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ScienceDirect
Phytochemistry 68 (2007) 571–579
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Peroxidase activity can dictate the *in vitro* lignin dehydrogenative polymer structure

Valérie Méchin *, Stéphanie Baumberger, Brigitte Pollet, Catherine Lapierre

UMR 206 Chimie Biologique, INRA/INA PG, F-78850 Thiverval Grignon, France

Received 5 October 2006; received in revised form 9 November 2006
Available online 21 December 2006

Abstract

The objective of this study was to assess the influence of the peroxidase/coniferyl alcohol (CA) ratio on the dehydrogenation polymer (DHP) synthesis. The soluble and unsoluble fractions of horseradish peroxidase (HRP)-catalyzed CA dehydrogenation mixtures were recovered in various proportions, depending on the polymerization mode (Zutropf ZT/Zulauf ZL) and HRP/CA ratio (1.6–1100 purpurogallin U $mmol^{-1}$). The ZL mode yielded 0–57%/initial CA of unsoluble condensed DHPs (thioacidolysis yields <200 μmol g^{-1}) with a proportion of uncondensed CA end groups increasing with the HRP/CA ratio (7.2–55.5%/total uncondensed CA). Systematically lower polymer yields (0–49%/initial CA) were obtained for the ZT mode. In that mode, a negative correlation was established between the β-O-4 content (thioacidolysis yields: 222–660 μmol g^{-1}) and the HRP/CA ratio. In both modes, decreasing the HRP/CA ratio below 18 U $mmol^{-1}$ favoured an end-wise polymerization process evidenced by the occurrence of tri-, tetra- and pentamers involving at least one β-O-4 bond. At low ratio, the unsoluble ZT DHP was found to better approximate natural lignins than DHPs previously synthesized with traditional methods. Besides its possible implication in lignin biosynthesis, peroxidase activity is a crucial parameter accounting for the structural variations of *in vitro* DHPs.

Keywords: Dehydrogenation polymers; Coniferyl alcohol; Horseradish peroxidase; Lignin structure; Glycerol β-aryl ether bonds

1. Introduction

Lignin plays an essential role in plant growth, development and uses. It improves water conduction through tracheary elements, limits pathogen attacks but also restricts the degradation of cell wall polysaccharides by enzymes, thus decreasing feeding value. Although studies on lignin biosynthesis have started in the early-50s (Higuchi, 1990), important findings occurred within the last few years. Facilitated by the combination of molecular biology, genetics, bioinformatics, biochemistry and physiology, these findings underlie the successive updated schemes proposed for the monolignol biosynthetic pathways (Boerjan et al., 2003; Barriere et al., 2004; Ralph, 2005; Sibout et al., 2005; Chiang, 2006). After their synthesis, the monolignols are thought to be transported under a glycosylated form to the cell wall where oxidative polymerization takes place (Steeves et al., 2001). This last step in lignin synthesis still raises questions but the involvement of peroxidases (among others proteins) to catalyze the production of monolignol radicals is quite widely admitted (Onnerud et al., 2002; Boerjan et al., 2003; Ros Barcelo et al., 2004). Formation of the polymer would result from radical couplings between two dehydrogenated compounds, the oxidized monomer and the growing polymer phenoxy radical (endwise polymerization) or two oligomer phenoxy radicals (bulk polymerization) (Brunow, 1998).

Given the colossal complexity of lignin in terms of biosynthesis, structure and interactions with the cell wall polysaccharidic network, the investigation of normal, mutants or transformant plants altered in their lignification profile is not enough for plainly deciphering lignin structure and properties. A variety of model systems have consequently

* Corresponding author. Tel.: +33 1 30 81 54 62; fax: +33 1 30 81 53 73.
E-mail address: mechin@grignon.inra.fr (V. Méchin).

0031-9422/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2006.11.024

been developed, including the *in vitro* oxidase-catalyzed polymerization of lignin precursors into dehydrogenation polymer (DHP) initiated by Freudenberg 50 years ago. As previously reviewed (Boerjan et al., 2003), much of what is known today about the radical coupling process and the parameters governing lignin structure, and more particularly the frequency of the different types of interunit bonds, is due to this system. It offers the main advantage that monolignol types, matrix components and polymerization conditions can be accurately adjusted, which enables a high level of control over the composition, structure and lignin–matrix interactions. As recently reviewed (Grabber, 2005), DHP lignins are supposed to not fully mimic the structure of plant lignins. DHPs are reported as highly condensed polymers (low proportion of β-O-4 linkages) and abnormally rich in coniferyl alcohol (CA) end-groups (15–40%), regardless of whether they are formed under bulk (Zulauf mode) or endwise (Zutropf mode) polymerization conditions (Terashima et al., 1996).

Two main points can be noticed when considering the literature relative to DHPs. The first one concerns the diversity of the parameters and polymerization conditions tested to better understand and possibly control the DHP synthesis: monolignol structure (Ralph et al., 1992, 1995; Ito et al., 2002; Fournand et al., 2003), pH (Terashima et al., 1996; Fournand et al., 2003; Grabber et al., 2003), solvent environment (Houtman, 1999), monolignol addition mode and rate (Saake et al., 1996; Terashima et al., 1996; Grabber et al., 2003), presence of soluble carbohydrates (Higuchi et al., 1971; Terashima et al., 1996; Cathala and Monties, 2001; Lairez et al., 2005) or of a macromolecular template (Terashima et al., 1996; Guan et al., 1997); polymerization under heterogeneous conditions, under homogeneous conditions (De Angelis et al., 1999) or at the air/water interface (Cathala et al., 2004); use of isolated enzymes, plant tissue culture or cell wall model systems (Whitmore, 1978; Fukuda, 1992; Grabber et al., 1996; Terashima et al., 2004); classical use of horseradish peroxidases (HRP) (Syrjanen and Brunow, 1998, 2000), specific peroxidases isolated from plants (Sasaki et al., 2004), enzymes other than peroxidases suspected to be involved in polymerization (Driouich et al., 1992; Sterjiades et al., 1992, 1993) or to orientate the stereospecificity of the process (Davin et al., 1997). The second point is the severe contrast between this diversity of parameters and conditions tested and the fact that little attention has been paid so far to the peroxidase activity, and more precisely to the peroxidase/monolignol ratio. Peroxidase is generally used in large excess with respect to the monolignols and uncontrolled variations of the peroxidase activity within the same study are not rare. If this parameter is sometimes taken into consideration, however (Tanahashi and Higuchi, 1981), systematic investigations of its influence on the DHP structure are lacking.

Our objective was to assess the influence of the peroxidase/monolignols ratio on the DHP synthesis. We investigated a wide range of ratios covering the values reported in the literature. DHPs were prepared both by the continuous Zutropf (ZT) and discontinuous Zulauf (ZL) methods in order to compare the ratio effect, respectively, on an endwise (EW) and bulk polymerization process. Combined analysis of the soluble oligomers and the insoluble polymer shows that it is possible to increase the proportion of β-O-4 bonds and to decrease that of coniferyl alcohol end-groups, thus to better approximate the structure of normal plant lignins. The influence of the HRP/CA ratio on the polymerization mechanisms is discussed, together with its implications on lignin synthesis *in vivo*.

2. Results

2.1. DHPs recovery and fractionation

Both the traditional Zutropf (ZT) and Zulauf (ZL) methods were implemented to prepare DHPs from CA, at a constant 0.340 mol l^{-1} CA concentration but with HRP/CA ratios ranging from 1.6 to 1100 purpurogallin U $mmol^{-1}$. This range covers not only the conditions mentioned in the literature but also unusually low ratios. Two distinct fractions, respectively, soluble and unsoluble in the reaction medium, could be recovered from the dehydrogenation mixtures in various proportions. The soluble and solvent-extractable fraction was found to consist in oligomers with polymerization degrees (PD) inferior to 10 (size exclusion chromatography, SEC and liquid chromatography–mass spectrometry, LC–MS analyses, Figs. 1 and 2), whereas the precipitate is assumed to correspond to a polymer fraction of higher PD. This polymer was recovered in a yield highly dependent on the polymerization mode (bulk or endwise) and HRP/CA ratio (Fig. 3). Above 9 U $mmol^{-1}$, all the reaction conditions lead to a polymer fraction with a yield ranging between 20 and 60 wt%, determined gravimetrically with respect to the total initial CA. Similar yields are described for ZT and ZL methods (Russell et al., 2000; Tobimatsu et al., 2006) whereas the yields of the DHPs synthesized with dialysis tubes do not exceed 10% (Tanahashi and Higuchi, 1981). Such low yields were also observed in the present study for the unusually low HRP/CA ratio (<4.5 and 1.6 U $mmol^{-1}$, respectively, for ZT and ZL DHPs). Conversely, SEC analysis of the soluble fraction indicates an increased proportion of dimers and oligomers when the HRP/CA ratio decreases (Fig. 1). Good agreement was found between SEC and LC combined with a diode array detector, both enabling the detection of dimers and trimers only below 9 U $mmol^{-1}$ (Figs. 1 and 4). It seems thus possible to adjust this ratio in order to avoid the precipitation of the DHP while favoring the formation of high DP soluble oligomers.

2.2. Structure of the oligomers

The oligomers were analyzed by LC combined with electrospray ionization (ESI) mass spectrometry, a method

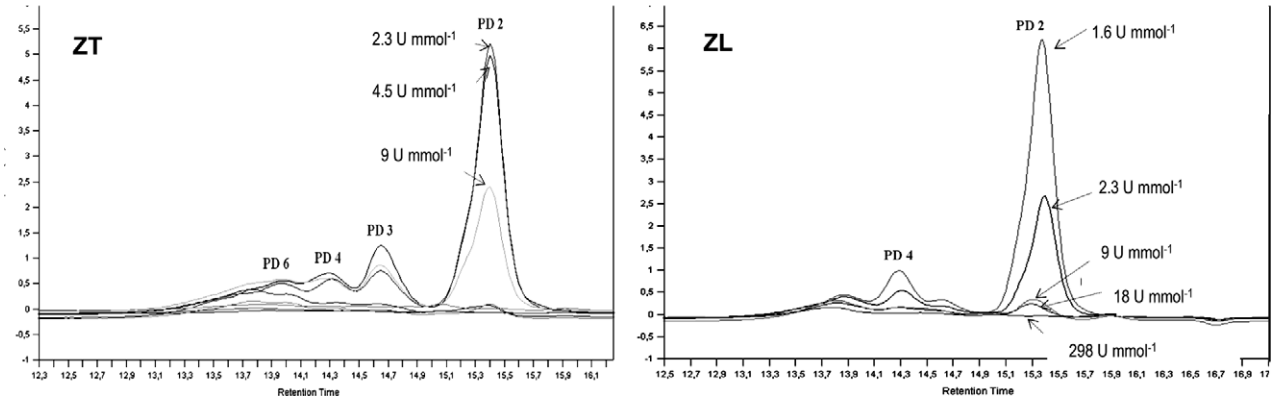

Fig. 1. Size exclusion chromatograms of the soluble fraction recovered from (ZT) Zutropf and (ZL) Zulauf polymerization mixtures as a function of the HRP/CA ratio. Chromatograms normalized with respect to the TMBA internal standard peak. (PD) Polymerization degrees determined from the apparent molar masses according to a calibration with polyethylene oxide standards and purified lignin model compounds.

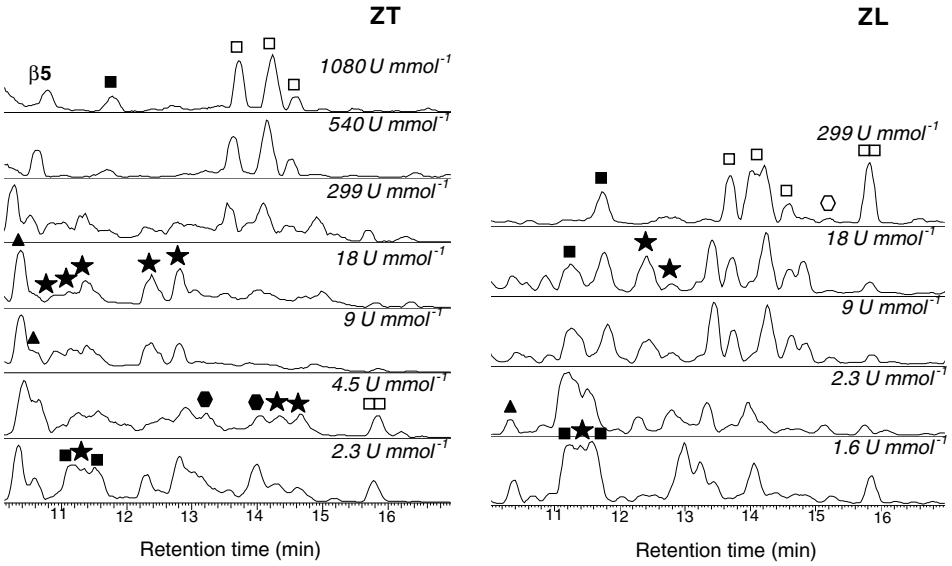

Fig. 2. LC-ESI chromatograms of the soluble fraction recovered from the (ZT) Zutropf and (ZL) Zulauf polymerization mixtures as a function of the HRP/CA ratio. ESI mass spectra detection showing the increasing proportion of uncondensed structures (full symbols) to the detriment of structures deprived of β-O-4 bonds (empty symbols): (▲) trimers, (■) tetramers, (★) pentamers, (⬣) hexamers.

with demonstrated potential for the analysis of natural or synthetic oligolignols (De Angelis et al., 1999; Evtuguin and Amado, 2003; Morreel et al., 2004). Although the quantitative determination of the oligomers is hindered by the co-elution of several compounds, LC–MS brought here the evidence that both the HRP/CA ratio and the method (ZT/ZL) deeply influence the very first steps of the polymerization. While CA dehydrodimers, trimers and tetramers are already detected with the photodiode-array (PDA) detector (Fig. 4), LC–MS was found more suitable for the analysis of the oligomers (Fig. 2). Indeed, our LC–MS conditions (acetonitrile/water/formic acid gradient and negative-mode ESI mass spectrometry) favor the detection of trimers and higher-PD oligomers whereas dimers get hardly ionized. A m/z screening up to 2000 uma enables the detection of negative ions ($[M-H]^-$) with molar mass corresponding to PD inferior or equal to 11 (mainly trimers: $m/z = 553$; tetramers: $m/z = 713$, 731, 749; pentamers: $m/z = 909$, 927; hexamers: $m/z = 1069$, 1105, 1123; octamers: $m/z = 1427$). In some cases, the $[M+HCOOH-H]^-$ adduct was also detected.

For each PD, the m/z increments between the $[M-H]^-$ values ($n \times 18$) directly reflects the number of β-O-4 bonds involved, since the formation of a β-O-4 bond implied the addition of a water molecule on the quinone methide (Brunow et al., 1998). It was thus possible to classify the oligomers into two groups according to the presence (full symbols, Fig. 2) or to the absence (empty symbols, Fig. 2) of β-O-4 bonds. A first glance at Fig. 2 shows that whatever the polymerization mode, the decrease in HRP/CA ratio increases the proportion of non-condensed structures (structures involving at least one β-O-4 bond) together with their polymerization degree. The relative position of the full symbols with respect to the empty ones

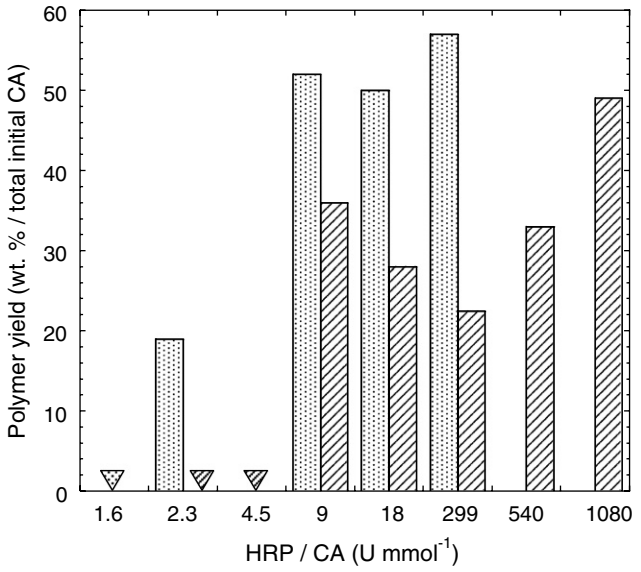

Fig. 3. Influence of the HRP/CA ratio on the polymer recovery yields for the (▨) Zutropf and (⬚) Zulauf polymerization mode. Arrows indicate that the amount of precipitation residue was too small for a gravimetric determination.

on the chromatogram indicates that the uncondensed compounds are systematically eluted before their condensed homologous. Between 1080 and 299 U mmol^{-1}, the oligomers arising from the ZT polymerization are mainly tetramers, one of them containing 1 β-O-4 bond ($m/z = 731$). In the case of ZL polymerization, additional compounds of higher PD (hexamers and octamers) are observed. The absence of odd-PD compounds suggests that these oligomers arise from the coupling of dehydrodimers, in agreement with Syrjanen and Brunow (2000). A 18 U mmol^{-1} ratio seems to be a turning point at which non-condensed oligomers of odd PD (trimers and pentamers involving one or two β-O-4) start to occur to the detriment of condensed tetra- ($m/z = 713$), hexa- ($m/z = 1069$) or octamers ($m/z = 1427$). Further decreasing the ratio generates non-condensed structures of higher PD ($\geqslant 6$) and/or higher frequency of β-O-4 bonds. At 2.3 U mmol^{-1}, 2 β-O-4 tetramers ($m/z = 749$) and hexamers ($m/z = 1105$) and even an hexamer contain 3 β-O-4 bonds ($m/z = 1123$) are detected. These oligomers have the particularity to be formed in an homogeneous phase, since no precipitate is formed at this ratio. Worth noting is the fact that the only trimers detected in the dehydrogenation media systematically contain 1 β-O-4 bond ($m/z = 553$), which shows that β-O-4 bonds are involved in the polymer at the very first steps of oligomerization, just after the dehydrodimerization step.

2.3. Structure of the polymer

Investigation of the polymer fraction was performed by thioacidolysis, a method already applied to DHPs to study the influence of various parameters on the polymer structure (Grabber et al., 1996, 2003; Terashima et al., 1996; Cathala and Monties, 2001; Onnerud et al., 2002). GC/MS analysis of the main thioethylated monomers under their silylated form provides the content of monomers only involved in β-O-4 structures in the sample together with the proportion of coniferyl alcohol end-groups units linked at C_4OH. Complementary SEC analysis of the thioacidolysis mixture without any derivatization informs on the size of the condensed lignin-derived domains and on the distribution of the β-O-4 bonds within the whole polymer (Fig. 5). Taken together, the results reflect the molecular organization of the DHP, in terms of condensation degree and branching level. The thioacidolysis yields obtained in this study vary between 105 μmol g^{-1} (ZL; 18 U mmol^{-1}) and

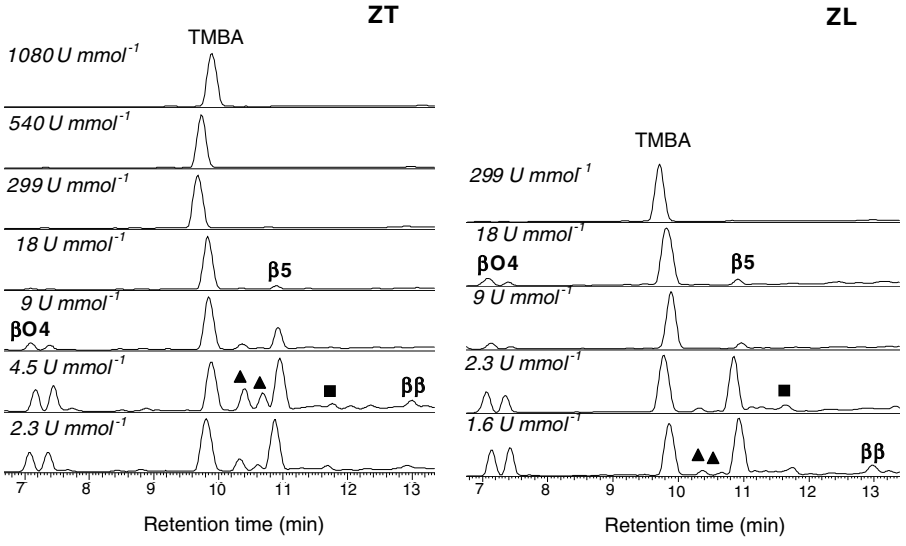

Fig. 4. LC-PDA chromatograms of the soluble fraction recovered from (ZT) Zutropf and (ZL) Zulauf polymerization mixtures as a function of the HRP/CA ratio. Progressive detection of the glycerol aryl ether (β-O-4 erythro and threo isomers), phenyl coumaran (β-5) and pinoresinol (β–β) dehydrodymers together with two trimers (▲) and one tetramer (■) involving each 1 β-O-4 bond.

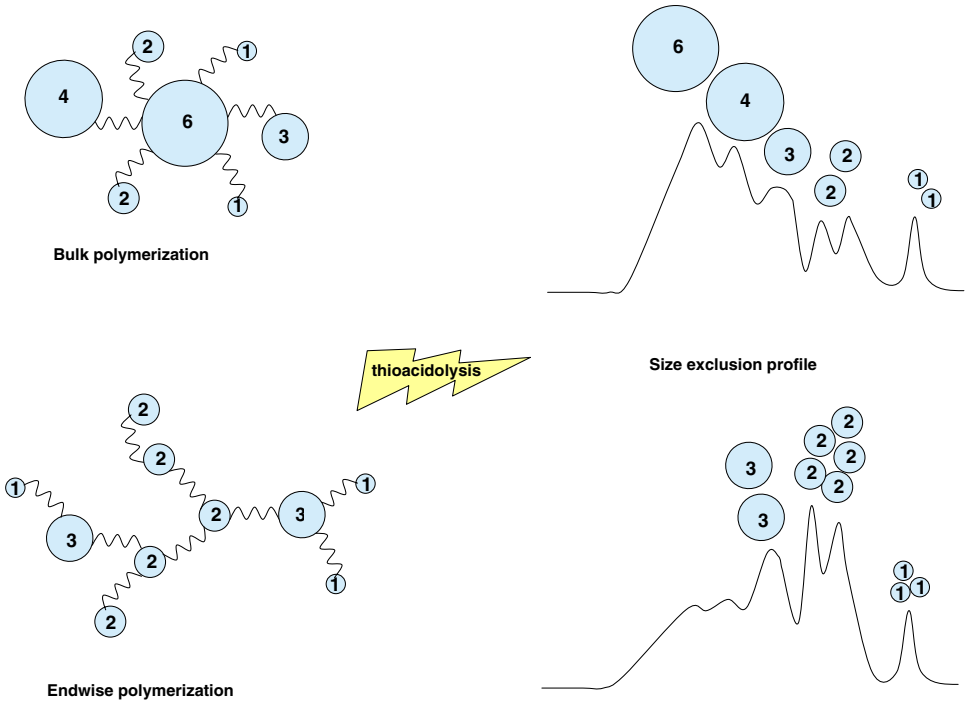

Fig. 5. Scheme for the SEC analysis of the thioacidolysis mixture considering a schematic bulk or endwise polymer.

659 μmol g^{-1} (ZT; 18 U $mmol^{-1}$), which corresponds, respectively, to 1.9% and 11.9% units only involved in β-O-4 bonds. In agreement with previous studies (Grabber et al., 2003) this parameter is systematically higher for the ZT DHPs than for their ZL counterparts which exhibit values inferior to 5%. A new result lies in the assessment of a negative linear correlation between the HRP/CA ratio and the % units only involved in β-O-4 bonds, in the case of ZT polymers (Fig. 6). Inversely the proportion of coniferyl alcohol end-groups was found to increase with the HRP/CA ratio. Thus, at 1100 U $mmol^{-1}$, a condensed branched polymer is obtained whereas low ratios lead to more non-condensed linear polymers. According to the molar mass distribution of the thioacidolysis products (Fig. 7), the β-O-4 bonds would be located at the periphery of the condensed polymer whereas they would be more regularly distributed within the non-condensed polymer (Fig. 5).

3. Discussion and conclusion

3.1. Influence of the enzyme/substrate ratio on the DHPs

Important differences in molecular organization are observed when the ZL mode is changed for the ZT mode or when the polymerization rate is further slowed down by a gradual delivery of the lignin precursor through a dialysis tube (Tanahashi and Higuchi, 1981). The fact that an HRP/CA ratio decrease leads to similar apparent effects than a reduction in CA diffusion rate is not surprising, since the same primary consequence is expected in both case: a lowered monolignol radical concentrations. It is generally stipulated that low monolignol radical concentrations favor cross-coupling reactions (coupling of a monomer radical with the growing polymer) rather than dehydrodimerization, thus leading to an increased proportion of β-O-4 bonds (Ralph et al., 2004). In agreement with this theory are (i) the occurrence of trimers preferentially at low HRP/CA ratios, both for the ZT and ZL procedure, higher ratio leading to compounds with even polymerization degree, (ii) the fact that all trimers detected contain one β-O-4 bond. However, it does not explain why β-ethers bonds are strongly favored in cross-coupling reactions. Another theory suggests that β-O-4 bonds would not result from the coupling between two phenoxy radicals, as other β-involving bonds (β–β and β-5) do, but from the coupling between a phenoxy radical and a phenoxide anion (Rolando et al., 1991). In that case, a low radical concentration, and subsequent phenate excess, could favor phenate-involving couplings, thus leading to an higher proportion of β-O-4 bonds.

3.2. Possibility to synthesize DHP close to lignin structure

In order to better understand lignin structure but also the mechanism governing its formation, the recovery of both a polymer and an oligomer fraction is of particular interest. Indeed, whereas the polymer fraction provides information on the macromolecular organization of lignins, soluble oligomers are useful to investigate the very first steps of lignin formation. Moreover, the main challenge of *in vitro* modelization is to get the highest content in β-O-4 bonds, primary criteria to assess the representativity of the DHP with respect to natural lignins. As stated by

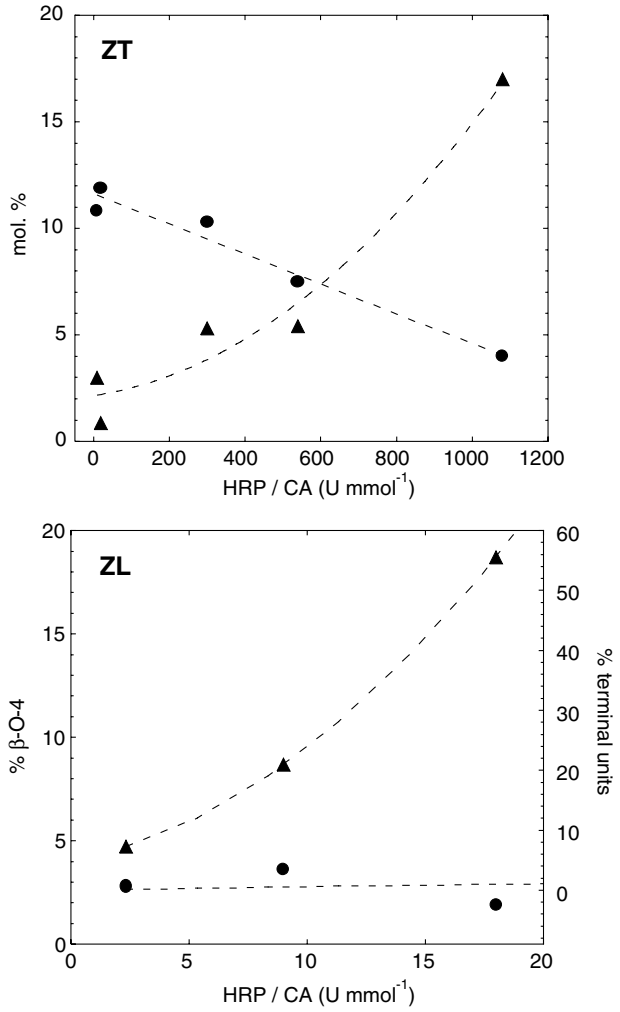

Fig. 6. Influence of the HRP/CA ratio on the (ZT) Zutropf and (ZL) Zulauf polymer structure. Total units only involved in β-O-4 bonds (● mol%/total polymer units) and frequency of terminal units (▲ mol%/total uncondensed units).

Grabber (2005) most of DHP syntheses were not able to satisfactory mimic natural lignin and DHP–CW appeared more adapted, except that synthesized lignins are more representative of lignin encountered in primary cell wall than in secondary lignified cell wall. Performing CA dehydrogenation by the ZT method at an acidic pH (5.5) belonging to the pH range proposed for the apoplast (Grignon and Sentenac, 1991) is expected to yield DHP structurally close to natural lignins. We reached maximum thioacidolysis yields of 660 μmol g^{-1}, which is not as high as the values observed for natural lignins (Rolando et al., 1992) or DHP synthesized in a polysaccharidic template (1034 μmol g^{-1} (Terashima et al., 1996); 949 μmol g^{-1} (Grabber et al., 2003)) but remains among the highest values reported for DHP prepared with the traditional ZT method (430 μmol g^{-1} (Grabber et al., 1996); 621 μmol g^{-1} (Terashima et al., 1996); 687 μmol g^{-1} (Cathala and Monties, 2001)). Lowering the pH down to 4 would probably increase the β-O-4 content (Fournand et al., 2003; Grabber et al., 2003), while leading to environmental conditions representative of only few lignified tissues. As far as we know, no study devoted to the impact of CA/peroxidase ratio on lignin structure exists. However, Terashima et al. (1996) while working on the impact of coniferin and pectin on DHPs synthesis also introduced variations in the CA/ or coniferin/peroxidase ratio. These variations were not discussed by the authors but we think that a part of the lignin structure modification observed could be attributed to the lowest CA/ or coniferin/peroxidase ratio and not only to the presence of pectin or coniferin. In another study, Tanahashi and Higuchi (1981) looked at the impact of peroxidase concentration on DHPs synthesis but their experimental design is too different from ours to allow any comparison. Indeed, peroxidase was kept in a dialyse membrane and was thus not limitative for DHPs synthesis.

In our ZT dehydrogenation conditions the HRP/CA ratio of 9 U $mmol^{-1}$ leads to a good compromise: precipitation of a polymer fraction rich in β-O-4 bonds (600 μmol g^{-1}), and occurrence of soluble non-condensed oligomers. With this system it is possible to recover about 4 mg polymer from 10 mg CA.

3.3. Implications for lignin biosynthesis

The strong influence of the HRP/CA ratio on the structure of the DHPs, and more particularly on their β-O-4 content, show that this parameter should be taken into

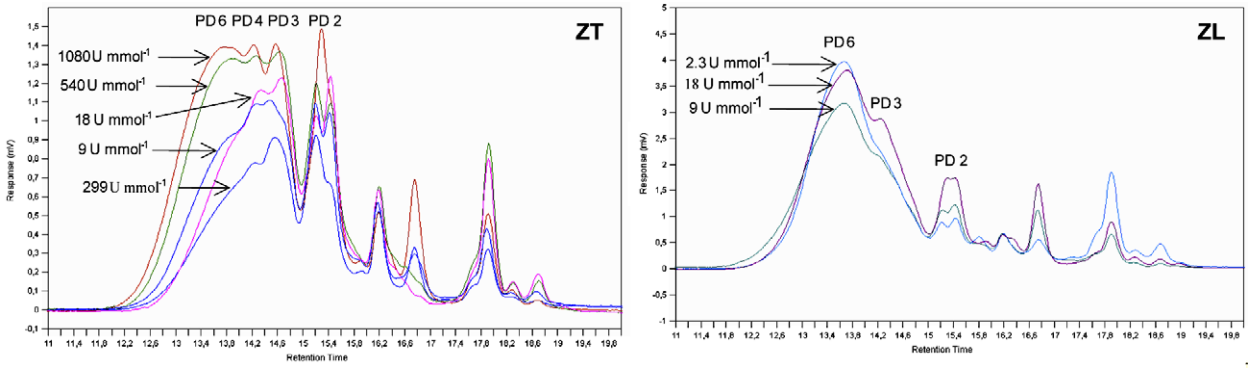

Fig. 7. Size exclusion chromatograms of the thioacidolysis products recovered from the (ZT) Zutropf and (ZL) Zulauf polymerization mixtures as a function of the HRP/CA ratio. Chromatograms normalized with respect to the dimer peak and polymerization degrees (PD) assigned as for Fig. 1.

account when investigating the influence of any other parameter or when comparing results obtained by different authors. Besides its direct practical consequence for *in vitro* modelization, the influence of the HRP/CA ratio may also have important implications for lignin biosynthesis. It is generally accepted today that peroxidases are involved in constitutive lignification, as enzymes able to oxidize *p*-OH cinnamyl alcohols and various phenolic substrates in the presence of H_2O_2. Lignins formed at the early stages of lignification have been repeatedly reported to be enriched in resistant condensed bonds (Terashima et al., 1993). Indeed, the highly lignified middle lamella region seems to be enriched in bulk-type lignin. This phenomenon has been related to monolignol concentration or type as middle lamella lignin is enriched in *p*-hydroxyphenyl units (Terashima et al., 1993). It has also been related to matrix effects (Wi et al., 2005), or to the involvement of laccase enzymes in addition to peroxidase (Sterjiades et al., 1993; Donaldson, 2001). The present results suggest that it could be also related to a high peroxidase activity in the middle lamella region, an hypothesis supported by the pattern distribution of these enzymes (Wi et al., 2005).

In addition, these enzymes have also been shown to play a key role in several stress-induced processes such as wound-healing and disease resistance (Lagrimini et al., 1993), processes involving perturbation of the phenolic metabolism and of the lignification. In the light of the present study, it can be expected that variations in peroxidase concentration, either temporal or spatial, may account not only for the lignin content but also for lignin structure, in the same way as the variations in monomer supply. In agreement is the fact that the peroxidase expression dramatically increases subsequent to a stress and that stress lignins are reported as highly condensed polymer with a high proportion of terminal units (Boudet et al., 1995; Cabane et al., 2004): a rapid and abundant supply in peroxidase would lead to a bulk-like polymerization as a consequence of increased radical-generating capacity.

As stated by Ralph (2005), "no simple unifying theory currently explains all of the features seen in lignin structural analyses, so researchers should still feel free to advance new concepts and hypotheses". We think our data could bring some new information concerning lignin biosynthesis and lignin structure. It clearly appears indeed that a parameter as simple as peroxidase availability can drastically modify the lignin structure while other parameters (monomer availability, pH, matrix environment, etc.) stay unchanged.

4. Experimental

4.1. Chemicals

Coniferyl alcohol was synthesized according to the method of Ludley and Ralph (1996). Horseradish peroxidase type II (HRPII, 180 purpurogallin U/mg) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The internal standard 3,4,5-trimethoxybenzoic acid (TMBA) was obtained from Fluka (Buchs, Switzerland) and hydrogen peroxide (30 wt% solution) from Acros (New Jersey, USA). Phosphate buffer was prepared from sodium dihydrogen phosphate ($NaH_2PO_4 \cdot 2H_2O$) and disodium hydrogen phosphate ($Na_2HPO_4 \cdot 2H_2O$) (analytical reagents; Prolabo, France).

4.2. Dehydrogenative polymerization of coniferyl alcohol

Four solutions were prepared for the polymerization.

Solution 1: 10 mg of coniferyl alcohol in 1 ml of acetone and 49 ml of phosphate buffer (10 mM, pH 5.5).
Solution 2: 10 mg TMBA in 50 ml of phosphate buffer.
Solution 3: 9 μl (1.5 equiv. compared to coniferyl alcohol) of hydrogen peroxide in 50 ml of water.
Solution 4: a variable quantity of HRPII in 50 ml of phosphate buffer in order to scan a range from 0 to 1080 purpurogallin U of HRPII per mmol of coniferyl alcohol.

Zulauf (ZL) synthesis: solutions 1, 3 and 4 were added simultaneously to 10 ml of solution 2 and the mixture was left to react for 10 h under magnetic stirring, at room temperature and in the dark.

Zutropf (ZT) synthesis: solutions 1, 3 and 4 were added to 10 ml of solution 2 through a 8 h period (0.1 ml/min flow rate) and the mixture was then left to react for another 2 h.

Both the Zulauf and Zutropf reaction media were centrifuged (60 min, 13,000 rpm) to separate the precipitate from the soluble fraction. The precipitate was washed three times with water before freeze-drying and gravimetric determination of the polymer yield (mass percent with respect to the initial CA). All the supernatants were pooled before extraction with a 50/50 v/v dichloromethane/ethyl acetate mixture and evaporation of the organic phase to dryness. Part of the soluble extracts residues were used to prepare tetrahydrofuran (stabilized THF, JT Baker) and methanol (CLHP grade, Carlo Erba) solutions, respectively, for size exclusion and LC–MS chromatography. All the solutions were ultrafiltered (GFP Acrodisc, Gelman, 0.45 μm) before injection.

4.3. Analysis of the dehydrogenation products

4.3.1. Thioacidolysis and GC/MS analysis of the unsoluble fraction

Thioacidolysis was performed according to Lapierre et al. (1986) on the unsoluble fraction recovered from the centrifugation step. The freeze-dried unsoluble fractions were placed in a screw-cap glass tube together with 10 ml of a dioxane/ethanethiol mixture (9:1, v/v) containing 0.2 M boron trifluoride etherate, for 4 h, at 100 °C (oil bath). After extraction of the lignin-derived monomers, the analysis of their trimethylsilyl derivatives (TMS) was

run on a polydimethylsiloxane capillary column (SPB1, Supelco, 30 × 0.25 m, 0.25 μm) GC/MS with an ion trap spectrometer detector (IE 70 eV, positive mode).

4.3.2. Steric exclusion chromatography (SEC) of both the unsoluble and soluble fractions

Both the extracted soluble fractions and the thioacidolysates of the unsoluble fractions (20 μL injection) were analyzed by SEC, using a PL-Gel column (Polymer Laboratories, 5 μm, 100 Å, 600 × 7.5 mm) with THF as an eluent (stabilized THF, JT Baker; 1 ml min^{-1}) and 280 nm UV detection. The PD was assigned according to the apparent molar masses of the compounds based on a calibration with polyethylene oxide standards (Igepal, Aldrich) and purified lignin model compounds (Baumberger et al., 2003).

4.3.3. LC/MS of the soluble fraction

The extracted soluble fractions were analyzed by reverse-phase LC with ESI-MS and PDA co-detection. The methanolic solutions were ultra filtrated (0.45 μm – GHP Acrodisc – Gelman) and injected on a C_{18} column (Highpurity, Thermo electron, 5 μm, 150 × 4.6 mm) using a 12–95 vol% aqueous acetonitrile, 1‰ HCOOH gradient (45 min) and 1 ml min^{-1} flow rate. Negative ion ESI-MS spectra (120–2000 *m/z*) were acquired using an ion trap spectrometer (Finnigan LCQ-DECA – Thermo Electron Corporation) setting the needle voltage at 4 kV and the desolvating capillary temperature at 350 °C. The dimers were assigned according to their retention time, PDA (190–600 nm) and mass spectra, compared to dimers previously purified from CA oxidation media (Fournand et al., 2003). The polymerization degree and amount of β-O-4 bonds of the oligomers were determined according to the mass of the deprotonated ion.

Acknowledgements

The authors sincerely acknowledge Dr. Bernard Cathala for help in synthesizing CA and Dr. Paul-Henri Ducrot for fruitful discussions.

References

Barriere, Y., Ralph, J., Mechin, V., Guillaumie, S., Grabber, J.H., Argillier, O., Chabbert, B., Lapierre, C., 2004. Genetic and molecular basis of grass cell wall biosynthesis and degradability II. Lessons from brown-midrib mutants. C.R. Biol. 327, 847–860.

Baumberger, S., Fournand, D., Pollet, B., Lapierre, C., Ducrot, P.-H., 2003. Peroxidase-catalyzed polymerization of monolignols: HPSEC investigation of the oligomerization step. In: Proceedings of the 12th ISWPC, Madison, 8–12 June, 2003, vol. III, pp. 165–168.

Boerjan, W., Ralph, J., Baucher, M., 2003. Lignin biosynthesis. Annu. Rev. Plant. Biol. 54, 519–546.

Boudet, A.M., Hawkins, S., Cabane, M., Ernst, D., Galliano, H., Heller, W., Lange, M., Sandermann Jr., H., Lapierre, C., 1995. Developmental and stress lignification. In: Bonnet-Mazimbert, M. (Ed.), Eurosilva: Contribution to Forest Tree Physiology. INRA, Paris, pp. 3–34.

Brunow, G., 1998. Oxidative coupling of phenols and the biosynthesis of lignin. In: Lewis, N.G., Sarkanen, S. (Eds.), Lignin and Lignan Biosynthesis. ACS, Washington, DC, pp. 131–147.

Brunow, G., Ammalahti, E., Niemi, T., Sipila, J., Simola, L.K., Kilpelainen, I., 1998. Labelling of a lignin from suspension cultures of *Picea abies*. Phytochemistry 47, 1495–1500.

Cabane, M., Pireaux, J.C., Leger, E., Weber, E., Dizengremel, P., Pollet, B., Lapierre, C., 2004. Condensed lignins are synthesized in poplar leaves exposed to ozone. Plant Physiol. 134, 586–594.

Cathala, B., Monties, B., 2001. Influence of pectins on the solubility and the molar mass distribution of dehydrogenative polymers (DHPs, lignin model compounds). Int. J. Biol. Macromol. 29, 45–51.

Cathala, B., Aguie-Beghin, V., Douillard, R., 2004. Coniferyl alcohol reactivity at the air/water interface. C.R. Biol. 327, 777–784.

Chiang, V.L., 2006. Monolignol biosynthesis and genetic engineering of lignin in trees, a review. Environ. Chem. Lett. 4, 143–146.

Davin, L.B., Wang, H.B., Crowell, A.L., Bedgar, D.L., Martin, D.M., Sarkanen, S., Lewis, N.G., 1997. Stereoselective bimolecular phenoxy radical coupling by an auxiliary (dirigent) protein without an active center. Science 275, 362–366.

De Angelis, F., Nicoletti, R., Spreti, N., Veri, F., 1999. A new *in vitro* model of lignin biosynthesis. Angew. Chem., Int. Ed. 38, 1283–1285.

Donaldson, L.A., 2001. Lignification and lignin topochemistry – an ultrastructural view. Phytochemistry 57, 859–873.

Driouich, A., Laine, A.C., Vian, B., Faye, L., 1992. Characterization and localization of laccase forms in stem and cell-cultures of sycamore. Plant J. 2, 13–24.

Evtuguin, D.V., Amado, F.M.L., 2003. Towards a better understanding of lignin assembly: the assessment of the sequential order of building blocks and inter-units linkages using electrospray ionisation mass spectrometry. In: Proceedings of the 12th ISWPC, Madison, 8–12 June, 2003, vol. I, pp. 277–280.

Fournand, D., Cathala, B., Lapierre, C., 2003. Initial steps of the peroxidase-catalyzed polymerization of coniferyl alcohol and/or sinapyl aldehyde: capillary zone electrophoresis study of pH effect. Phytochemistry 62, 139–146.

Fukuda, H., 1992. Tracheary element formation as a model system of cell differentiation. Int. Rev. Cytol. 136, 289–332.

Grabber, J.H., 2005. How do lignin composition, structure, and cross-linking affect degradability? A review of cell wall model studies. Crop Sci. 45, 820–831.

Grabber, J.H., Ralph, J., Hatfield, R.D., Quideau, S., Kuster, T., Pell, A.N., 1996. Dehydrogenation polymer-cell wall complexes as a model for lignified grass walls. J. Agric. Food Chem. 44, 1453–1459.

Grabber, J.H., Hatfield, R.D., Ralph, J., 2003. Apoplastic pH and monolignol addition rate effects on lignin formation and cell wall degradability in maize. J. Agric. Food Chem. 51, 4984–4989.

Grignon, C., Sentenac, H., 1991. pH and ionic conditions in the apoplast. Annu. Rev. Plant Physiol. Plant Mol. Biol. 42, 103–128.

Guan, S.Y., Mylnar, J., Sarkanen, S., 1997. Dehydrogenative polymerization of coniferyl alcohol on macromolecular lignin templates. Phytochemistry 45, 911–918.

Higuchi, T., 1990. Lignin biochemistry: biosynthesis and biodegradation. Wood Sci. Technol. 24, 23–63.

Higuchi, T., Ogino, K., Tanahashi, M., 1971. Effect of polysaccharides on dehydropolymerization of coniferyl alcohol. Wood Res. 51, 1–11.

Houtman, C.J., 1999. What factors control dimerization of coniferyl alcohol? Holzforschung 53, 585–589.

Ito, T., Kawai, S., Ohashi, H., Higuchi, T., 2002. Characterization of new thioacidolysis products of sinapyl aldehyde and coniferyl aldehyde. J. Wood Sci. 48, 409–413.

Lagrimini, L.M., Vaughn, J., Erb, W.A., Miller, S.A., 1993. Peroxidase overproduction in tomato: wound-induced polyphenol deposition and disease resistance. HortScience 28, 218–221.

Lairez, D., Cathala, B., Monties, B., Bedos-Belval, F., Duran, H., Gorrichon, L., 2005. Aggregation during coniferyl alcohol polymer-

ization in pectin solution: a biomimetic approach of the first steps of lignification. Biomacromolecules 6, 763–774.

Lapierre, C., Monties, B., Rolando, C., 1986. Preparative thioacidolysis of spruce lignin: isolation and identification of main monomeric products. Holzforschung 40, 47–50.

Ludley, F.H., Ralph, J., 1996. Improved preparation of coniferyl and sinapyl alcohols. J. Agric. Food Chem. 44, 2942–2943.

Morreel, K., Ralph, J., Kim, H., Lu, F., Goeminne, G., Ralph, S., Messens, E., Boerjan, W., 2004. Profiling of oligolignols reveals monolignol coupling conditions in lignifying poplar xylem. Plant Physiol. 136, 3537–3549.

Onnerud, H., Zhang, L., Gellerstedt, G., Henriksson, G., 2002. Polymerization of monolignols by redox shuttle-mediated enzymatic oxidation: a new model in lignin biosynthesis I. Plant Cell. 14, 1953–1962.

Ralph, J., 2005. Elucidation of new pathways in normal and perturbed lignification. In: Proceedings of the 13th ISWFPC, Auckland, New Zealand, 16–19 May 2005, vol. I, pp. 3–13.

Ralph, J., Helm, R.F., Quideau, S., Hatfield, R.D., 1992. Lignin feruloyl ester cross-links in grasses.1. Incorporation of feruloyl esters into coniferyl alcohol dehydrogenation polymers. J. Chem. Soc., Perkin Trans. 1, 2961–2969.

Ralph, J., Grabber, J.H., Hatfield, R.D., 1995. Lignin-ferulate cross-links in grasses – active incorporation of ferulate polysaccharide esters into ryegrass lignins. Carbohydr. Res. 275, 167–178.

Ralph, J., Lundquist, K., Brunow, G., Lu, F., Kim, H., Schatz, P., Marita, J.M., Hatfield, R.D., Ralph, S.A., Christensen, J.H., Boerjan, W., 2004. Lignins: natural polymers from oxidative coupling of 4-hydroxyphenyl-propanoids. Phytochem. Rev. 3, 29–60.

Rolando, C., Schneider, S., Pinson, J., Lapierre, C., Monties, B., Catesson, A.M., Goldberg, R., 1991. Mechanistical studies on the polymerization of coniferyl alcohol. In: Gaspar, T., Penel, C. (Eds.), Molecular and Physiological Aspects of Plant Peroxidases. Presse de l'Université de Genève, Genève, pp. 131–147.

Rolando, C., Monties, B., Lapierre, C., 1992. Thioacidolysis. In: Dence, C., Lin, S.Y. (Eds.), Methods in Lignin Chemistry. Springer, Heidelberg, pp. 334–349.

Ros Barcelo, A., Gomez Ros, L.V., Gabaldon, C., Lopez-Serrano, M., Pomar, F., Carrion, J.S., Pedreno, M.A., 2004. Basic peroxidases: the gateway for lignin evolution? Phytochem. Rev. 3, 61–78.

Russell, W.R., Forrester, A.R., Chesson, A., 2000. Predicting the macromolecular structure and properties of lignin and comparison with synthetically produced polymers. Holzforschung 54, 505–510.

Saake, B., Argyropoulos, D.S., Beinhoff, O., Faix, O., 1996. A comparison of lignin polymer models (DHPs) and lignins by P-31 NMR spectroscopy. Phytochemistry 43, 499–507.

Sasaki, S., Nishida, T., Tsutsumi, Y., Kondo, R., 2004. Lignin dehydrogenative polymerization mechanism: a poplar cell wall peroxidase directly oxidizes polymer lignin and produces *in vitro* dehydrogenative polymer rich in b-*O*-4 linkage. FEBS Lett. 562, 197–201.

Sibout, R., Eudes, A., Mouille, G., Pollet, B., Lapierre, C., Jouanin, L., Seguin, A., 2005. Cinnamyl alcohol dehydrogenase-C and -D are the primary genes involved in lignin biosynthesis in the floral stem of Arabidopsis. Plant Cell. 17, 2059–2076.

Steeves, V., Forster, H., Pommer, U., Savidge, R., 2001. Coniferyl alcohol metabolism in conifers – I. Glucosidic turnover of cinnamyl aldehydes by UDPG: coniferyl alcohol glucosyltransferase from pine cambium. Phytochemistry 57, 1085–1093.

Sterjiades, R., Dean, J.F., Eriksson, K.E., 1992. Laccase from sycamore maple (*Acer pseudoplatanus*) polymerizes monolignols. Plant Physiol. 99, 1162–1168.

Sterjiades, R., Dean, J.F.D., Gamble, G., Himmelsbach, D.S., Eriksson, K.E.L., 1993. Extracellular laccases and peroxidases from sycamore maple (*Acer pseudoplatanus*) cell-suspension cultures: reactions with monolignols and lignin model compounds. Planta 190, 75–87.

Syrjanen, K., Brunow, G., 1998. Oxidative cross-coupling of *p*-hydroxycinnamic alcohols with dimeric arylglycerol beta-aryl ether lignin model compounds. The effect of oxidation potentials. J. Chem. Soc., Perkin Trans. I 1, 3425–3429.

Syrjanen, K., Brunow, G., 2000. Regioselectivity in lignin biosynthesis. The influence of dimerization and cross-coupling. J. Chem. Soc., Perkin Trans. 1, 183–187.

Tanahashi, M., Higuchi, T., 1981. Dehydrogenative polymerization of monolignols by peroxidase and H_2O_2 in a dialysis tube. I. Preparation of highly polymerized DHPs. Wood Res. 67, 29–42.

Terashima, N., Fukushima, K., He, L.F., Takabe, K., 1993. Comprehensive model of the lignified plant cell wall. In: Jung, H.G., Buxton, D.R., Hatfield, R.D., Ralph, J. (Eds.), Forage Cell Wall Structure and Digestibility, Am. Soc. Agron., Madison, WI, USA, pp. 247–270.

Terashima, N., Atalla, R.H., Ralph, S.A., Landucci, L.L., Lapierre, C., Monties, B., 1996. New preparations of lignin polymer models under conditions that approximate cell wall lignification. 2. Structural characterization of the models by thioacidolysis. Holzforschung 50, 9–14.

Terashima, N., Awano, T., Takabe, K., Yoshida, M., 2004. Formation of macromolecular lignin in ginkgo xylem cell walls as observed by field emission scanning electron microscopy. C.R. Biol. 327, 903–910.

Tobimatsu, Y., Takano, T., Kamitakahara, H., Nakatsubo, F., 2006. Studies on the dehydrogenative polymerizations of monolignol ?-glycosides. Part 2: Horseradish peroxidase-catalyzed dehydrogenative polymerization of isoconiferin. Holzforschung 60, 513–518.

Whitmore, F.W., 1978. Lignin-carbohydrate complex formed in isolated cell walls of callus. Phytochemistry 17, 421–425.

Wi, S.G., Singh, A.P., Lee, K.H., Kim, Y.S., 2005. The pattern of distribution of pectin, peroxidase and lignin in the middle lamella of secondary xylem fibres in alfalfa (*Medicago sativa*). Ann. Bot. 95, 863–868.