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Isolation and characterization of *Populus* isoperoxidases involved in the last step of lignin formation

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Abstract. Peroxidases (EC 1.11.1.7) from *Populus* × *euramericana* were investigated during the dormant and growing seasons using histochemical and biochemical methods. The activities of syringaldazine oxidase and *p*-paraphenylenediamine-pyrocatechol oxidase in sections of branches were maximal during spring in both phloem and young xylem. Cytoplasmic and cell-wall peroxidase activities from different lignified tissues were estimated in vitro. Pronounced differences were noticed between fractions isolated during spring and winter. Gel electrophoresis showed the presence of an anionic fast-migrating isoperoxidase group with a high syringaldazine-oxidase activity. The isoenzymes of this group were different in winter and in spring. The properties of these isoperoxidases (kinetic constants, pH optimum, resistance to heat) were investigated after isolation by ion-exchange chromatography.

Key words: Lignification – Peroxidase (lignin biosynthesis) – *Populus*.

Introduction

Many enzymes are involved in lignification processes (Gross 1980). Peroxidases (EC 1.11.1.7) are known to act on the last step of lignin biosynthesis, i.e. the polymerization of substituted cinnamyl alcohols in the presence of hydrogen peroxide (Harkin and Obst 1973). The formation of hydrogen peroxide from NADH can also be mediated by cell-wall peroxidases (Elstner and Heupel 1976; Gross et al. 1977). Peroxidases are also involved in many other physiological processes (Gaspar et al. 1982). Thus, it is of interest to determine the nature and the properties of the isoperoxidases involved in the polymerization of lignin monomers.

The peroxidases may be recognized by the simultaneous use of histochemical and biochemical techniques. In the former case, a substrate specific for the peroxidases involved in lignification processes can be used i.e. syringaldazine (Harkin and Obst 1973) which has a special affinity for lignifying cell wall peroxidases (Catesson et al. 1978). In the latter case, the development of peroxidase activity can be followed in various lignified tissues during periods of dormancy and lignification. The formation of wood throughout the year has been studied by Nelson (1978) using cherry and walnut. Heartwood and sapwood peroxidase activities, estimated by H_2O_2 decomposition, were reported to be higher in winter than in spring. In contrast, histochemical investigations showed that oxidation of syringaldazine and other substrates was maximal in phloem and sapwood cell walls during spring. A comparison of biochemical and histochemical results is difficult since the peroxidase substrates used in each investigation method were different.

The aim of our work was to compare in situ and in vitro the properties of peroxidase activites from tissues isolated during periods of dormancy and lignification. Our experiments were performed with branches from *Populus* × *euramericana*, the peroxidases of which have already been studied by Goldberg et al. (1983). Peroxidase activities were estimated with syringaldazine and with *p*-phenylenediamine-pyrocatechol, which is oxidized by a number of isoperoxidases but allows histochemical as well as biochemical investigations (Imberty et al. 1984). Peroxidases of phloem (containing many fibers), "young xylem" (produced during the year) and "old xylem" (one or two years

Abbreviations: PPD-PC = *p*-phenylenediamine-pyrocatechol; syr-oxidase = syringaldazine-oxidase

old) were separated by cytoplasmic and various cell-wall enzymes.

Material and methods

Plant material. Poplar trees (*Populus* × *euramericana* (Dode) Guignier, cv. I. 214) were grown in natural conditions. One- or two-year-old branches were cut in winter and in spring.

Histochemical assays. For *p*-phenylene diamine-pyrocatechol (PPD-PC)-oxidase localization, hand sections collected in 0.1 M 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)-HCl buffer pH 7.6 at room temperature were incubated for 5 min in the following medium: $7 \cdot 10^{-4}$ M PPD, $9 \cdot 10^{-3}$ M PC, $2.5 \cdot 10^{-3}$ M H_2O_2 and 0.1 M Tris-HCl buffer pH 7.6. The sections were rinsed with distilled water and observed by light microscope; for syr-oxidase localization, the method was adapted from Goldberg et al. (1981). The sections were incubated in 0.1% alcoholic syringaldazine and $1.25 \cdot 10^{-3}$ M H_2O_2 for 5 min.

Preparation of enzymatic fractions. After branches were peeled, phloem, young xylem and old xylem were separated, ground in liquid nitrogen and lyophilized. Each tissue was then further ground in 0.1 M Na-K phosphate buffer pH 6.0 with a Polytron (Bioblock, Paris, France). Enzymatic extraction was performed according to Ridge and Osborne (1970) and Parish (1975) with an additional step: all enzymatic extracts were reduced to a small volume in an Amicon ultrafiltration cell (membrane PM 10; Amicon, Lexington, USA). Three fractions were successively solubilized by buffer, 1 M NaCl and cellulase-pectinase treatment to yield: "cytoplasmic" enzymes (S), lightly- and strongly-bound enzymes, so called "ionically" (E) and "covalently" (C) bound enzymes.

Spectrophotometric assays. For PPD-PC oxidation, the assay mixture contained in 4 ml final volume: $3.5 \cdot 10^{-4}$ M PPD, $4.5 \cdot 10^{-3}$ M PC, $1.125 \cdot 10^{-3}$ M H_2O_2, enzyme extract (50 or 100 µl) and 0.1 M Tris-HCl buffer pH 7.6. The oxidation time course was recorded at 557 nm. Syringaldazine was dissolved in methanol (3.6 mg ml^{-1}) and then mixed with dioxane (1:2, v/v). The assay mixture contained 50 µl syringaldazine solution ($4.16 \cdot 10^{-5}$ M final concentration), $1.125 \cdot 10^{-3}$ M H_2O_2, enzyme extract and 0.1 M Na-K phosphate buffer pH 6.0 in 4 ml final volume. The oxidation time course was recorded at 530 nm.

Electrophoretic assays. The isoperoxidases were separated by electrophoresis on 7.5% polyacrylamide disc gel according to Ornstein and Davis (1964). The isoenzymes were stained by immersing the gels in an incubation mixture containing for PPD-PC-oxidases: $7 \cdot 10^{-4}$ M PPD, $9 \cdot 10^{-3}$ M PC, $2.5 \cdot 10^{-3}$ M H_2O_2 and 0.1 M Tris-HCl buffer pH 7.6 in 6 ml final volume. For syr-oxidase staining, the incubation medium contained 3 ml syringaldazine solution described above ($3.3 \cdot 10^{-3}$ M), $2.5 \cdot 10^{-3}$ M H_2O_2 and 3 ml 0.1 M Na-K phosphate buffer pH 6.0. The staining due to peroxidase activities was estimated with a densitometer (Vernon, Paris, France).

Chromatographic assays. Phloem cytoplasmic peroxidases of the lignification season (spring) were submitted to ion-exchange chromatography on diethylaminoethyl-sepharose CL-6B (Pharmacia; Uppsala, Sweden) columns (20 cm long, 1.7 cm diameter) equilibrated with 0.01 M Na-K phosphate buffer pH 7.2. Sample (approx. 13 mg protein) was loaded on the exchanger and the column was washed with 0.01 M Na-K phosphate buffer pH 7.2. Bound peroxidases were eluted with a NaCl gradient (300 ml) at pH 7.2 (0–0.5 M). Fractions (5 ml) were collected and analysed for their protein content and their PPD-PC- and syr-oxidase activities. Appropriate fractions were pooled, dialysed against H_2O and analysed for their isoenzyme pattern and kinetic characteristics.

Heat stability. Heat treatments were performed on buffer solutions heated to a specified temperature before injections of diluted enzymes. After defined incubation periods, the solutions were rapidly cooled by immersing the tubes in an ice-water bath. Samples were then assayed as described above.

Protein determination. Proteins were estimated according to Lowry et al. (1951).

Results

Peroxidase activities in active and dormant branch tissues. Histochemical localization of PPD-PC- and syr-oxidases is represented in Table 1. Only cell-wall activities could be detected by light-microscope methods. Syr-oxidase activities were noticed only on lignifying cell walls (phloem fibers and xylem), whereas PPD-PC-oxidase activities were found in all tissues. All peroxidase activities were higher during the growing season (spring: S) than during the dormant season (winter: W), except in old xylem cell walls.

Peroxidase activities of active and dormant tissues are represented in Fig. 1. During the dormant season (Fig. 1 A, A′), cytoplasmic as well as cell-wall activities were maximal in the phloem. The lowest activities were obtained in young xylem. The most obvious difference noticed between tissues isolated in spring and winter (Fig. 1 B, B′) was an increase of PPD-PC-oxidase activities in phloem and young xylem during the spring. With syringaldazine, only cell-wall activities of young xylem (i.e. lignifying cell walls) increased slightly during the growing season. In old xylem, with both substrates, cytoplasmic and cell-wall peroxidases were more active in winter than in spring. These results indicate changes of phloem and xylem peroxidase activities during the annual cycle of the tree.

Isoenzyme patterns. Isoperoxidase pattern of cytoplasmic and cell-wall peroxidase fractions obtained in winter and in spring were then compared. The enzymes, separated on polyacrylamide gels, were stained with PPD-PC (Fig. 2). In all patterns, three groups of isoenzymes could be recognized: "cationic" peroxidase (Ca) which did not migrate and two anionic peroxidasic groups, a slowly migrating one (A_I) and a fast-migrating one (A_{II}). Appreciable differences, restricted to the A_{II} group, were noticed between "spring" and "winter" peroxi-

Table 1. Histochemical localization of PPD-PC- and Syr-oxidase activities in the dormant (winter: W) and growing (spring: S) seasons. The number of + signs represents the intensity of the reaction as judged by observation by light microscope

Substrate	Tissue							
	Phloem				Xylem (young)		Xylem (old)	
	Parenchyma		Fibers					
	W	S	W	S	W	S	W	S
PPD-PC	+++	++++	+	++++	+	+++	++	++
Syr-oxidase	0	0	0	++	+	+++	++	++

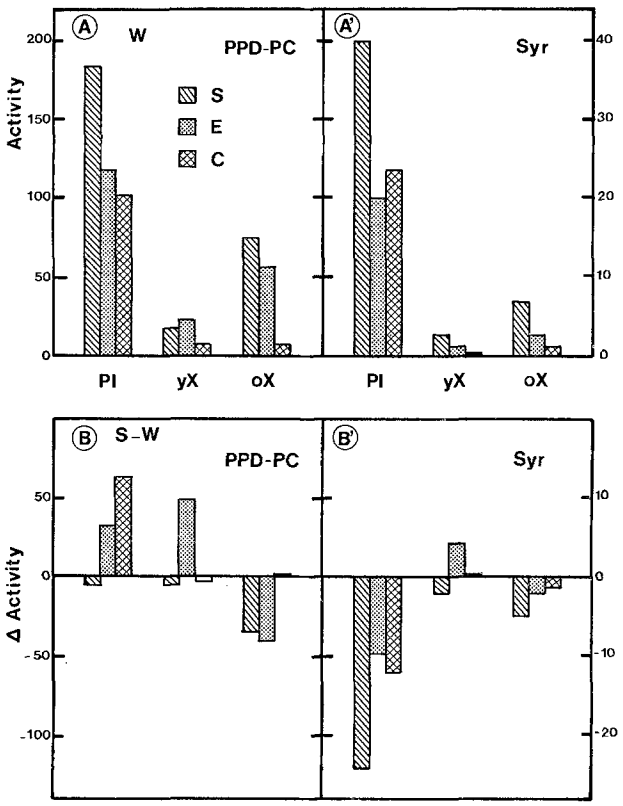

Fig. 1. A Activities of PPD-PC oxidase in winter of different tissues (*Pl*, phloem; *yX*, young xylem; *oX* old xylem) peroxidases separated into cytoplasmic (*S*), ionically bound (*E*) and covalently bound (*C*) enzymes. The activity was expressed as $\Delta\ DO_{557nm} \cdot min^{-1} \cdot g^{-1}$ dried material. **A'** Syr-oxidase activities in winter of the same peroxidase fractions. The activity was expressed as $\Delta\ DO_{530nm}\ min^{-1}\ g^{-1}$ dry material. **B** and **B'** represent, respectively, the differences of PPD-PC- and syr-oxidase activities between winter and spring. A positive value indicates a greater peroxidase activity in lignifying tissue (spring) than in dormant tissue. A negative value indicates a lower activity in spring

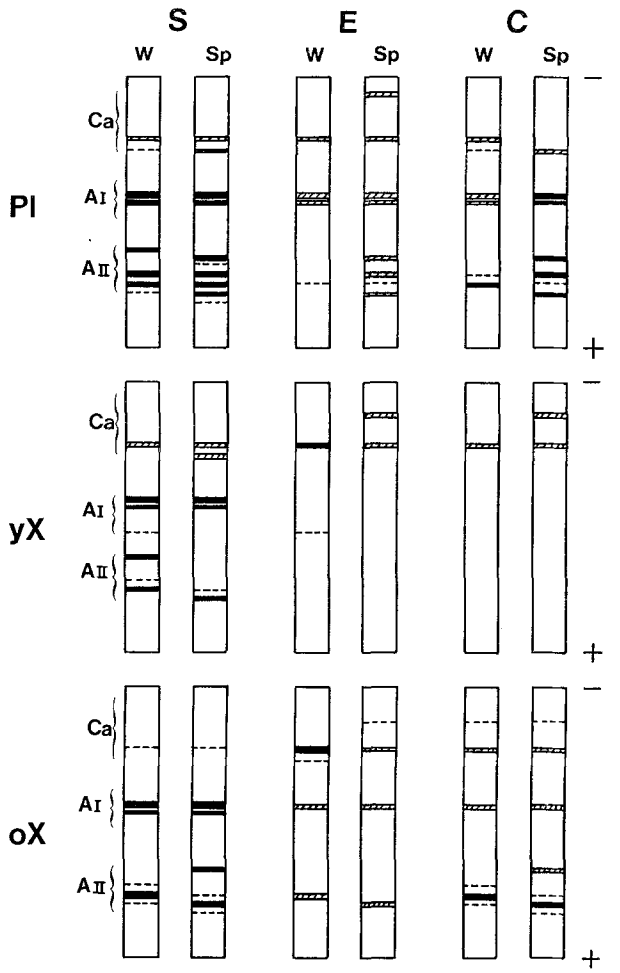

Fig. 2. Isoenzyme patterns of each peroxidasic fraction (symbols see Fig. 1) in lignification (*Sp*, spring) and dormant (*W* winter) seasons. The isoperoxidases were stained with PPD-PC; *Ca* "cationic" isoperoxidases; A_I anionic low-migrating and A_{II} anionic fast-migrating isoenzymes

dases. Some A_{II} isoenzymes appeared specific for winter and spring tissues. The apparent lack of A_{II} isoperoxidases in cell-wall fractions of young xylem might be caused by the very weak activity of these fractions. When gels were incubated in a syr-oxidase-staining medium, only isoperoxidases A_{II} were visible (no reaction in young xylem was observed because of its low peroxidase activity). The A_{II} isoperoxidases are different in active and dormant tissues and are able to oxidize syr-

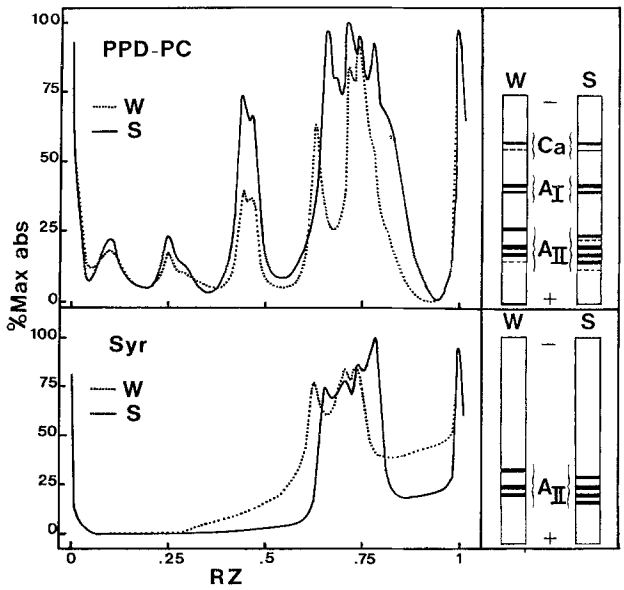

Fig. 3. Isoenzyme patterns of phloem cytoplasmic peroxidases stained with PPD-PC and syringaldazine. The stainings were estimated densitometrically and comparisons made between spring (*S*) and winter (*W*). Three isoperoxidase groups appeared: *Ca*, cationic; A_I, anionic low-migrating and A_{II}, anionic fast-migrating isoenzymes

ingaldazine. This A_{II} group could contain the isoperoxidases involved in lignification. In order to characterize these isoenzymes, the most active fraction (cytoplasmic phloem) was then investigated. It may appear paradoxical to choose phloem fractions for lignification studies but phloem contains many fibers. The cytoplasmic isoperoxidase patterns of winter and spring phloem stained with PPD-PC and with syringaldazine are represented in Fig. 3. Both Ca and A_I isoperoxidases exhibited only quantitative differences whereas the A_{II} isoperoxidase group contained obviously different isoenzymes (the first band RZ = 0.1 appearing with PPD-PC as substrate is only an artefact).

Separation of the isoenzymes by ion-exchange chromatography. In order to isolate A_{II} "spring" isoperoxidases, phloem cytoplasmic peroxidases of the growing season were separated by anion-exchange chromatography (Fig. 4). Three peaks of peroxidase activity were found with PPD-PC but only one with syringaldazine. The unbound peroxidases ("cationic column": Cc) only oxidized PPD-PC. The first peroxidases eluted by the NaCl gradient presented only PPD-PC-oxidase activities and were called A_Ic; the main peak presented both PPD-PC- and syr-oxidase activities and was called A_{II}c. Fractions of each peak were pooled, dialysed against H_2O and reduced to a small volume. The A_{II}c specific activity was twice the specific activity of the phloem cytoplasmic fraction.

Properties of isoperoxidase fractions obtained after ion-exchange chromatography. The isoenzyme composition of each group was checked with PPD-PC (Fig. 5). Whereas Cc exhibited a slight contamination by A_Ic isoenzymes, both A_Ic and A_{II}c exhibited well-separated bands. Kinetic constants of the three peroxidase groups and of the phloem cytoplasmic fraction are shown in Table 2. With PPD-PC, A_Ic isoperoxidases exhibited the highest affinity. In contrast, with syringaldazine the highest affinity was obtained with isoperoxidases A_{II}c. The K_m for H_2O_2 (with PPD-PC as substrate) did

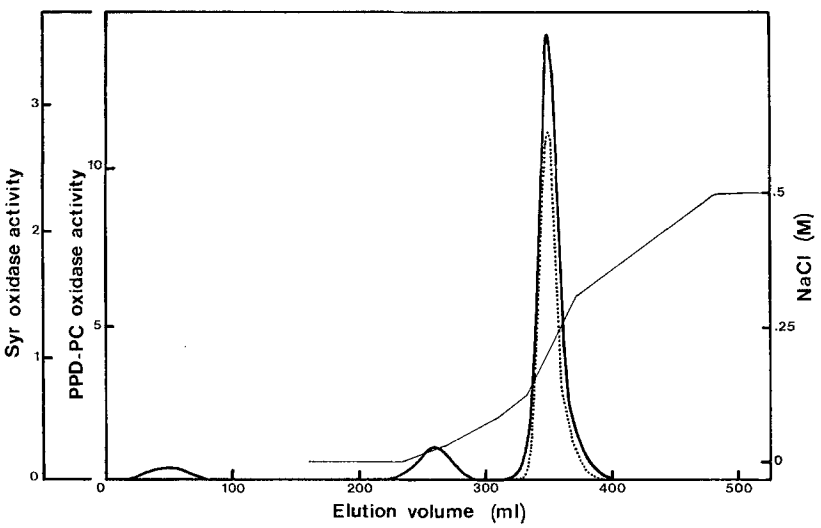

Fig. 4. Ion-exchange chromatogram of spring cytoplasmic phloem peroxidases on diethylaminoethyl-sepharose CL-6B (20 cm long, 1.7 cm inner diameter). After sample loading (approx. 13 mg protein) the column was washed with 0.01 M Na-K phosphate buffer pH 7.2, after which bound peroxidases were eluted by a NaCl gradient (0–0.5 M, 300 ml). Collected fractions were anlysed for their PP-PC-oxidase activity (——) expressed as DO_{557nm} min^{-1} g^{-1} DW and for their syr-oxidase activity (·····) expressed as DO_{530nm} min^{-1} g^{-1} DW

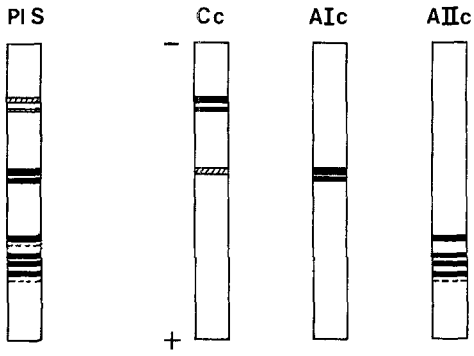

Fig. 5. Isoenzyme patterns of phloem cytoplasmic peroxidase in spring (*Pl S*) and of the fractions collected after ion-exchange chromatography. *Cc*, "cationic column"; *AIc*, "anionic I column"; *AIIc*, "anionic II column". The peroxidases were revealed using PPD-PC as substrate

Table 2. Michaelis constants (10^{-4}M) of spring phloem cytoplasmic peroxidases (Pl S) and of the three groups of peroxidase after anion-exchange chromatography: Cc, AIc, AIIc

Peroxidase fraction	Substrate		
	PPD-PC	Syringaldazine	H_2O_2
Pl S	5.3	10	4.1
Cc	16.7	–	3.3
AIc	1.6	–	3.3
AIIc	6.2	1	4.2

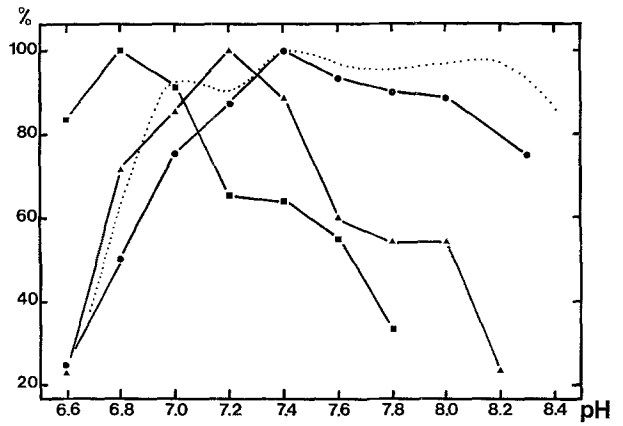

Fig. 6. Effect of pH on the oxidation of PPD-PC by phloem cytoplasmic peroxidases (·····), Cc (▲–▲), AIc (■–■) and AIIc (●–●). pH assays were performed with 0.1 M Tris-HCl buffer. The activities are expressed as per cent of the maximal activity for each fraction

not show any significant differences among all the fractions.

The effect of pH on the oxidation rates of PPD-PC by the different isoperoxidase groups is illustrated in Fig. 6. Phloem cytoplasmic peroxidases showed a broad pH optimum extending from 7.0 to at least 8.2. Each isoenzyme group showed stricter optima: 6.8 for A_Ic, 7.2 for Cc, and A_{II}c which contained several isoperoxidases showed a pH optimum extending from 7.4 to 8.2.

Each isoenzyme group exhibited different heat sensitivities with PPD-PC as substrate. After 12 min at 80° C, only 40% of the initial activity remained in A_Ic fraction, whereas A_{II}c exhibited about 70% of activity.

Discussion

Histochemical and biochemical investigations on peroxidase activities show important differences between tissues isolated during the growing and the dormant seasons. In section of branches, both PPD-PC and syr-oxidase activities are maximal during the lignification season (except in old xylem). Differences were also observed in vitro but are more difficult to analyse. The increase of old xylem activity noticed in winter had already been observed by Nelson (1978) who noticed a parallel increase in other physiological parameters. Ethylene production and nitrogen content of heartwood in cherry and walnut are maximal in the dormancy season. Nelson (1978) suggested that heartwood formation was maximal during dormancy and minimal when cambial activity was the greatest. Shain and Mackau (1973) had previously reported that oxygen uptake was maximal during winter in heartwood of *Pinus radiata*. In young poplar branches, "old" xylem (one or two years old) seems to show the same annual behaviour as heartwood of cherry, walnut and *Pinus*. In contrast, a decrease of phloem syr-oxidase activities during spring was observed only in vitro. These discrepancies between in vivo and in vitro enzyme activities have been observed by Nelson (1978).

In each tissue, cytoplasmic peroxidases present a high syr-oxidase activity, although peroxidases involved in lignification processes are known to be localized in cell walls (Gross 1980). Mäder et al. (1975) suggested that the isoperoxidases involved in the polymerization of lignin monomers were localized in the free space of the wall. The dilute buffer used for the extraction of the "cytoplasmic" enzymes could then solubilize these isoperoxidases.

The quantitative differences observed between spring and winter can be correlated with qualitative difference in the isoperoxidase patterns. Similar development of isoperoxidase patterns during the year had already been demonstrated in needles from *Picea abies* (Esterbauer et al. 1978) but with no relevance to lignification processes.

The poplar isoperoxidases which show syr-oxi-

dase activity and differences between dormant and growing seasons are anionic fast-migrating enzymes (group A_{II}). This electrophoretic behaviour allows a comparison with the G_I group of *Nicotiana tabacum* (Mäder et al. 1975). These anionic fast-migrating isoperoxidases of tobacco were also reported to be specially involved in the polymerization of lignin monomers (Mäder et al. 1980).

Another characteristic of A_{II} isoperoxidases may be related to lignification. These isoperoxidases present a very high resistance to heat treatment; 70% of the initial activity still remains after 12 min at 80° C. Similarly, in parallel histochemical observations, sections of branches showed a PPD-PC-oxidase activity restricted to lignifying cell walls after 30 min at 100° C.

In conclusion, the properties of A_{II} isoperoxidases (isoenzyme differences between winter and spring, affinity for syringaldazine, resistance to heat treatment) indicate that these isoenzymes are involved in the polymerization of substituted cinnamyl alcohols to lignin.

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