radial vessel area could be caused by reduced cambial vacuolation.

Finally, the observed decrease of wood increment caused by calcium deficiency was an important finding of the present study. Previously, Schulte-Baukloh & Fromm (1993) observed by microautoradiography that poplars grown under calcium deficiency showed a reduced phloem loading rate in the leaves as well as decreased phloem unloading of photoassimilates into the cambial tissue of the stem. To prove the hypothesis that cambial cells are not sufficiently provided with carbon under calcium stress, sugar analysis showed that, specifically in the bark, the concentrations of glucose, sucrose and fructose decreased under Ca^{2+} deficiency (Fig. 8a). However, all sugars increased in the xylem during Ca^{2+} deficiency (Fig. 8b), indicating that the structural and chemical changes in Ca^{2+}-deficient poplars are not caused by a lack of carbon. Therefore, Ca^{2+} seems to have a direct effect on wood structure and cell wall chemistry.

Taken together, the results shown here indicate a possible regulatory role of Ca^{2+} during wood formation; however, the molecular control points that mediate and facilitate the effect of calcium on xylem development are far from clear. For a further disclosure of the role of calcium in xylogenesis and assimilate transport, further molecular studies on Ca^{2+} channels and receptors in the xylem are planned in combination with immunocytological tools.

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