Plant Physiol. (1994) 106: 53–60

Apoplastic Peroxidases and Lignification in Needles of Norway Spruce (*Picea abies* L.)[1]

Andrea Polle*, Tilman Otter, and Friederike Seifert

Institut für Forstbotanik und Baumphysiologie, Professur für Baumphysiologie, Albert-Ludwigs-Universität Freiburg, Am Flughafen 17, D-79085 Freiburg, Germany (A.P., T.O.); and Fraunhofer Institut für Atmosphärische Umweltforschung, Kreuzeckbahnstrasse 19, D-82467 Garmisch-Partenkirchen, Germany (F.S.)

The objective of the present study was to investigate the correlation of soluble apoplastic peroxidase activity with lignification in needles of field-grown Norway spruce (*Picea abies* L.) trees. Apoplastic peroxidases (EC 1.11.1.7) were obtained by vacuum infiltration of needles. The lignin content of isolated cell walls was determined by the acetyl bromide method. Accumulation of lignin and seasonal variations of apoplastic peroxidase activities were studied in the first year of needle development. The major phase of lignification started after bud break and was terminated about 4 weeks later. This phase correlated with a transient increase in apoplastic guaiacol and coniferyl alcohol peroxidase activity. NADH oxidase activity, which is thought to sustain peroxidase activity by production of H_2O_2, peaked sharply after bud break and decreased during the lignification period. Histochemical localization of peroxidase with guaiacol indicated that high activities were present in lignifying cell walls. In mature needles, lignin was localized in walls of most needle tissues including mesophyll cells, and corresponded to 80 to 130 μmol lignin monomers/g needle dry weight. Isoelectric focusing of apoplastic washing fluids and activity staining with guaiacol showed the presence of strongly alkaline peroxidases (isoelectric point ≥ 9) in all developmental stages investigated. New isozymes with isoelectric points of 7.1 and 8.1 appeared during the major phase of lignification. These isozymes disappeared after lignification was terminated. A strong increase in peroxidase activity in autumn was associated with the appearance of acidic peroxidases (isoelectric point ≤ 3). These results suggest that soluble alkaline apoplastic peroxidases participate in lignin formation. Soluble acidic apoplastic peroxidases were apparently unrelated to developmentally regulated lignification in spruce needles.

Lignin is an aromatic polymer composed mainly of cinnamyl alcohols such as *p*-coumaryl, coniferyl, and sinapyl alcohol (Lewis and Yamamoto, 1990). The incorporation of lignin into cell walls results in structural rigidity and durability of plant tissues and, therefore, is important in evergreen leaves that persist for many years. In mature needles of Norway spruce (*Picea abies* L.) lignin accounts for about 5 to 10% of dry matter (Miksche and Yasuda, 1977).

The enzymatic reactions involved in the last step of lignification are not yet fully understood (Lewis and Yamamoto, 1990; O'Malley et al., 1993), but it is generally believed that monomeric precursors of lignin are enzymatically dehydrogenated in the cell wall to phenoxy radicals. These radicals polymerize spontaneously, yielding a complex net of cross-links among monolignols, proteins, and polysaccharides. Peroxidases (donor:H_2O_2 oxidoreductase, EC 1.11.1.7) have been implicated in these cross-linking reactions for two reasons: (a) they have been localized in cell walls of lignifying tissues, and (b) they catalyze the production of lignin-like products in vitro (Lewis and Yamamoto, 1990). However, conflicting results have been reported as to whether specific anionic (acidic) or cationic (alkaline) peroxidase isozymes are involved in lignification. For example, an anionic peroxidase with affinity for syringaldazine was present in cell walls of poplar stems during the differentiation of xylem and phloem vessels but not in dormant tissues (Goldberg et al., 1983; Imberty et al., 1985). However, a direct correlation of peroxidase activity with lignin content was not established in these studies. Cell walls of tobacco contained an acidic peroxidase with affinity for coniferyl alcohol (Mäder, 1976; Mäder et al., 1977). Overexpression of this isozyme in the apoplastic space of transgenic tobacco and tomato plants resulted in elevated lignin content (Lagrimini, 1991; Lagrimini et al., 1993).

In contrast to these results, differentiation of tracheary elements from mesophyll cells of *Zinnia elegans* was associated with changes in wall-associated cationic and soluble peroxidases (Fukuda and Komamine, 1982; Church and Galston, 1988; Sato et al., 1993). An accumulation of lignin-like material in the culture medium of castor bean cells that had been challenged with pectic elicitors was positively correlated with the appearance of new extracellular, cationic peroxidases (Bruce and West, 1989). However, an increase in the activities of acidic peroxidases was also found (Bruce and West, 1989). Nonlignified peach mesocarp contained only acidic peroxidases, whereas lignifying endocarp also contained alkaline peroxidases (Abeles and Biles, 1991). These examples show that the specific peroxidases involved in vivo in normal developmentally regulated lignification have not been identified unequivocally.

Lignification by peroxidases requires H_2O_2 as co-substrate. H_2O_2 has been localized histochemically in lignifying tissue (Olson and Varner, 1993) and may be formed in situ by

[1] This work was conducted in the Forschungsschwerpunkt "Physiologie der Bäume" with financial support from the Deutsche Forschungsgemeinschaft.

* Corresponding author; fax 49–761–808349.

Abbreviation: pI, isoelectric point.

peroxidases acting as NADH oxidases (Gross et al., 1977). Cationic peroxidases are apparently more efficient than anionic peroxidases in catalyzing NADH oxidation, whereas anionic peroxidases have higher affinity for coniferyl alcohol oxidation than cationic peroxidases (Mäder et al., 1986). Therefore, it has been speculated that cationic and anionic peroxidases interact in lignification by production and consumption of H_2O_2 required for formation of phenoxy radicals (Mäder et al., 1986). Little is known about this system in lignifying tissues. In flax fibers, lignification was positively correlated with an increase in cell-wall-associated NADH oxidase and peroxidase activities (McDougall, 1991, 1992). However, peroxidase activities were not determined with natural substrates, and it was not possible to attribute specific roles to different isozymes.

Spruce needles contain strongly acidic (pI ≤ 3) and strongly alkaline (pI ≥ 9) apoplastic peroxidases that catalyze both H_2O_2 production by oxidation of NADH and oxidation of coniferyl alcohol by consumption of H_2O_2 (Polle and Chakrabarti, 1994). Because these peroxidase activities have been investigated only in mature, fully lignified needles, their role in the phase of active lignification is unknown. To investigate whether cationic or anionic peroxidase activities are correlated with normal developmentally regulated lignification in spruce, soluble apoplastic peroxidase activities, their isozyme patterns, and lignin accumulation were analyzed during needle maturation. The activities of apoplastic peroxidases were determined with substrates important for lignification, i.e. coniferyl alcohol, guaiacol, syringaldazine, and NADH. Biochemical and histochemical methods were employed to study the temporal and spatial correlation of peroxidase activities with lignin production.

MATERIALS AND METHODS

Plant Material and Field Sites

Needles were obtained from about 60- to 100-year-old, healthy Norway spruce (*Picea abies* L. Karst.) trees. Needles were collected in 1991/1992 at the foot of the Katzenstein Mountain (735 m above sea level, 11°4.1′ E, 47°28.7′ N, Bavarian Alps, Bavaria, Germany) and in 1992/1993 on top of the Schauinsland Mountain (1230 m above sea level, 7°54′ E, 47°55′ N, Black Forest, Baden Württemberg, Germany). Both sites are located at similar latitudes about 350 km apart. In the period of needle emergence and maturation from May to October, mean climate and pollution were SO_2 0.6/2.7 ppb, NO_x 2.5/3.9 ppb, O_3 31.1/52.3 ppb, temperature 13.7/10.2°C, and sum of precipitation 658/721 mm at Katzenstein/Schauinsland Mountains, respectively. Nutrient deficiencies were not apparent in needles from Katzenstein or Schauinsland (Polle et al., 1992; Wolfart, 1992). At each site, five spruce trees were sampled. Needles were collected at about 8 AM and transported to the laboratory within 15 to 45 min. Apoplastic washing fluids were immediately extracted. Samples of needles were kept frozen at −80°C until extraction of total needle peroxidase, cell walls, and lignin.

Extraction and Analyses of Peroxidase Activities and Protein

Needles were powdered in liquid nitrogen. Frozen needle powder (400 mg) was added to 20 mL of extraction buffer containing 100 mM KH_2PO_4/K_2HPO_4, pH 7.8, 0.5% Triton X-100, and 2% insoluble PVP. The mixture was homogenized for 1 min and centrifuged for 20 min at 48,000*g*. The supernatant was passed over Sephadex G-25 (PD-10 columns, Pharmacia, Freiburg, Germany) with 100 mM KH_2PO_4/K_2HPO_4, pH 7.8, to remove low mol wt compounds before analyses of enzymatic activities.

Apoplastic washing fluids were prepared by vacuum infiltration of fresh needles. Five grams of needles were thoroughly cut at the base and the tip and then washed with distilled water. The needles were infiltrated at −70 kPa for 5 min. The vacuum was applied and released slowly. To extract soluble and ionically bound peroxidases, 50 mM Mes, pH 6.0, containing 1 M NaCl was used as infiltration buffer. Contamination of apoplastic washing fluids by symplastic compounds was estimated from the presence of glutathione in apoplastic washing fluids. Glutathione concentrations were determined by HPLC as described by Schupp and Rennenberg (1988). Before analyses of protein and enzyme activities, apoplastic washing fluids were passed over Sephadex G-25 (NAP 5 columns, Pharmacia) with 20 mM Mes/KOH (pH 6.5).

Peroxidase activities (EC 1.11.1.7) were determined spectrophotometrically with guaiacol at 436 nm, coniferyl alcohol at 262 nm, syringaldazine at 530 nm, and NADH at 340 nm as described elsewhere (Imberty et al., 1985; Ishida et al., 1987; Pedreno et al., 1989; Polle et al., 1990). Reaction rates in the absence of H_2O_2 were negligible. Enzymatic activities for guaiacol peroxidase, coniferyl alcohol peroxidase, and NADH oxidase were calculated using extinction coefficients of 25.5, 2.2, and 6.33 $mM^{-1}\ cm^{-1}$, respectively. The extinction coefficient of syringaldazine was not available and activity, therefore, is expressed as ΔE/min. Protein was determined with bicinchoninic acid reagent kit (Pierce, Oud-Beijerland, The Netherlands) and BSA as standard.

Apoplastic washing fluids were separated under nondenaturing conditions on a Phastsystem (Pharmacia) by IEF in a pH gradient from 3 to 9 at 15°C (precast IEF gels pH 3 to 9 were from Pharmacia). To localize peroxidase isozymes, gels were incubated in staining buffer (50 mM KH_2PO_4/K_2HPO_4, pH 5.3, 5 mM H_2O_2, 10 mM guaiacol) for 10 min. Cross-sections obtained by hand-cutting from fresh needles were incubated in staining buffer and the localization of peroxidase activity was observed under a light microscope (Zeiss MC-63, Oberkochen, Germany). Staining was not observed in the absence of H_2O_2.

Extraction of Cell Walls and Determination of Lignin

Dry needles (72 h at 80°C) were ground to a fine powder in a micro-dismembrator (Braun, Melsungen, Germany) for 2 min at a frequency of 2000 oscillations min^{-1}. To obtain cell walls, 400 mg of needle powder was stirred for 1 h in 20 mL of 80% methanol and centrifuged for 10 min at 6000 rpm. The pellet was subjected to the following washing steps

according to Strack et al. (1988): 1× (1 M NaCl, 0.5% Triton X-100), 2× distilled water, 2× 100% methanol, 2× 100% acetone (each step in 10 mL, 30 min). The remaining material was called "fraction I" cell walls. After drying, fraction I cell walls represent total cell wall dry mass. The cell walls were subjected to alkaline hydrolysis in 2 M NaOH under N_2 for 1 h. This step serves to solubilize esterified phenolics that would interfere with the lignin assay. The resulting material, called "fraction II," was washed with distilled water (two times), dried at 80°C, and weighed. Fraction II represents de-esterified cell walls. Aliquots of 2.5 mg of fraction II powder were used for lignin determination with acetyl bromide according to Morrison (1972). Standard curves were produced with freshly prepared solutions of coniferyl alcohol and used to calculate the concentration of lignin monomers extracted from fraction II cell walls. Lignin was also detected in cross-sections of spruce needles after staining with phloroglucinol/HCl.

Statistical Analyses

Five individual trees were analyzed, each in three to five replicates at each sampling date. The SD of the mean of these replicates was less than 5%. These means were used to calculate mean values indicated in Figures 1 and 2, c and d ($n = 5$, ±SE).

RESULTS

After bud break, 1.1 ± 0.5% of total guaiacol peroxidase activity from spruce needles was found in apoplastic washing fluids (Fig. 1a). The glutathione content in apoplastic washing fluids, which was determined to assess symplastic contamination, accounted for less than 0.05% of the total glutathione content of needles. In August and September, apoplastic peroxidase increased to 3.3 ± 1.1% of total peroxidase activity, whereas the glutathione content in apoplastic washing fluids accounted for less than 0.03% of total glutathione. Thus, apoplastic peroxidase activity was significantly higher than the level of symplastic contamination.

Guaiacol peroxidase activity in total needle extracts showed no significant variations in the period of time from bud break early in June until August (Fig. 1a). By contrast, apoplastic guaiacol peroxidase activity increased transiently in June regardless of whether the activity was related to fresh weight (Fig. 1a) or dry weight of the needles (Fig. 1b) or to protein content of the apoplastic washing fluids (Fig. 1b). In August, peroxidase activity increased in both total needle extracts and apoplastic washing fluids (Fig. 1a).

To investigate how fluctuations in peroxidase activities were related to lignification, the accumulation of dry matter, cell wall material, and lignin was determined (Fig. 2, a and b). Additionally, apoplastic peroxidase activities were measured with substrates important for lignification, i.e. coniferyl alcohol and NADH (Fig. 2, c and d). The study was conducted for 2 years at two locations to determine whether the observed fluctuations were independent from site-specific parameters such as soil, pollution, and climate.

Lignification started immediately after bud break and was accomplished 4 weeks later (Fig. 2b). In the period of time

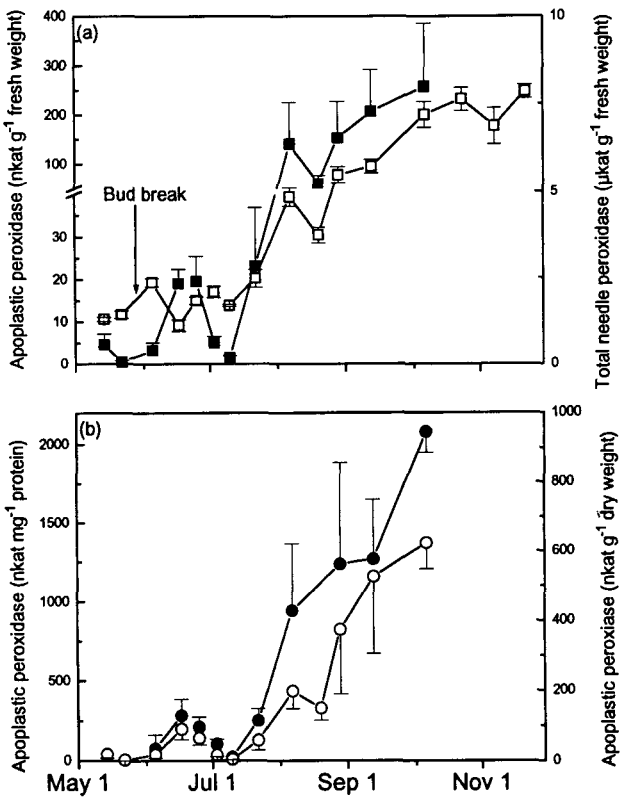

Figure 1. Developmental changes of guaiacol peroxidase activities in spruce needles. a, Peroxidase in apoplastic washing fluids (■) and total needle extracts (□) on the basis fresh weight. b, Apoplastic peroxidase activity on the basis of dry weight (○) and apoplastic protein (●). Data indicate means (±SE, $n = 5$).

following bud break, the lignin content increased 10- to 20-fold (Fig. 2b). The increase in dry mass of de-esterified cell walls (fraction II, Fig. 2a) was similar to the increase in lignin monomers (Fig. 2b). By contrast, total cell wall dry mass (fraction I) changed only 1.2- to 1.4-fold (Fig. 2a). Since dry matter of needles continued to increase after the major phase of lignification was terminated, the amount of lignin per gram of dry matter decreased later in the season (Fig. 2, a and b). However, on the basis of cell walls a small decrease in lignin was also observed. Lignin degradation in healthy tissues appears unlikely. A slow incorporation of other less-absorbing compounds into lignin would also result in an apparent decrease of lignin. This requires further investigation. Maximum lignin contents found in cell walls corresponded to about 300 and 170 µmol lignin monomers extractable/g cell walls at Katzenstein and Schauinsland, respectively.

When lignification started in young needles, the activity of apoplastic coniferyl alcohol peroxidase was high, accounting for about 100 and 200 nkat/g dry weight at Katzenstein and Schauinsland, respectively (Fig. 2c). After lignin accumulation was 50 to 75% complete, apoplastic coniferyl alcohol peroxidase activity started to decrease gradually and showed a minimum when net lignin production was finished. The time course of apoplastic guaiacol peroxidase activity (Fig.

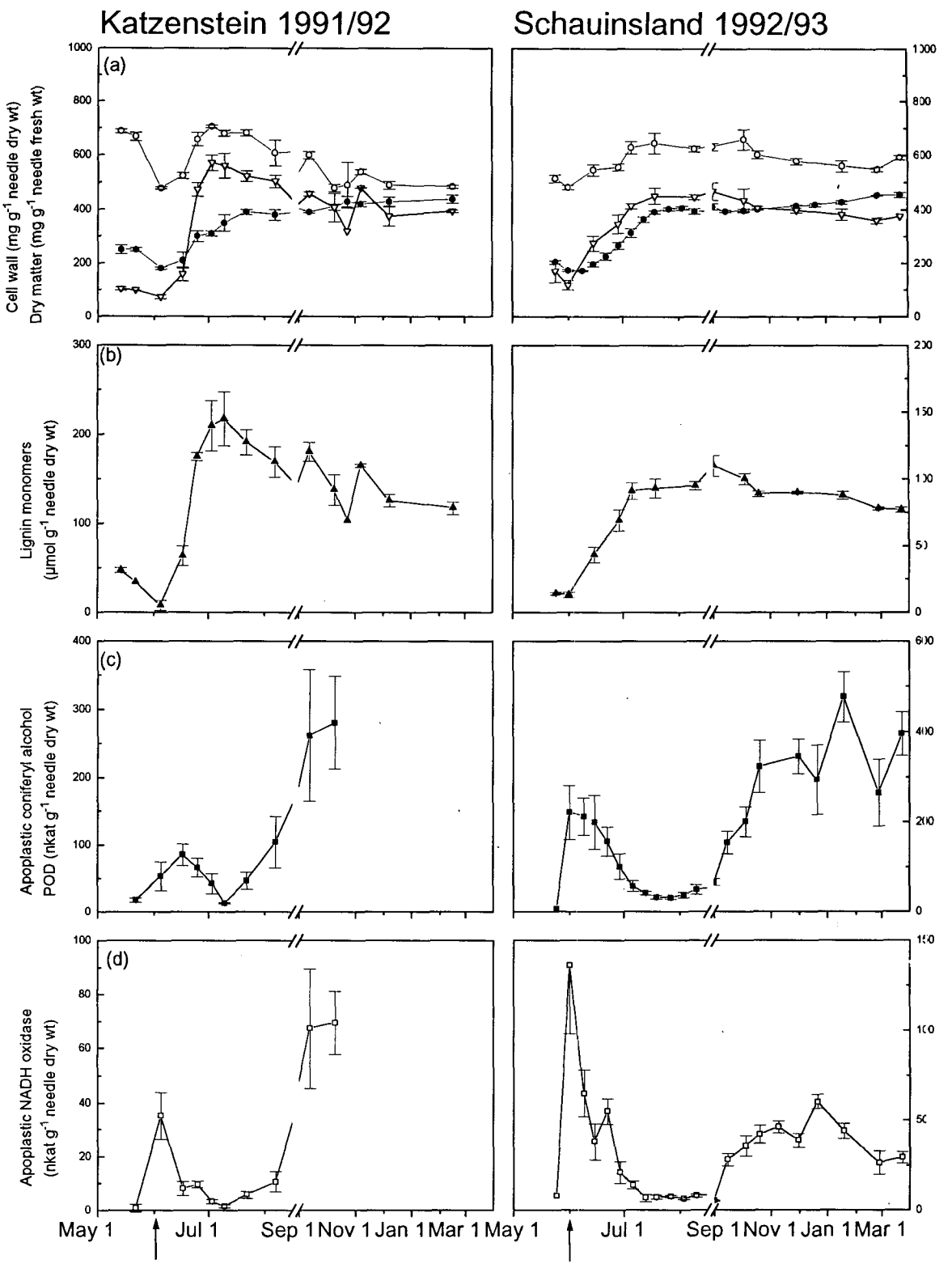

Figure 2. Accumulation of dry matter (a, ●), cell wall material (a, O, fraction I cell walls), and de-esterified cell wall material (a, ▽, fraction II cell walls), lignin monomers (b, ▲), apoplastic coniferyl alcohol peroxidase (c, ■), and apoplastic NADH oxidase activity (d, □). Data indicate means (±SE, $n = 5$ for peroxidase activity determinations, $n = 3$ for cell-wall-related parameters). Data were related to needle dry mass. Lignin monomers were detected after solubilization of de-esterified cell walls (fraction II) by acetyl bromide. The concentration of lignin monomers was calculated using coniferyl alcohol as standard. Arrows at the bottom indicate bud break.

1b) was similar to that of coniferyl alcohol peroxidase activity (Fig. 2c). This was also observed at Schauinsland Mountain (not shown). These observations suggest similar affinities of apoplastic peroxidases for the guaiacyl substrates coniferyl alcohol and guaiacol. By contrast, NADH oxidase activity peaked sharply at bud break and decreased as lignin increased (Fig. 2d). Apoplastic syringaldazine peroxidase activity was not detected above the level of symplastic contamination in the present study. In extracts of mature, 1-year-old needles, syringaldazine peroxidase activity amounted to 10.9 $\Delta E\ min^{-1}\ g^{-1}$ fresh weight, of which 0.0001% was found in apoplastic washing fluids (Polle and Chakrabarti, 1994).

Activities of apoplastic peroxidases with guaiacol, coniferyl alcohol, or NADH as substrates increased again later in the season (Figs. 1 and 2, c and d). The maximum attained in autumn was generally higher than the peak in June. These high peroxidase activities were obviously not associated with lignification and appeared to be maintained over the following years (Table I). The present results indicate that soluble apoplastic peroxidase activity and lignin production were positively correlated because the increase in lignin was high when coniferyl alcohol peroxidase activity was also high. The lignin production rate appeared to decrease as the peroxidase activity decreased. To investigate whether lignification and peroxidase activity also showed spatial coincidence, cross-sections of needles were examined by light microscopy after staining for peroxidase activity by guaiacol and for lignin by acidic phloroglucinol. Syringaldazine was checked initially as a peroxidase substrate but gave no reasonable color development, in contrast to that reported previously by Harkin and Obst (1973). Four characteristic developmental stages in the major phase of lignification are illustrated in Figure 3. A major portion of peroxidase activity indicated by brown staining was always associated with the vascular system (Fig. 3, A, C, E, and F). At the first stage (Fig. 3A), young needles emerging from the buds were investigated. The young needles had approximately two-thirds of their full length of 20 mm and contained 260 μg Chl/g fresh weight. In the next stages analyzed, Chl increased from 630, to 713, to 823 ± 84 μg Chl/g fresh weight, whereas needle length did not increase further.

In young needles emerging from buds, radial walls of the endodermis (Fig. 3A, arrow), xylem tracheids, transfusion tracheids, and epidermal cells, especially in stomatal regions, stained strongly with guaiacol (Fig. 3A). These walls were lignified at the next developmental stage investigated (Fig. 3D), whereas initially, lignin was detected only in walls of stomatal cells and in xylem tracheids (Fig. 3B). At the second stage, endodermis and hypodermis, which were the next to be lignified (Fig. 3F), showed high peroxidase activity (Fig. 3C). At the third developmental stage, peroxidase activity was still generally high in the vascular system except for in xylem tracheids (Fig. 3E), which were fully differentiated at this time (Fig. 3F). It seemed remarkable that peroxidase activity could be located in walls of mesophyll cells (Fig. 3E, arrow), but at the fourth stage, which was investigated at the beginning of July, walls of mesophyll cells were also lignified (Fig. 3H). At this time biochemical investigations indicated a minimum in apoplastic peroxidase activity and a maximum in lignin content of the needles (Figs. 1 and 2, c and d). According to histochemical studies, lignification continued into autumn (not shown). Since few cell types were concerned, i.e. mainly walls of resin ducts and sclerenchymatic cells in the central cylinder, the contribution of these tissues to total lignin on a weight basis was probably not important and, therefore, not detected in biochemical studies (Fig. 2c). The observed increase in peroxidase activity in autumn was associated with high peroxidase staining in hypodermis and the vascular system, especially in the phloem, but not in xylem tracheids, which showed no or little peroxidase activity (not shown).

To investigate whether the transient increase in apoplastic peroxidase activity observed in the phase of maximum lignin accumulation was associated with specific peroxidase isozymes, apoplastic washing fluids were subjected to IEF in a pH gradient from 3 to 9. Figure 4 shows that apoplastic washing fluids from buds and young needles of both Katzenstein and Schauinsland Mountains initially contained only strongly alkaline peroxidases (pI ≥ 9). Since peroxidase activity focused at the end of the gel, it remains unknown whether the observed band contained more than one isozyme and what the true pI value of alkaline peroxidase(s) was. In the major phase of lignification in June, new isozymes with slightly alkaline to neutral pI values appeared (Fig. 4, a and b). At Katzenstein, the pI values of these new peroxidase isozymes were 8.1, 7.6 (weak), and 7.0 (Fig. 4a, lanes 6–17, 6–25, 7–03, and 7–18). New isozymes were also found in needles of spruce trees grown at Schauinsland (Fig. 4b, lanes 6–15, 6–22, and 7–06), which had, within the accuracy of the method (±0.1), the same pI values as peroxidases from Katzenstein, i.e. 8.1 and 7.1. The neutral to slightly alkaline peroxidase isozymes disappeared when lignin accumulation was terminated. The increase in apoplastic peroxidase activity in autumn was associated with an increase in strongly alkaline (pI ≥ 9) and strongly acidic (pI ≤ 3) peroxidases (Fig. 4, a and b). The presence of these isoperoxidases in apoplastic washing fluids of mature spruce needles has been reported previously (Polle and Chakrabarti, 1994; Polle and Junkermann, 1994).

Table I. *Apoplastic peroxidase activities in seven needle age classes of field-grown spruce trees*

Samples were collected in autumn 1992 from five individual trees. Figures in the table indicate means (±SD, $n = 5$).

Needle Age	Peroxidase Activity		
	Guaiacol	Coniferyl alcohol	NADH
years	*nkat g^{-1} dry wt*		
1	553 ± 107	533 ± 67	60 ± 9
3	952 ± 185	664 ± 111	63 ± 13
5	669 ± 210	765 ± 91	79 ± 16
7	816 ± 516	446 ± 216	92 ± 29

DISCUSSION

The present study shows that peroxidases of apoplastic washing fluids from lignifying needles oxidized the guaiacyl substrates guaiacol and coniferyl alcohol. Guaiacyl residues

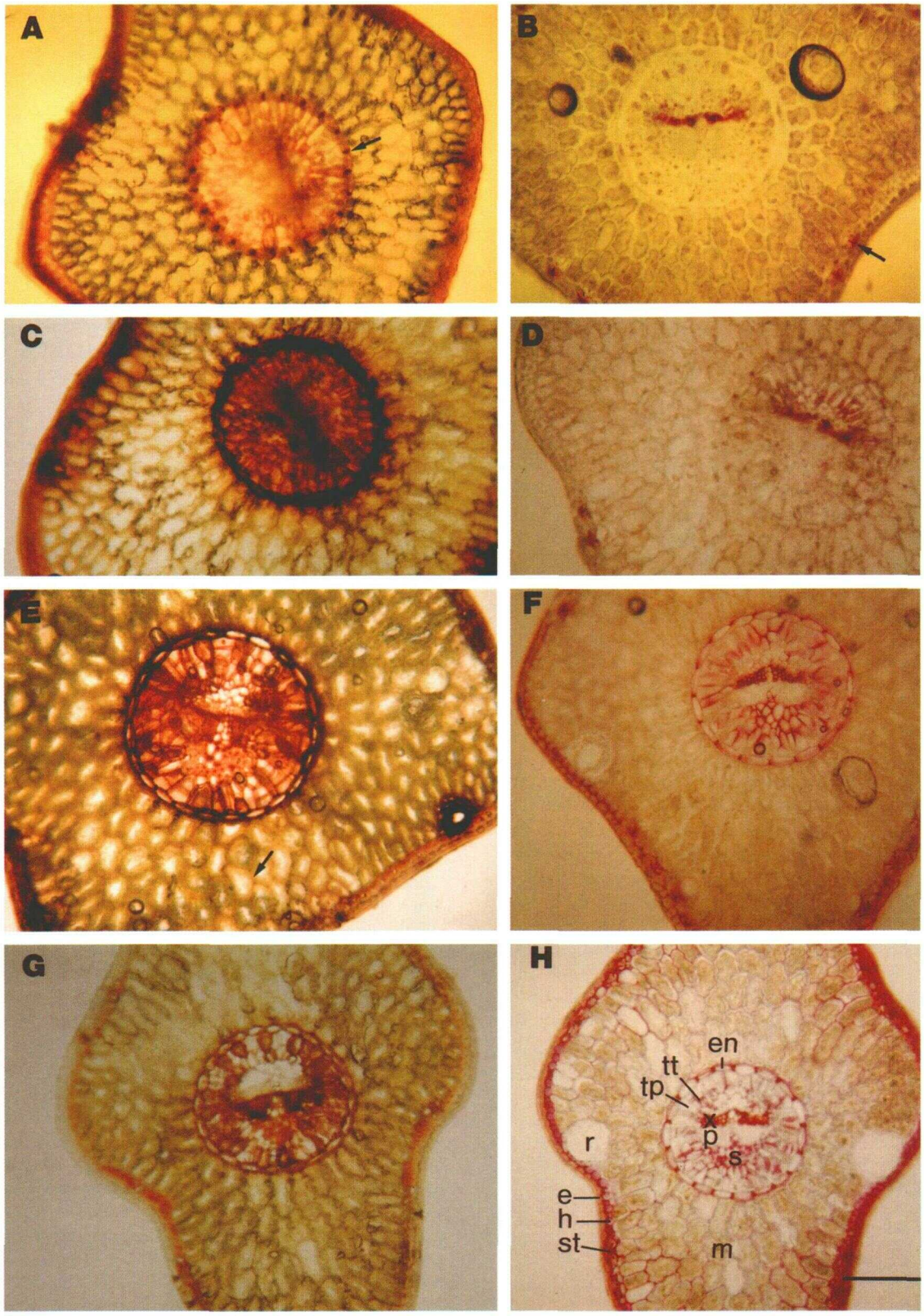

Figure 3. Localization of guaiacol peroxidase activity (A, C, E, and G) and lignin (B, D, F, and H) in four developmental stages in the major phase of lignification. Samples were collected in 1991 at Katzenstein Mountain at the following dates: June 5 (A and B), June 17 (C and D), June 25 (E and F), and July 3 (G and H). Cross-sections were hand-cut from fresh needles and stained as described in "Materials and Methods." e, Epidermis; h, hypodermis, st, stomata; m, mesophyll, r, resin duct; en, endodermis; tt, transfusion tracheids; tp, transfusion parenchyma; p, phloem, x, xylem; s, sclerenchymatic cells. Arrows, See text. The bar corresponds to a length of 200 μm.

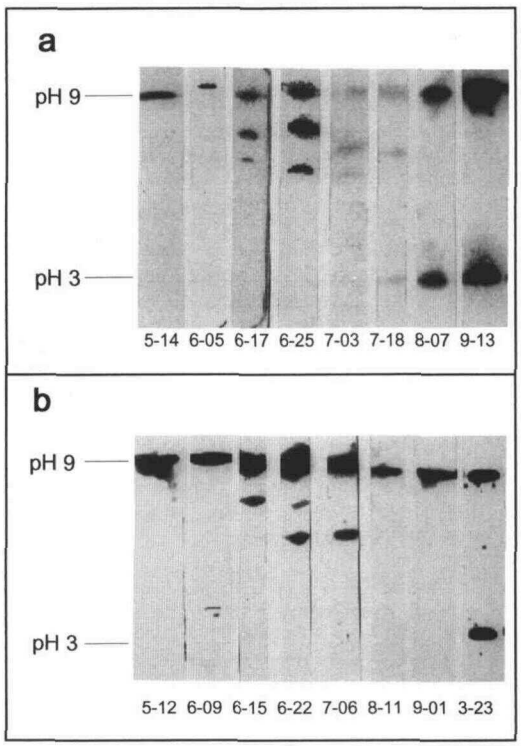

Figure 4. IEF of apoplastic washing fluids in a pH gradient from 3 to 9 and activity staining of guaiacol peroxidases during maturation of needles collected at Katzenstein (a) and Schauinsland (b). Sampling dates are indicated below corresponding lanes. Volumes of 12 μL of desalted, apoplastic washing fluid were applied to each lane. A typical example is shown for one tree at each location.

are typical components of gymnosperm woods. The composition of needle lignin is similar to softwood lignin, containing predominately guaiacyl units and lacking dimeric aromatic esters typical of syringyl-guaiacyl lignin of angiosperm wood (Miksche and Yasuda, 1977). The apoplastic peroxidases had only negligible activity with syringaldazine, which is widely used as an analog of sinapyl alcohol (Lewis and Yamamoto, 1990). Therefore, this compound may not be an important substrate of lignifying peroxidases in conifers.

In the present study guaiacol was used for histochemical localization of peroxidase activity. By a combination of histochemical and biochemical methods, it was shown that guaiacol and coniferyl alcohol peroxidase activities were temporally and spatially correlated with lignin production in spruce needles. Cinnamyl alcohol dehydrogenase from spruce needles also shows a transient increase after bud break (Galliano et al., 1993), suggesting a co-regulation of enzymes involved in production and polymerization of monolignols. This view is also supported by recent results obtained from lignifying bean hypocotyls (Smith et al., 1994). In those tissues, cationic peroxidases were localized in the xylem at sites of secondary thickening by immunogold staining (Smith et al., 1994). At the same time, enzymes involved in the supply with the phenylpropanoids Phe ammonia-lyase and cinnamate-4-hydroxylase were localized in the cytosol and in Golgi vesicles, respectively (Smith et al., 1994).

The present data show that apoplastic washing fluids from lignifying needles contained new isozymes that were present only during lignification (Fig. 4). The pI values of apoplastic peroxidases from young, developing needles were neutral to alkaline (Fig. 4). Acidic, apoplastic peroxidases were found only in mature needles (Fig. 4), and their presence was apparently unrelated to lignification. This was unexpected because acidic peroxidases have previously been associated with cinnamyl alcohol oxidation, whereas alkaline peroxidases were thought to be involved in defense reactions (Gaspar et al., 1985) or in the production of H_2O_2 as co-substrate for lignification (Mäder and Amberg-Fischer, 1982). In the present study, apoplastic NADH oxidase activity showed no clear correlation with lignification (Fig. 2d). Therefore, the source of H_2O_2 for peroxidase activity in the process of lignification remains uncertain.

In contrast to our results, lignifying fibers of poplar wood in spring contained higher activities of anionic (acidic) peroxidases than dormant tissues in winter (Imberty et al., 1985). However, the degree of lignification of these tissues was not determined. McDougall (1992) determined the percentage of lignification in flax by counting the number of lignified fibers. He found that wall-associated peroxidase and NADH oxidase activities increased as the degree of lignification increased. NADH oxidase and peroxidase activities showed similar time dependencies, but the appearance of specific isozymes was not observed (McDougall, 1991, 1992). It was not investigated whether peroxidase activities decreased after termination of lignification.

The determination of "total" lignin is difficult because of the complexity and heterogeneity of this cell wall component. Since different techniques for lignin determination may give conflicting results, Lewis and Yamamoto (1990) suggested that the presence of lignin should be confirmed by using at least two independent methods. Both of the two methods employed in the present study, i.e. phoroglucinol staining and acetyl bromide, revealed similar time courses for lignin accumulation. The observation that lignin was present in walls of mesophyll cells was unexpected. However, green suspension cultures of spruce cells also deposited lignin-like material into their culture medium (Simola et al., 1992).

Histochemical localization of peroxidase activity and lignin content showed that high peroxidase activity was already present before lignin polymers could be detected (Fig. 3). A small delay between the appearance of lignin and activities of apoplastic peroxidases can also be inferred from Figure 2, b and c. A difference in time between the detection of lignin and peroxidase activities may have been caused by difficulties in detecting growing, incomplete lignin structures. It is known that alkaline hydrolysis of cell walls removes phenolics such as coumaric acid and ferulic acid that are covalently attached to walls but apparently not incorporated into the three-dimensional lignin network (Strack et al., 1988). We suspect that smaller precursor units of lignin might have been removed by the method of lignin isolation used in the present paper.

Recently, laccase and laccase-like activity were associated with lignification (O'Malley et al., 1993). For instance, in stems of conifers a cell-wall-bound oxidase was detected that utilized O_2 to oxidize coniferyl alcohol to a lignin-like product

(Savidge and Udagama-Randeniya, 1992). This coniferyl alcohol oxidase activity increased at the initiation and disappeared with the termination of lignification (Savidge and Udagama-Randeniya, 1992). Thus, it had the same time course in relation to lignification as the apoplastic peroxidase activity in needles investigated in the present study. Considering both of these observations, it seems as if both laccase and peroxidase contribute to lignification in spruce as originally proposed by Freudenberg (1968). The presence of laccase activity in spruce needles and a causal relationship between any of these enzymes and lignin production remains to be demonstrated.

ACKNOWLEDGMENTS

We are grateful Dr. R. Sladkovic (Fraunhofer Institut für Atmosphärische Umweltforschung, Garmisch-Partenkirchen) and K. Uhse (Umweltbundesamt, Offenbach) for providing data on climate and pollution at Katzenstein and Schauinsland Mountains, respectively. We thank Prof. Dr. H. Rennenberg for critical reading of the manuscript.

Received March 7, 1994; accepted May 23, 1994.
Copyright Clearance Center: 0032-0889/94/106/0053/08.

LITERATURE CITED

Abeles F, Biles C (1991) Characterization of peroxidases in lignifying peach fruit endocarp. Plant Physiol **95:** 269–273

Bruce R, West CA (1989) Elicitation of lignin biosynthesis and isoperoxidase activity by pectic fragments in suspension cultures of castor bean. Plant Physiol **91:** 889–897

Church DL, Galston AW (1988) 4-Coumarate:coenzyme A ligase and isoperoxidase expression in *Zinnia* mesophyll cells induced to differentiate into tracheary elements. Plant Physiol **88:** 679–684

Freudenberg K (1968) The constitution and biosynthesis of lignin. *In* K Freudenberg, AC Neish, eds, Constitution and Biosynthesis of Lignin. Springer Verlag, Heidelberg, Germany, pp 47–116

Fukuda H, Komamine A (1982) Lignin synthesis and its related enzymes as markers of tracheary-element differentiation in single cells isolated from the mesophyll of *Zinnia elegans*. Plant Cell Physiol **24:** 403–414

Galliano H, Heller W, Sandermann H (1993) Ozone-induction and purification of spruce cinnamyl alcohol dehydrogenase. Phytochemistry **32:** 557–563

Gaspar T, Penel C, Castillo F, Greppin H (1985) A two-step control of basic and acidic peroxidases and its significance for growth and development. Physiol Plant **64:** 418–423

Goldberg R, Catesson AM, Czaninski Y (1983) Some properties of syringaldazine oxidase, a peroxidase specifically involved in the lignification process. Z Pflanzenphysiol **110:** 259–266

Gross GG, Janse C, Elstner EF (1977) Involvement of malate, monophenols, and the superoxide radical in hydrogen peroxide formation by isolated cell walls from horseradish (*Armoracia lapathifolia* Gilib). Planta **136:** 271–276

Harkin JM, Obst JR (1973) Lignification in trees: indication of exclusive peroxidase participation. Science **180:** 296–297

Imberty A, Goldberg R, Catesson A-M (1985) Isolation and characterization of *Populus* isoperoxidases involved in the last step of lignification. Planta **164:** 221–226

Ishida A, Ookubo K, Ono K (1987) Formation of hydrogen peroxide by NAD(P)H oxidation with isolated cell wall-associated peroxidases from cultured liverwort cell, *Marchantia polymorpha* L. Plant Cell Physiol **28:** 723–726

Lagrimini LM (1991) Wound-induced deposition of polyphenols in transgenic plants overexpressing peroxidase. Plant Physiol **96:** 577–583

Lagrimini LM, Vaughn J, Erb A, Miller SA (1993) Peroxidase overproduction in tomato: wound-induced polyphenol deposition and disease resistance. HortScience **28:** 218–221

Lewis NG, Yamamoto E (1990) Lignin: occurrence, biogenesis and biodegradation. Annu Rev Plant Physiol Plant Mol Biol **41:** 455–496

Mäder M (1976) Die Lokalisation der Peroxidase-Isoenzymgruppe G_1 in der Zellwand von Tabak-Geweben. Planta **131:** 11–15

Mäder M, Amberg-Fischer V (1982) Role of peroxidase in lignification of tobacco cells. Oxidation of nicotinamid dinucleotide and formation of hydrogen peroxide by cell wall peroxidases. Plant Physiol **70:** 1128–1131

Mäder M, Nessel A, Bopp M (1977) Über die physiologische Bedeutung der Peroxidase-Isoenzymgruppen des Tabaks anhand einiger biochemischer Eigenschaften. II. pH Optima, Michealis Konstanten, maximale Oxidationsraten. Z Pflanzenphysiol **82:** 247–260

Mäder M, Nessel A, Schloss P (1986) Cell compartmentation and specific roles of isoenzymes. *In* H Greppin, C Penel, T Gaspar, eds, Molecular and Physiological Aspects of Plant Peroxidases. University Press, Geneva, Switzerland, pp 246–260

McDougall GJ (1991) Cell wall-associated peroxidases and lignification during growth of flax fibres. J Plant Physiol **139:** 182–186

McDougall GJ (1992) Changes in cell wall-associated peroxidases during the lignification of flax fibers. Phytochemistry **31:** 3385–3389

Miksche GE, Yasuda S (1977) Über die Lignine der Blätter und Nadeln einiger Angiospermen and Gymnospermen. Holzforschung **31:** 57–59

Morrison IM (1972) A semi-micro method for the determination of lignin and its use in predicting the digestibility of forage crops. J Sci Food Agric **23:** 455–463

Olson PD, Varner JE (1993) Hydrogen peroxide and lignification. Plant J **4:** 887–892

O'Malley DM, Whetten R, Bao W, Chen CL, Sederoff RR (1993) The role of laccase in lignification. Plant J **4:** 751–757

Pedreno M, RosBarcelo A, Sabater F, Munoz R (1989) Control by pH of cell wall peroxidase activity involved in lignification. Plant Cell Physiol **30:** 237–241

Polle A, Chakrabarti K (1994) Soluble apoplastic peroxidase activities and lignin contents in needles of Norway spruce (*Picea abies* L.) as affected by manganese deficiency. Tree Physiol (in press)

Polle A, Chakrabarti K, Chakrabarti S, Seifert F, Schramel P, Rennenberg H (1992) Antioxidants and manganese deficiency in needles of Norway spruce (*Picea abies* L.) trees. Plant Physiol **99:** 1084–1089

Polle A, Chakrabarti K, Schürmann W, Rennenberg H (1990) Composition and properties of hydrogen peroxide decomposing systems in extracellular and total extracts from needles of Norway spruce (*Picea abies* L. Karst.) Plant Physiol **94:** 314–319

Polle A, Junkermann W (1994) Inhibition of apoplastic and symplastic peroxidase activity from Norway spruce by the photooxidant hydroxymethyl hydroperoxide. Plant Physiol **104:** 617–621

Sato Y, Sugiyama M, Gorecki RJ, Fukuda H, Komamine A (1993) Interrelationship between lignin deposition and the activities of peroxidase isozymes in differentiating tracheary elements of *Zinnia*. Planta **189:** 584–589

Savidge R, Udagama-Randeniya P (1992) Cell wall-bound coniferyl alcohol oxidase associated with lignification in conifers. Phytochemistry **31:** 2959–2966

Schupp R, Rennenberg H (1988) Diurnal changes in the glutathione content of spruce needles (*Picea abies* L.). Plant Sci **57:** 113–117

Simola LK, Lemmetyinen J, Santanen A (1992) Lignin release and photomixotrophism in suspension cultures of *Picea abies*. Physiol Plant **84:** 374–379

Smith CG, Rodgers MW, Zimmerlin A, Ferdinando D, Bolwell GP (1994) Tissue and subcellular immunolocalisation of enzymes of lignin synthesis in differentiating and wounded hypocotyl tissue of French bean (*Phaseolus vulgaris* L.). Planta **192:** 155–164

Strack D, Heilemann J, Wray V, Dirks H (1988) Structures and accumulation patterns of soluble and insoluble phenolics from Norway spruce needles. Phytochemistry **28:** 2071–2078

Wolfart A (1992) Mehrjährige Studien zur Morphologie und Physiologie von Freiland-Fichten (*Picea abies* (L.) Karst.) im Hochschwarzwald. PhD thesis. University Freiburg, Germany