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Changes in Dehydrodiferulic Acids and Peroxidase Activity against Ferulic Acid Associated with Cell Walls during Growth of *Pinus pinaster* Hypocotyl[1]

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Hydroxycinnamic acids associated with hypocotyl cell walls of dark-grown seedlings of *Pinus pinaster* Aiton were extracted with 1 N NaOH and identified by gas chromatography-mass spectrometry. The main hydroxycinnamic acid found was ferulic acid. Diferulic acid dehydrodimers were also found, with the 8,8-coupled isomer (compound 11) being the dehydrodiferulate present in the highest amount. However, the 5,5-coupled isomer, commonly referred to as diferulic acid, was not detected. Two truxillic acids, 4–4′-dihydroxy-3–3′-dimethoxy-α-truxillic acids I and II, were tentatively identified. The 8,8-coupled dehydrodiferulic acid (compound 11) was the phenolic acid that showed the most conspicuous changes with hypocotyl age as well as along the hypocotyl axis. Peroxidase activity against ferulic acid was found in the apoplastic fluid as well as being ionically and covalently bound to the cell walls. The peroxidase activity increased with hypocotyl age as well as from the subapical toward the basal region of the hypocotyls. A key role in the cell-wall stiffening of 8,8 but not 5,5 dimerization of ferulic acid catalyzed by cell-wall peroxidases is proposed.

The polysaccharides of plant cell walls contain hydroxycinnamic acids that are present as an ester-linked side chain. Ferulic acid has been identified as being ester linked to arabinoxylans in monocotyledonous plants (Kato and Nevins, 1985; Hartley and Ford, 1989; Hartley et al., 1990b) and to pectic arabinans and galactans in dicotyledonous plants (Fry, 1982). Ferulic and *p*-coumaric acids have been identified as major hydroxycinnamic components bound to gymnosperm secondary cell walls (Strack et al., 1987).

Ferulic acid is able to undergo dimerization through peroxidase-mediated oxidative coupling to produce dehydrodiferulic acid, which cross-links matrix polysaccharides. The 5,5-coupled dehydrodiferulic acid, commonly referred to as diferulic acid, had been the only dehydrodiferulate reported as a cross-link between cell-wall polysaccharides (Kato and Nevins, 1985; Wallace and Fry, 1994). Recently, however, Ralph et al. (1994) identified new dehydrodiferulates coupled through 8,5, 8,8, 8-*O*-4, and 4-*O*-5 formed by peroxidases in grass walls. Cyclodimers formed photochemically from wall-bound *p*-coumaric and ferulic acid (truxillic and truxinic acid types) were also reported in monocotyledonous and dicotyledonous plants as a mechanism for cross-linking polysaccharide chains (Hartley et al., 1988, 1990a; Eraso and Hartley, 1990).

A key role of cell-wall peroxidases in the stiffening of the cell wall through the formation of biphenyl bridges between wall polymers and, consequently, in the cessation of cell elongation has been postulated (Fry, 1986). Peroxidases have been found to be soluble in the apoplastic fluid and linked by ionic or covalent bonds to *Pinus pinaster* wall (Sánchez et al., 1995).

The purpose of this work was to identify and quantify phenolic acids associated with pine hypocotyl walls at different stages of growth and to study peroxidase activity associated with pine cell walls in order to establish a relationship among hydroxycinnamic acids, cell-wall-associated peroxidases, and growth cessation.

MATERIALS AND METHODS

Plant Material

Seeds of *Pinus pinaster* Aiton were soaked in running tap water for 24 h and germinated and grown at 25°C in darkness (Sánchez et al., 1995). Whole hypocotyls were harvested at 7, 10, 13, and 16 d after soaking. Ten-day-old hypocotyls were divided into four 5-mm sections from the cotyledonary node toward the base and the sections were named I, II, III, and IV, as the distance from the cotyledonary node increased.

Cell-Wall Preparation and Hydroxycinnamic Acid Extraction

Whole hypocotyls or hypocotyl segments were immediately killed by boiling in methanol for 10 min and stored in methanol until further use. The rehydrated material was homogenized, treated with pronase and α-amylase from hog pancreas (Sigma), and washed with water, acetone, methanol:chloroform (1:1, v/v), and ethyl ether before air drying. The dry residue was considered to be the cell-wall preparation (Lorences and Zarra, 1986).

Hydroxycinnamic acid ester linked to polysaccharides was released from pine hypocotyl cell walls as described by

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Abbreviations: FA-FA, 4–4′-dihydroxy-3–3′-dimethoxy-α-truxillic acid; TMS, trimethylsilylated.

Eraso and Hartley (1990). Milled walls (100–50 mg) were shaken under N_2 with 1 N NaOH (5 mL) at 25°C for 24 h. Sinapic acid (100 μg), not present in this material, was added as internal standard. The suspension was centrifuged and the residue was washed with H_2O (2 × 1 mL). The supernatant and washings were combined, acidified to pH 2.5 with 6 N HCl, and extracted with diethyl ether (4 × 4 mL). The combined ether extract was evaporated under vacuum and the residue was dried under a stream of N_2. The dry residue was silylated by the addition of 20 μL of *N,O*-bis(trimethylsilyl)trifluroacetamide plus 20 μL of pyridine and shaken for 15 min.

All handling of phenolic solutions and their derivatives was carried out under white fluorescent light to avoid UV radiation, which causes *cis-trans*-isomerization of substituted cinnamic acid (Hartley and Jones, 1975).

Phenolic Acid Analysis

TMS derivatives (1 μL) were analyzed by GC-MS on a fused silica column (PTE-5, 30 m × 0.32 mm i.d., film thickness 0.25 μm, Supelco [Bellefonte, PA]). The column was held at 150°C for 2 min, programmed at 10°C min^{-1} to 300°C, and held for 30 min. Samples were injected using the splitless mode. For quantitative determination of phenolic acids, TMS derivatives were separated by GC as described above and detected by flame ionization. The same column and chromatographic conditions were used, except that the column oven temperature was programmed at 5°C min^{-1}. The response factors for 8,8- and 8,5-dehydrodiferulates used for their quantification were those given by Ralph et al. (1994).

Protein Extraction

Apoplastic peroxidases from intact hypocotyls at all ages or from 10-d-old hypocotyl regions were collected from intercellular and wall spaces by vacuum infiltration and low-speed centrifugation (Fry, 1988; Sánchez et al., 1995). After that the hypocotyls (10 g) were homogenized in 50 mM NaCl (50 mL) at 4°C with a Polytron (Kinematica, Luzern, Switzerland) and filtered through Miracloth (Calbiochem), and the residue, after washing with acetone at −20°C and ice-cold distilled water, was considered the cell-wall preparation. Wall proteins were extracted with 1 M LiCl (50 mL) at 4°C for 24 h with magnetic stirring. The suspension was filtered (Whatman GF/A) and dialyzed against distilled water, and the filtrate was concentrated (10 mL) with an ultrafiltration cell (PM 10, Amicon, Beverly, MA). This fraction was considered to be the ionically bound protein. The residue was exhaustively washed with water and treated with a mixture of 0.5% cellulase (EC 3.2.1.4) and 2.5% pectinase (EC 3.12.1.15), both from *Aspergillus niger* (Sigma), in 0.1 M sodium acetate buffer, pH 5.0, for 24 h at 25°C with shaking. The suspension was centrifuged at 10,000*g* for 30 min and the supernatant was dialyzed against distilled water and concentrated (10 mL). This fraction was considered to be the covalently bound protein fraction.

Protein content of the different extracts was measured as described by Bradford (1976) using BSA as the standard.

Peroxidase Assay

Peroxidase activity was measured using ferulic acid as substrate (Takahama et al., 1992). The oxidation of ferulic acid was measured spectrophotometrically following the absorbance decrease at 310 nm (extinction coefficient = 11.3 mM^{-1} cm^{-1}) in a reaction mixture (total volume 1 mL) containing 40 μM ferulic acid, 90 mM sodium-phosphate buffer, pH 4.0 to 5.5, 10 μg of protein extract, and 0.5 mM hydrogen peroxide. Apoplastic and covalently bound peroxidases showed maximum activity at pH 5.5 and 4.0, respectively. However, the ionically bound peroxidase showed two maxima at pH 5.5 and 6.5, and it was higher at pH 5.5.

RESULTS AND DISCUSSION

Phenolic Acids

The ester-linked phenolic acids were released from pine cell walls by treatment with 1 N NaOH and analyzed by GC-MS (Table I). Their release by NaOH hydrolysis at room temperature is in agreement with their association with cell walls through ester linkages probably linked to pectic polysaccharides, as has been shown for dicots (Fry, 1983). The retention time relative to both sinapic and 5,5-coupled dehydrodiferulic acid, as well as the mass fragmentation pattern, were used for qualitative analysis of phenolic acids. *Trans-* and *cis*-ferulic acid and *trans-p*-coumaric acid were identified as the major monomeric phenolic components in pine hypocotyl cell walls. These hydroxycinnamic acids are commonly found to be ester and/or ether linked to primary cell walls of higher plants (Bacic et al., 1988). Some hydroxybenzoic acids such as *p*-hydroxybenzoic, vanillic, protocatechuic acids, and aldehydes such as vanillin and syringaldehyde were also detected, although the amounts of these were very low (data not shown).

Phenolic dimers were also found and identified by relative retention times and GC-MS to be some of the new dehydrodiferulates recently reported by Ralph et al. (1994) in grass cell walls. Comparison with the relative retention times of these dehydrodiferulates to the 5,5-coupled dehydrodiferulic acid (used in a reference sample), coupled with the mass spectra reported for their fully TMS derivatives, allowed us to identify them as compounds **9**, **10**, and **11** (Fig. 1). These three dehydrodiferulates were first reported by Ralph et al. (1994) in grass cell walls, and to our knowledge this is the first report for gymnosperm cell walls. The identification by GC-MS of compound **9** corresponds to the presence in the cell walls of the ester-linked compound **5** formed via 8,5 dimerization. The detection by GC-MS of compounds **10**, derived by the extraction procedure from compound **6** and/or **7**, and **11** from **7** and/or **8**, shows the presence in the cell wall of compounds **6** and/or **7**, and **7** and/or **8**, respectively. Although it is not possible to assess which of these three dehydrodiferulates (**6**, **7**, or **8**) are present in the cell walls, all of the three compounds are

Table I. *Main phenolic acids associated with pine hypocotyl cell walls identified by full-scan GC-MS of their TMS derivatives*

Phenolic Acid	RRT[a] (SA)	RRT (DFA)	Major Ions (relative abundance)
cis-Ferulic acid	0.74	0.35	73 (100), 219 (36), 249 (53), 293 (25), 308 (36), 323 (37), 338 (M, 68)
trans-*p*-Coumaric acid	0.75	0.36	73 (100), 219 (94), 249 (41), 293 (54), 308 (M, 51)
trans-Ferulic acid (1)[b]	0.88	0.43	73 (100), 219 (32), 249 (53), 293 (32), 308 (49), 323 (53), 338 (M, 97)
8,8-Diferulic acid (11)[b]	1.5	0.76	73 (100), 147 (60), 273 (18), 467 (42), 556 (36), 659 (4), 674 (M, 2), 675 (1)
8,8-Diferulic acid (10)[b]	1.6	0.77	73 (100), 147 (23), 209 (24), 389 (2), 467 (7), 556 (1), 659 (1), 674 (M, 2)
8,5-Diferulic acid (9)[b]	1.73	0.84	73 (100), 147 (80), 393 (4), 496 (7), 674 (M, 7), 675 (4)
FA-FA I	1.61	0.78	73 (100), 219 (31), 249 (43), 293 (23), 308 (18), 323 (15), 338 (M-338, 56)
FA-FA II	1.74	0.81	73 (100), 219 (29), 249 (39), 293 (15), 308 (16), 323 (22), 338 (M-338, 45)

[a] RRT, Relative retention time to sinapic acid (SA) and 5,5-diferulic acid (DFA). [b] Compound number as referred to in Figure 1.

formed through an 8,8-dimerization mechanism (Ralph et al., 1994). It seems clear that of the five possible types of dimerization, only two, the 8,5 and 8,8 coupling, are operative in pine hypocotyl cell walls.

However, the 5,5-coupled dehydrodiferulate commonly reported for monocot and dicot cell walls has not been found in our material. The absence of 5,5-coupled diferulic acid cannot be caused by its destruction during the extraction and derivatization procedure, because when it was exogenously added to wall preparations its TMS derivative was detected by GC-MS (data not shown).

Two stereocyclodimers of ferulic acid were also tentatively identified. These compounds had similar mass spectra and showed the same major ions as the mass spectrum of the TMS derivative of *trans*-ferulic acid. The ratios of the relative quantities of m/z 338 to 308 and 249 to 219 of both isomers I and II were 3.1, 2.8 and 1.4, 1.34, respectively. These high ratios indicate that these compounds are FA-FA dimers (head-to-tail dimerization) (Ford and Hartley, 1989; Hartley et al., 1990b). These stereodimers were named FA-FA I and FA-FA II by their retention times. Truxillic acids have been reported as wall components for Gramineae (Hartley et al., 1990a) and Leguminosae (Eraso and Hartley, 1990) walls, but they have not yet been reported for other taxonomic groups. It has been suggested that such dimers are produced in vivo by photodimerization (Hartley et al., 1988, 1990b), but its presence in walls of etiolated pine seedlings suggests that light might not be necessary for its cyclodimerization, at least in our experimental material.

The quantitative analysis of the phenolic acids associated with pine cell walls and their changes with hypocotyl age, as well as along the hypocotyl axis, was also performed using GLC equipped with a flame-ionization detector. The amount of ferulic acid ester linked to wall components, probably pectins (Fry, 1982), did not show significant differences with hypocotyl age, at least as expressed on a cell-wall dry weight basis (Fig. 2). Thus, it seems that the ferulic acid incorporation to wall polymers is balanced with the synthesis of new polymers. A similar situation was found when its content was studied along the hypocotyl, with the exception of the basal region, where its content was much higher (Fig. 3). This increase in the amount of ferulic acid ester linked to the wall components in the basal region, which has already ceased to grow (Lorences et al., 1990), might be caused by wall differentiation. The amount of *p*-coumaric acid released from the cell wall was low and did not present any important change with hypocotyl age or along the hypocotyl axis.

However, the situation in the dehydrodiferulate components was different, with the most conspicuous changes being in the 8,8-coupled dehydrodiferulic acid, compound **11**. This dehydrodiferulic acid increased with hypocotyl age (Fig. 2) as well as from region I through region IV (Fig. 3). Compound **10** (8,8-dehydrodiferulate) was detected only in the basal region of 10-d-old hypocotyls (Fig. 3). Its amount in the whole hypocotyl increased from d 10 to d 16 (Fig. 2). Compound **9** (8,5-dehydrodiferulate) was detected only in 13- and 16-d-old hypocotyls (Fig. 2). Although the diferulic content of cell walls appears to be somewhat low, Neukom (1976) suggested that even low levels would be enough to cause appreciable cross-linking of wall polysaccharides, modifying wall extensibility (Wallace and Fry, 1994). In fact, if the amount of dehydrodiferulic acid ester linked to pine cell walls (Figs. 2 and 3) is compared with the changes in growth capacity of pine hypocotyls with age (Lorences and Zarra, 1986) and along the hypocotyl axis (Lorences et al., 1990), an inverse relationship is found. Thus, it seems feasible that the formation of dehydrodimers between feruloyl residues ester linked to pectic polymers increases wall cross-linking, decreasing cell-wall capacity for extension. This mechanism has been proposed for dicots (Fry, 1986) and monocots (Kamisaka et al., 1990) through the formation of 5,5-dehydrodiferulic bridges. However, the absence of the 5,5-coupled dehydrodiferulate in pine hypocotyl cell walls and the presence of the other dehydrodiferulates, mainly 8,8-coupled, leads to the idea that the 8,8-coupled dehydrodimers must be the main agent responsible for such cross-linking. Furthermore, the presence of different dehydrodiferulates in greater

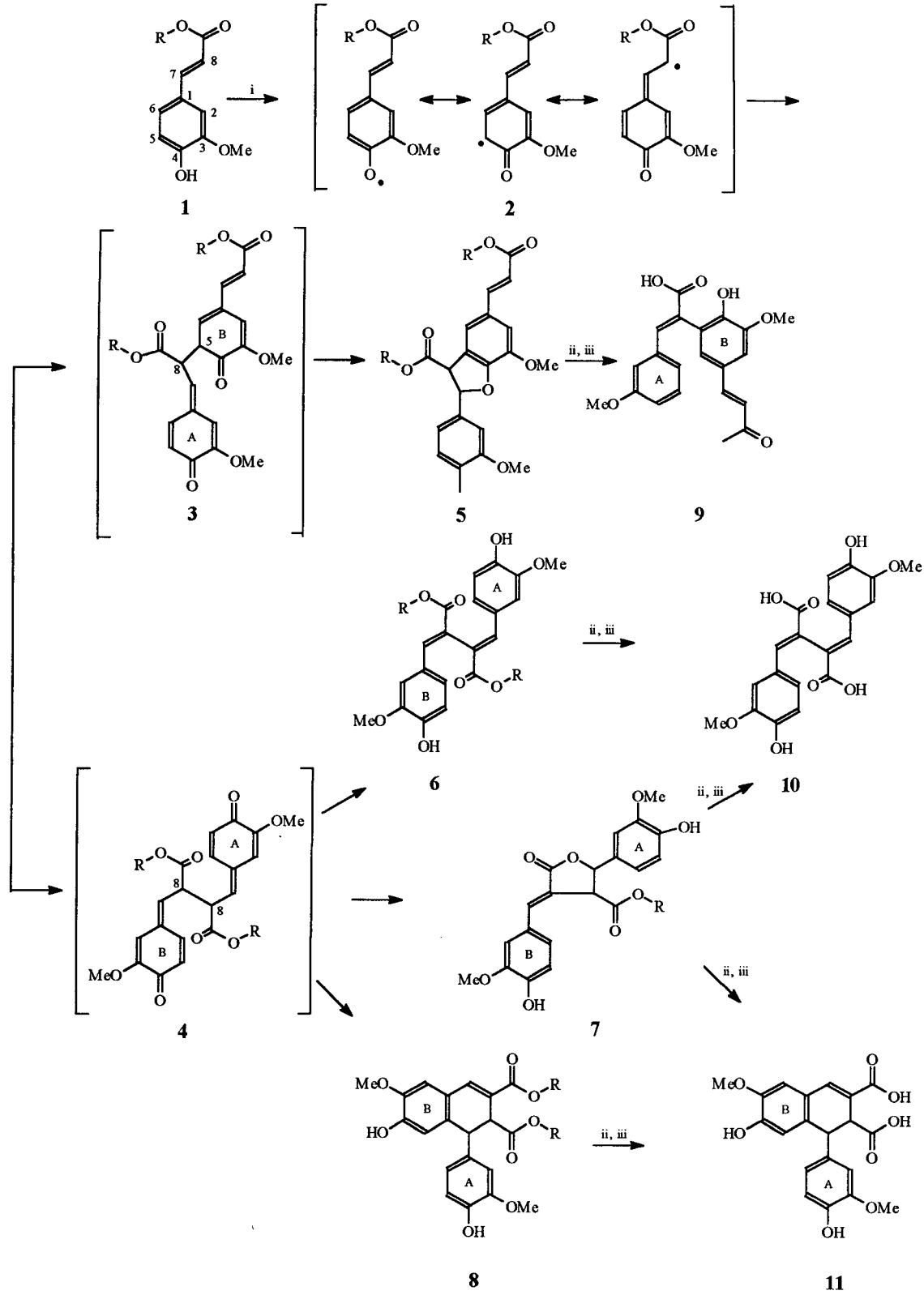

Figure 1. General chemistry of formation and saponification of dehydrodiferulates detected in pine hypocotyl cell walls. Dimerization of ferulate esters via phenoxy radical **2** gives intermediates **3** and **4**, which react in the cell wall to form dehydroferulate esters **5** to **8**. During chemical analysis involving saponification and HCl acidification, esters **5** to **8** are converted to dehydrodiferulic acids **9** to **11**. Only 8,5- and 8,8-dimerization involved in the formation of intermediates **3** and **4** are shown. Reagents and conditions are as follows: i, peroxidase; ii, NaOH; iii, HCl. (Adapted from Ralph et al., 1994.)

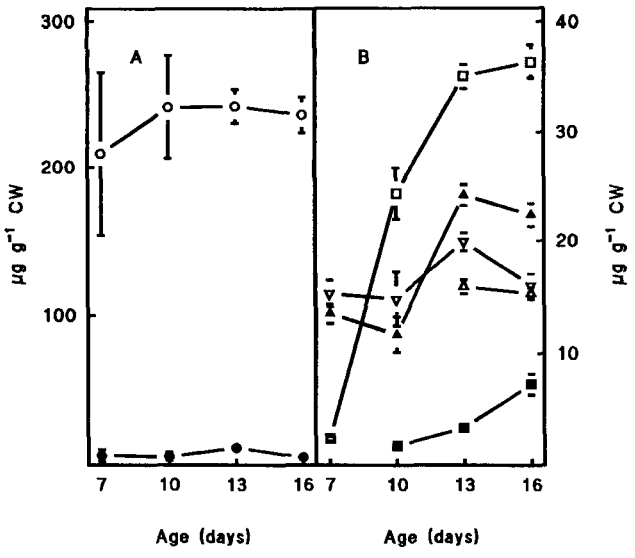

Figure 2. Changes in the amount of hydroxycinnamic acids released by alkali extraction from cell walls with pine hypocotyl age. A, Monomeric hydroxycinnamic acids; B, dimeric hydroxycinnamic acids. ○, *cis* + *trans*-Ferulic acid; ●, *p*-coumaric acid; □, compound 11; ■, compound 10; △, compound 9; ▲, FA-FA I; and ▽, FA-FA II.

amounts than the 5,5-coupled ones in grass cell walls (Ralph, 1994) is in agreement with that hypothesis.

Peroxidase Activity

Biochemical interest in hydroxycinnamic acids ester linked to wall polysaccharides is due to the fact that they apparently undergo oxidative coupling in vivo to yield cross-linked polysaccharides. Such cross-linking is thought to decrease extensibility of cell walls, leading to the cessation of cell growth. The oxidative coupling was carried out by peroxidases associated with plant cell walls (Wallace and Fry, 1994). The peroxidase activity associated with pine hypocotyl cell wall as a possible agent responsible for ferulic acid dimerization was also studied. Peroxidase activity against ferulic acid has been found to be associated with pine cell walls to different extents, freely soluble in the apoplastic fluid, as well as ionically and covalently bound to cell walls (Figs. 4 and 5). Most of the peroxidase activity was present in the covalent fraction, whereas the lowest activity was found in the apoplast fluid. The peroxidase activity in the three fractions, apoplastic and ionically and covalently bound, increased with hypocotyl age (Fig. 4) and as hypocotyl growth decreased (Lorences and Zarra, 1986; Sánchez et al., 1995). When the peroxidase activity was studied along the hypocotyl axis, its activity in the three fractions increased from the subapical (II) through the basal region (Fig. 5). Because the growth capacity decreased from the apical to the basal region of the hypocotyl (Lorences et al., 1990), it is possible to assume a negative relationship of peroxidase activity toward ferulic acid and growth capacity. A similar relationship between peroxidase and growth along the plant axis (Goldberg et al., 1987), as well as with plant age (Valero et al., 1991), has already been proposed for dicot axes, suggesting a role for peroxidases in the cell-wall stiffening. Furthermore, the ability of pine peroxidases to catalyze wall cross-linking through ferulic acid esterified to polysaccharides has already been shown (Whitmore, 1976).

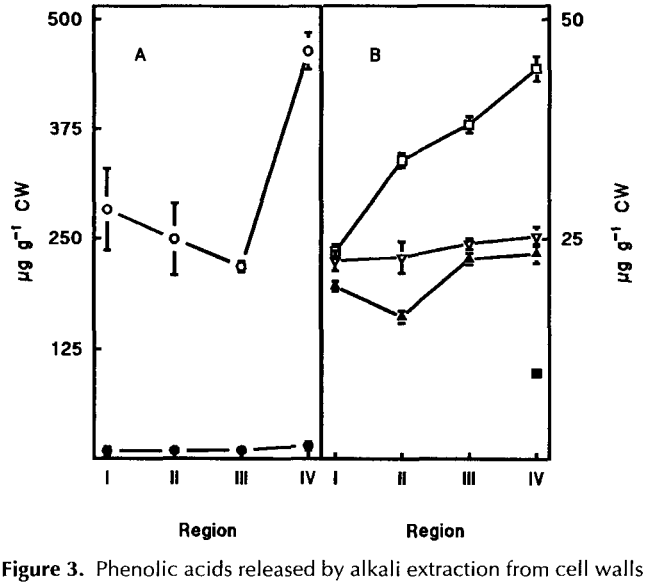

Figure 3. Phenolic acids released by alkali extraction from cell walls of different hypocotyl regions. Ten-day-old pine hypocotyls were divided into four 5-mm regions and named I, II, III, and IV, as the distance from the cotyledonary node increased. A, Hydroxycinnamic acids; B, dehydrodiferulic acids. ○, *cis* + *trans*-Ferulic acid; ●, *p*-coumaric acid; □, compound 11; ■, compound 10; ▲, FA-FA I; and ▽, FA-FA II.

CONCLUSIONS

We have presented evidence that dimerization of ferulic acid in pine hypocotyl cell walls takes place primarily

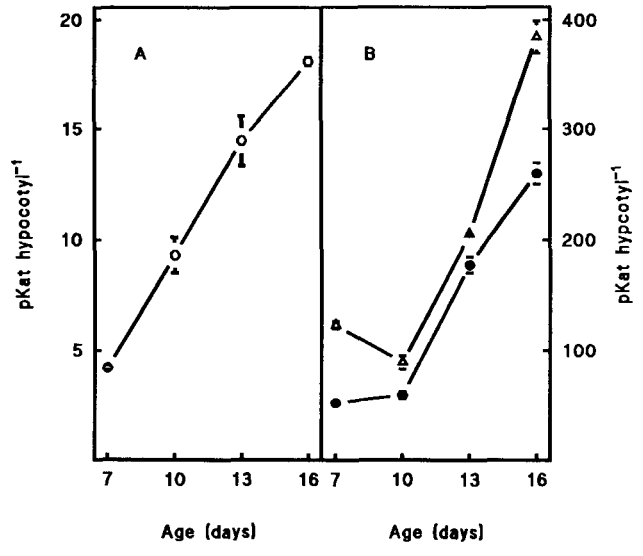

Figure 4. Changes in peroxidase activity with hypocotyl age. Shown are apoplastic (○), ionically bound (●), and covalently bound (△) peroxidase activities. Values shown are means of three different experiments.

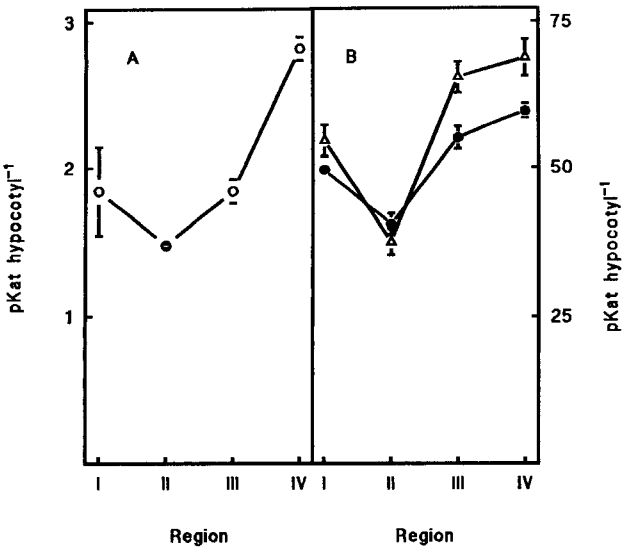

Figure 5. Changes in peroxidase activity along the hypocotyl axis. Shown are apoplastic (○), ionically bound (●), and covalently bound (△) peroxidase activities. Values shown are means of three different experiments.

through 8,8- and 8,5-coupling mechanisms and not through 5,5, as has been proposed for other higher plants. The capacity of peroxidases associated with pine cell walls to oxidize ferulic acid and the inverse relationship found between the content of ester-linked dehydrodiferulates (mainly 8,8 coupled) and growth capacity strongly support dimerization of ester-linked feruloyl as an important factor in the wall stiffening, causing growth cessation.

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