Hormonal Regulation of Cell Development and Polyphenol Biosynthesis in Cultured *Populus trichocarpa* Cells

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ABSTRACT

Hormonal Regulation of Polyphenol Biosynthesis in Cultured *Populus trichocarpa* Cells

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The purpose of this study was to investigate the regulation of polyphenol biosynthesis in plant cell walls using *Populus trichocarpa* as a model woody angiosperm. When 2,4-dichlorophenoxyacetic acid (2,4-D) was present in the culture medium, cells grew most rapidly and did not form tracheids. An estimated 15% of the cell wall material was composed of polyphenol. If the tissues were placed on media containing α-naphthaleneacetic acid (NAA) or NAA plus a cytokinin, growth rate was slower, polyphenol content increased, and tracheids formed. Cells transferred from NAA plus BAP to medium containing 2,4-D gradually produced less polyphenol and lost the ability to differentiate into tracheids. After tissues were returned to media containing NAA plus BAP, these hormone effects were reversed. Polyphenol content was directly proportional to the cytokinin concentration in the medium, while the effect of cytokinin on tracheid formation was biphasic.

The polyphenol in cultured tissues resembled wood lignin in several ways. 1. Histological staining with classical lignin reagents suggested that tracheids and some parenchyma-like cells contained lignin. 2. Alkaline copper hydrolysis and permanganate oxidation of purified methylated cell walls yielded the same methoxylated benzoic acid products as wood. 3. A small amount of C6-C3 product was recovered

after thioacidolysis of walls from tracheid-forming tissues, suggesting that some polyphenol was derived from phenylpropanoid units.

PAL activity correlated with level of tracheid development in cultured tissues. Anionic peroxidases were present in all tissues, but, with syringaldazine as substrate, peroxidase activity in ionic and medium fractions was greater in tissues that produced tracheids.

Several cloned *P. trichocarpa* cell lines were isolated which contained higher or lower than average polyphenol levels that remained stable. When highly habituated clones were cultured with cytokinin, they grew slowly and their oxidized walls yielded less *p*-hydroxybenzoic acid and more syringic acid. Less habituated clones grew faster with added cytokinin and yielded more *p*-hydroxybenzoic acid.

Shoots could be regenerated from several clones. Plants from a stable low-polyphenol cell line were grown in the greenhouse. Analysis of wood in a 2-year old stem from this plant indicated that it contained lower lignin than the parental tree.

CHAPTER 1

Introduction

Lignin is a major structural component in the cell walls of vascular plants. It is a three dimensional, amorphous polymer with an apparently random distribution of stable carbon-carbon and ether bonds (Adler, 1977). Lignin contributes about 20 to 30% of the dry mass of wood, and, after cellulose, represents the second most abundant natural polymer. In the plant, it serves to provide strength and rigidity to the cell wall and a barrier to disease. The proportion of phenylpropanoid subunits in lignin can vary considerably with species, cell type and stage of development (Gross, 1977). The means by which lignin content and composition are controlled in cell walls are poorly understood.

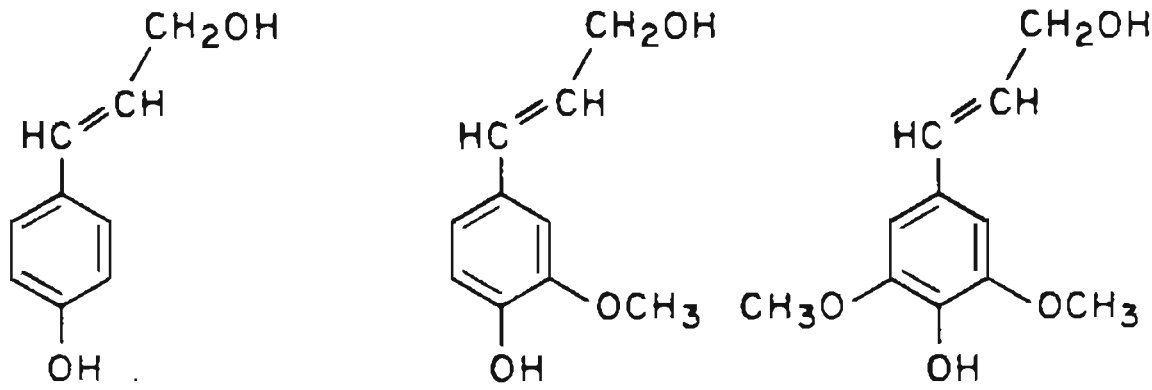
The purpose of this chapter is to describe what is known about lignin structure, its biosynthesis and its presence in cultured tissues. The focus of experiments described in subsequent chapters is on regulation of the type of phenolics found in cultured *Populus trichocarpa* tissues and how changes in their content and composition responded to changes in hormone treatment. Three approaches were used in assessing hormonal regulation of polyphenol biosynthesis. First, polyphenol content and composition were measured in purified cell walls from tissues cultured on various hormone treatments, and results from the different treatments were compared. Second, activities of two enzymes involved in lignin biosynthesis (phenylalanine ammonia lyase and peroxidase) were measured and correlated with hormonal treatments. In the third approach, clonal analysis of variant cell lines monitored the hormonal effects on polyphenol in variant cell lines of *P. trichocarpa* . These studies provide a better understanding of the relationship between polyphenol

content and composition and the differentiation of lignified cells. Furthermore, the generation of low lignin plants from *P. trichocarpa* clones with lower polyphenol levels would be useful in paper production, and could lead to a more simplified pulping and bleaching processes.

The structure of lignin

Lignin is formed in cell walls by the enzyme-mediated, free-radical polymerization of three cinnamyl alcohols: *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Figure 1.1). The relative proportions of these alcohols in lignin depend on plant species and tissue type (Freudenberg, 1968; Sarkanen and Hergert, 1971). Higuchi *et al.* (1977) has defined three major groups of lignins: guaiacyl lignin found in most conifers, lycopods, ferns and horsetails; guaiacyl-syringyl lignin found primarily in dicotyledenous angiosperms and a few gymnosperms; and guaiacyl-syringyl-*p*-hydroxyphenyl lignin found in grasses and other monocots, compression wood of conifers, and many members of the Salicaceae family.

Guaiacyl lignin is composed principally of coniferyl alcohol, with small amounts of *p*-coumaryl and sinapyl residues. Freudenberg *et al.* (1962) estimated that the relative proportions of *p*-coumaryl, coniferyl and sinapyl alcohol in spruce lignin were 14 : 80 : 6, respectively, while Erickson and Miksche (1974) gave a somewhat higher estimate for coniferyl alcohol units (5 : 95 : 1). Beech wood, a representative hardwood species, contains guaiacyl-syringyl lignin. Freudenberg (1968) estimated that this wood was composed of *p*-coumaryl, coniferyl and sinapyl alcohol units in a ratio of about 5 : 49 : 46. Guaiacyl-syringyl-*p*-hydroxyphenyl lignin from grasses is thought to be composed of approximately equal proportions of



p-COUMARYL ALCOHOL

CONIFERYL ALCOHOL

SINAPYL ALCOHOL

Figure 1.1 Lignin precursors: substituted cinnamyl alcohols.

all three cinnamyl alcohols (Higichi *et al.*, 1977). Alternatively, the presence of *p*-hydroxyphenyl groups may be attributed, at least in part, to *p*-coumaric acid esterified with lignin (Higuchi *et al.*, 1967).

Because lignin is extensively polymerized to other cell wall components, isolation of total lignin in a chemically unaltered state is a major problem in lignin chemistry. Many early investigators used such preparations as "dioxane lignin", "alkali lignin", "phenol lignin" or "Klason lignin". These lignins are classified according to their method of isolation, and they are more or less modified from the natural state. For example, Klason lignin (Klason, 1908) is the acid-insoluble material that remains after treatment of lignified tissue with cold 72% sulfuric acid followed by refluxing the diluted acid solution (Pearl, 1967; Effland, 1977). Although this material is a vastly modified form of lignin, the insoluble residue has been used as an estimate of the lignin content in plant tissues (Venverloo, 1969). "Brauns' native lignin" is a lignin preparation obtained by extracting ground plant tissues with ethanol (Brauns, 1962). A very small amount of low molecular weight material is obtained after purification from the ethanol extract, and is unlikely to be representative of the bulk of lignin. Industrially produced lignins from either the sulfite or kraft pulping processes are also significantly modified from the natural state (Lundquist *et al.*, 1977).

Milled wood lignin (MWL) is a much less modified lignin preparation. It is extracted from ball-milled plant tissues using neutral solvents (Bjorkman, 1956; Bjorkman and Person, 1957). About 30 to 50% of the lignin in ball-milled wood can be extracted using 9:1 dioxane-water, and this lignin typically contains a few percent carbohydrate (Lundquist and Simonson, 1975). The average molecular weight of MWL is 15,000-16,000 (Chang *et al.*, 1975). Residual cellulose may be

removed from MWL by treatment with cellulases (Pew and Weyna, 1962). These lignins are generally believed to be representative of the bulk lignin in plants (Adler, 1977), although they contain a somewhat increased hydroxyl content as a result of the milling process (Chang *et al.*, 1975).

A synthetic model lignin, generally referred to as DHP (dehydrogenation polymerizate) (Freudenberg, 1965), can be synthesized from coniferyl, sinapyl or *p*-coumaryl alcohols in a system containing phosphate buffer, H_2O_2 and peroxidase (Sarkanen and Ludwig, 1971; Kirk *et al.*, 1975). A very slow addition of alcohol results in a higher molecular weight polymer, while fast addition leads to lower molecular weight compounds. It has been shown by spectroscopic and chemical methods that DHPs contain the same types of intermolecular linkages as those found in natural lignins (Kirk *et al.*, 1975). These DHPs are insoluble in water and have an average molecular weight similar to that of MWL. DHPs can also be formed from cinnamic acids alone or in combination with cinnamyl alcohols (Shimada *et al.*, 1971; Nakamura and Higuchi, 1978).

Since carbohydrate-free lignin preparations are nearly impossible to attain, it is assumed that lignin and carbohydrates are covalently bound (Lai and Sarkanen, 1971, Kosiková *et al.*, 1979, Hemmingson, 1979). Linkages between lignin and carbohydrates have been studied by forming copolymers between cinnamyl alcohols and sugars (Katayama *et al.*, 1980a, b). Experiments with model compounds have demonstrated that quinone methides, which are intermediates in the formation of lignin, are able to add to carbohydrate hydroxyl groups (Freudenberg and Harkin, 1960).

Oxidation is a common method for studying lignin composition, even though this method only leads to partial degradation of the lignin complex (Bland *et al.*, 1950;

Leopold, 1952). When lignin is oxidized with nitrobenzene in alkali, the products are benzyl aldehydes containing methoxylation patterns identical to those found in the lignin-producing cinnamyl alcohols. After permanganate oxidation (Erickson *et al.*, 1973), methylated gymnosperm lignin yields methoxybenzoic acids, including veratric, dehydrodiveratric, meta and isohemipinic acids. Additionally, angiosperm lignins yield 3,4,5-trimethoxybenzoic and anisic acids as well as various dicarboxylic acids and other dimers and trimers (Adler, 1977). Because the lignin molecule is not completely depolymerized by oxidation, such methods are more useful for obtaining information concerning lignin structure and composition rather than for quantitative determination of lignin.

The lignin structure has been deduced by various types of hydrolysis in which the three-carbon side chain is preserved. These methods provide information about the types of bonds formed between monomeric subunits. Hibbert's work involving ethanolysis (reviewed in Kratzl and Claus, 1962) and pressure hydrogenation of wood provided much information on the structure of lignin. Additionally, both model compounds (dimers, trimers and tetramers) and lignin preparations were degraded by catalytic hydrogenolysis (Sakakibara, 1977), acidolysis (Pepper *et al.*, 1959; Adler *et al.*, 1966; Lundquist, 1970; Lundquist and Kirk, 1971), and thioacetic acid treatment (Nimz *et al.*, 1971; Nimz, 1974). Using these methods, hydrolysis products were isolated, quantitated, their structures deduced, and the mechanisms for their cleavages were established. Based on yields from crude and purified degradation products, frequencies of predominant bond types were calculated (Freudenberg, 1965; Adler, 1977; Sakakibara, 1980).

Studies of spruce MWL have indicated that the polymerized guiaicylpropane units are joined by both ether and carbon-carbon linkages (Adler, 1977). The ether

linkages include guaiacylglycerol-ß-aryl ether (approximately 50% of the total number of bonds) and lesser proportions of guaiacylglycerol-α-aryl ether bonds (Figure 1.2). Guaiacyl propane units joined to both ether and carbon-carbon bonds as well as biphenyl, diphenyl and ß-ß linked structures have also been found. Guaiacyl and syringyl lignins share a number of common structures (Larson and Miksche, 1971; Nimz, 1974; Adler, 1977). Guaiacyl and *p*-hydroxyphenyl units, however, have more bonding options available for polymerization on their ring structures than do syringyl residues. Since methoxyl groups block both the three and five positions on syringyl rings, ring-bound ether and side chain linkages are likely on these compounds, whereas carbon-carbon linkages are not.

Adler (1977) proposed a scheme which depicted a representative portion of a conifer lignin model. Somewhat later, Sakakibara (1980) developed a slightly different model for softwood lignin. Nimz (1974) proposed a similar model for a representative portion of hardwood (beech) lignin (Figure 1.3).

Model structures have been confirmed by spectroscopic methods. These methods include ultraviolet (Aulin-Erdtman, 1949; Goldschmid, 1954), infrared (Smith 1955b, Sarkanen *et al.*, 1967), two-dimensional Fourier transform ^{1}H-NMR (Hatfield *et al.*, 1987), and Raman spectroscopy (Atalla and Agarwal, 1985; Agarwal and Atalla, 1986). Further support for the various structural proposals has come from the application of ^{1}H-NMR (Lundquist, 1979, 1980), ^{13}C-NMR (Lüdemann and Nimz, 1973; Nimz *et al.*, 1981; Newman *et al.*, 1986; Tollier *et al.*, 1986; Lewis *et al.*, 1987). The chemical shifts of nearly all the carbon atoms in the structure shown in Figure 1.3 have been determined from the ^{13}C-NMR spectra of more than 50 model compounds (Lüdemann and Nimz, 1974).

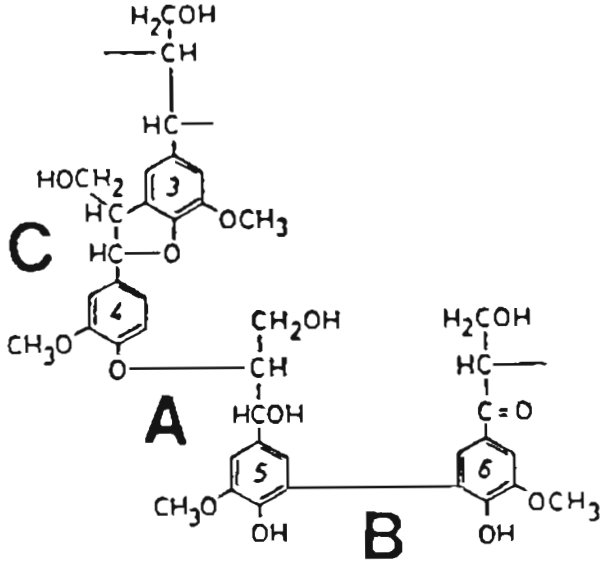


Figure 1.2 Major linkages between phenylpropanoid units in softwood lignin: aryl-glycerol-ß-aryl ether (**A**, 48%); biphenyl linkages (**B**, 9.5-12%); phenylcoumarin structures (**C**, 9-12%) (Adler, 1977).

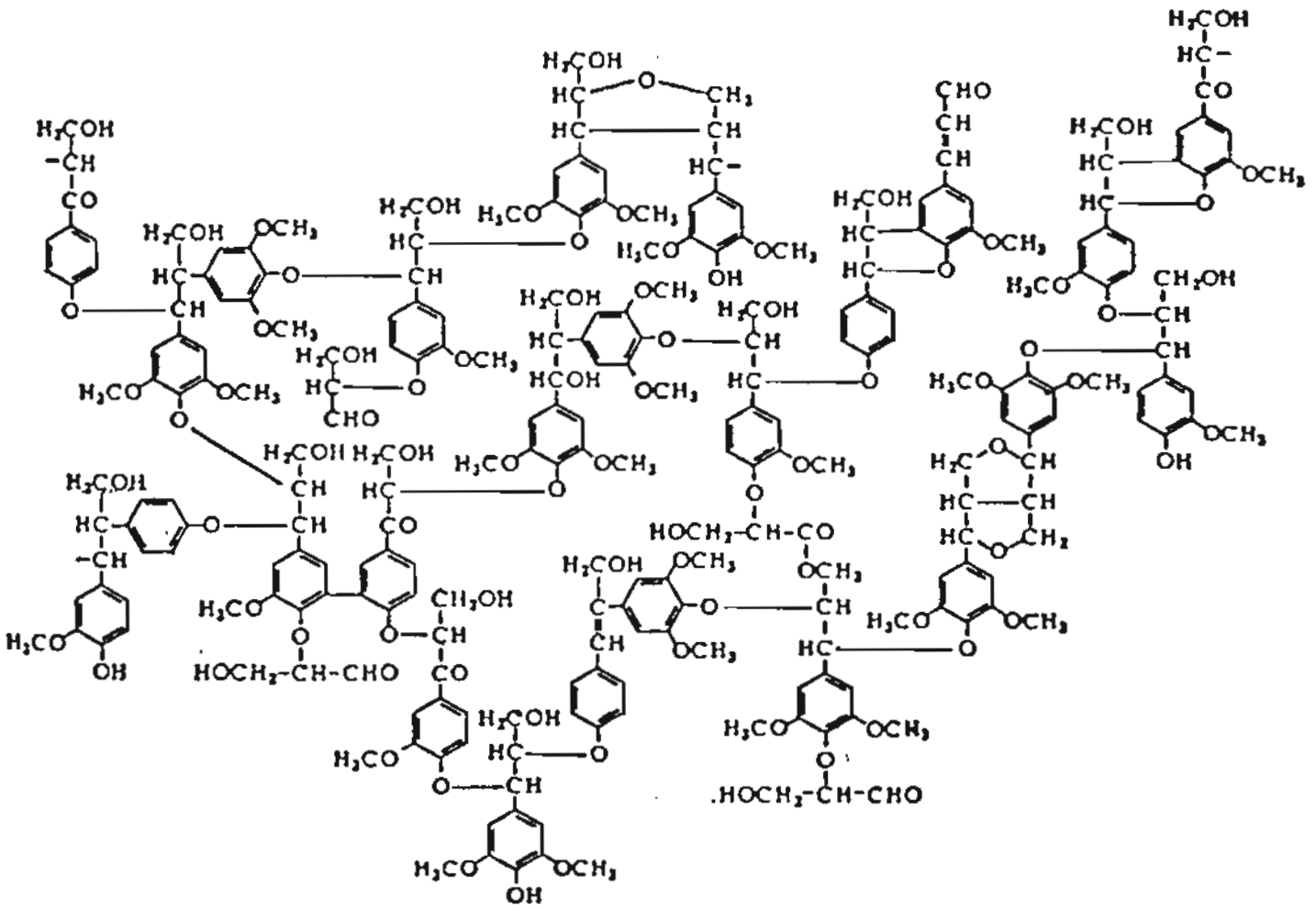


Figure 1.3 A possible structure for Poplar lignin (revised, after Nimz, 1974).

Tissue distribution of lignin

The relative proportions of phenylpropanoid units in lignin vary with plant species and cell type. Ultraviolet microspectrophotometry studies by Fergus *et al.* (1969), Fergus and Goring (1970b) and Musha and Goring (1975) indicated that, in birch wood, various types of lignin are compartmented. Ray-cell lignin was found to be rich in syringyl residues, while vessels, cell corners and the middle lamella were composed mainly of guaiacyl lignin. Hardell *et al.* (1980) oxidized birch samples enriched in either middle lamella, fibers, ray cells or vessels, and obtained results that were in agreement with those from UV microspectrophotometry. Chang and Sarkanen (1973) analyzed four hardwoods, and found that the percentage of syringylpropane units in these lignin preparations varied from 26% to 60%. Tollier *et al.* (1986) found nonhomogeneity and a large variation in lignin monomeric units and carbohydrate content among six hardwood species.

The composition of lignin can vary in a given plant depending on the season and/or stage of development. For example, Higuchi (1957) compared the composition of younger and older parts of bamboo stalks. While younger stalks showed lignin with few syringyl groups and a low methoxyl content, lignin in older sections contained both a high syringyl content and a greater number of methoxyl groups. Early wood of *Robinia* and *Populus* contained predominately guaiacyl lignin, whereas late wood consisted mainly of syringyl lignin (Fukuda, 1983). Similar results were shown in birch wood (Eom *et al.*, 1987). It has also been found that vessels complete their differentiation earlier than the surrounding fibers (Terashima *et al.*, 1986). Deposition of guaiacyl lignin preceeded that of the syringyl type.

A radioautographic study of cottonwood (Selah *et al.*, 1967) showed three different stages of lignification. Tritiated ferulic acid was incorporated first into the

lignin of cell corners in xylem tissues nearest the cambium. Lignification then extended along the middle lamella and radial walls, with regions between adjacent cells lignifying last. Terashima *et al.* (1979) fed [Ring-U-^{14}C-5-^{3}H]double-labeled ferulic acid to pine and poplar cuttings over a three week period of time. They demonstrated that MWL prepared from tissues that were only beginning to lignify contained a more condensed lignin, containing more carbon-carbon linkages at C5, as measured by a lower ratio of ^{3}H to ^{14}C, when compared with MWL from fully lignified tissues.

Other cell wall phenolics

Brown (1966) has suggested that lignin models consisting solely of phenylpropane units are too simple to be representative of many lignins. Smith (1955a) first demonstrated the occurrence of esters of *p*-coumaric acid in sugar cane and of *p*-hydroxybenzoic acid in aspen lignins. Phenolic esters have been noted in the cell walls of many plants (Okabe and Kratzl, 1956; Higuchi *et al.,* 1967; Shimada *et al.,* 1971; Venverloo, 1971; Harris and Hartley, 1976; Scalbert *et al.*, 1986). For example, Smith (1955b) subjected *Populus tremuloides* native lignin to base hydrolysis, and obtained more than 10% of the total estimated lignin as *p*-hydroxybenzoic acid. Lesser amounts of vanillic, syringic and ferulic acids were also recovered. The results obtained by Pearl *et al.* (1957, 1960) indicate that the occurrence of *p*-hydroxybenzoate groups is a common characteristic of *Populus* lignin. Joseleau and Kesraoui (1986) showed that poplar enzyme extracts can be used *in vitro* to produce a DHP-sugar polymer possessing the same type of linkages as those found in isolated poplar wood. It was shown that the majority of the carbohydrates were attached to the phenolic polymer by glycosidic bonds.

Since ferulic acid can be alkylated at both the carbonyl (esters) and the phenolic hydroxyl (ethers and glycosides), it may form cross-links between carbohydrates and other compounds (Fry, 1986). Lignin-carbohydrate complexes were isolated from numerous plant species (McNiel *et al.*, 1984). Azuma *et al.* (1985) demonstrated the presence of alkaline stable as well as alkaline labile linkages between carbohydrate and lignin in beech wood. Fry (1983) has noted that primary walls from spinach cells contain ferulic acid esters which can be released by digestion with Driselase, a mixture of fungal exo- and endo-glyconases lacking esterase activity. He found evidence that the ferulate is linked to pectins. It has been postulated that phenolic polysaccharide-bound esters may provide free-radical initiation sites for the oxidative polymerization of cinnamyl alcohols into lignin (Lewis *et al.*, 1987). Ferulic acid dimers have been isolated from cell walls of wheat germ (Markwalder and Neukom, 1976) and *Lolium multiflorum* (Hartley and Jones, 1976). Diferulates, which are joined as biphenyls, may serve to cross-link carbohydrates in these cell walls, thus rendering them insoluble and contributing to the rigidity of the cell wall (Fry, 1979).

The cell walls of Angiosperms contain extensin, an hydroxyproline-rich glycoprotein that is covalently bound to the primary cell wall (McNiel *et al.*, 1984). Extensin is a rod-like molecule consisting of a protein core and a sheath of ß-linked arabinofuranosides attached to hydroxyproline residues (Fry, 1982). Epstein and Lamport (1984) showed that tyrosine dimers occur as intramolecular crosslinks in extensin. Isodityrosine (IDT) probably contributes to insolubility of extensin and to the stability of the protein. Since IDT is not found in soluble extensins, the most likely mechanism for the synthesis of IDT residues is by the

peroxidase-catalyzed oxidative coupling of tyrosyl residues after their incorporation into the cell wall (O'Niel and Selvendran, 1980).

Lignin biosynthesis

The phenylpropanoid and lignin pathways.

Lignin is synthesized in plant cells by phenylpropanoid pathway enzymes (Higuchi *et al.*, 1977; Grisebach, 1981). The details of this pathway have largely been worked out by using radiolabled tracers (Hanson and Havir, 1979; Gross, 1979) and enzymatic studies (Higuchi, 1981). In this scheme, L-phenylalanine is deaminated to produce cinnamic acid. Substituted cinnamic acids can enter different biosynthetic pathways leading, not only to lignin, but also to flavanoids, stilbenes, benzoic acids and other phenolic compounds (Grisebach, 1979; Knogge and Weissenbock, 1986).

The first enzyme of the phenylpropanoid pathway (Figure 1.4), phenylalanine ammonia-lyase (PAL), catalyzes the *trans* elimination of ammonia from L-phenylalanine to form *trans*-cinnamic acid (Camm and Towers, 1979). Cinnamic acid is then converted, by a sequence of hydroxylation and methylation reactions, to *p*-coumaric, ferulic or sinapic acid. Hydroxylation occurs first in the *para* position, and is mediated by the microsomal mixed function oxidase, cinnamic acid 4-hydroxylase (Pflander *et al.*, 1977). *p*-Coumaric acid may then be further hydroxylated in the 3-position by the action of hydroxylases to form caffeic acid. This is followed by the methylation of the 3-hydroxyl group to form ferulic acid. The latter reaction is catalyzed by a methyltransferase using S-adenosylmethionine as the methyl donor. Hydroxylation and methylation of ferulic acid leads to formation of sinapic acid (Shimada *et al.*, 1970; Poulton *et al.*, 1976).

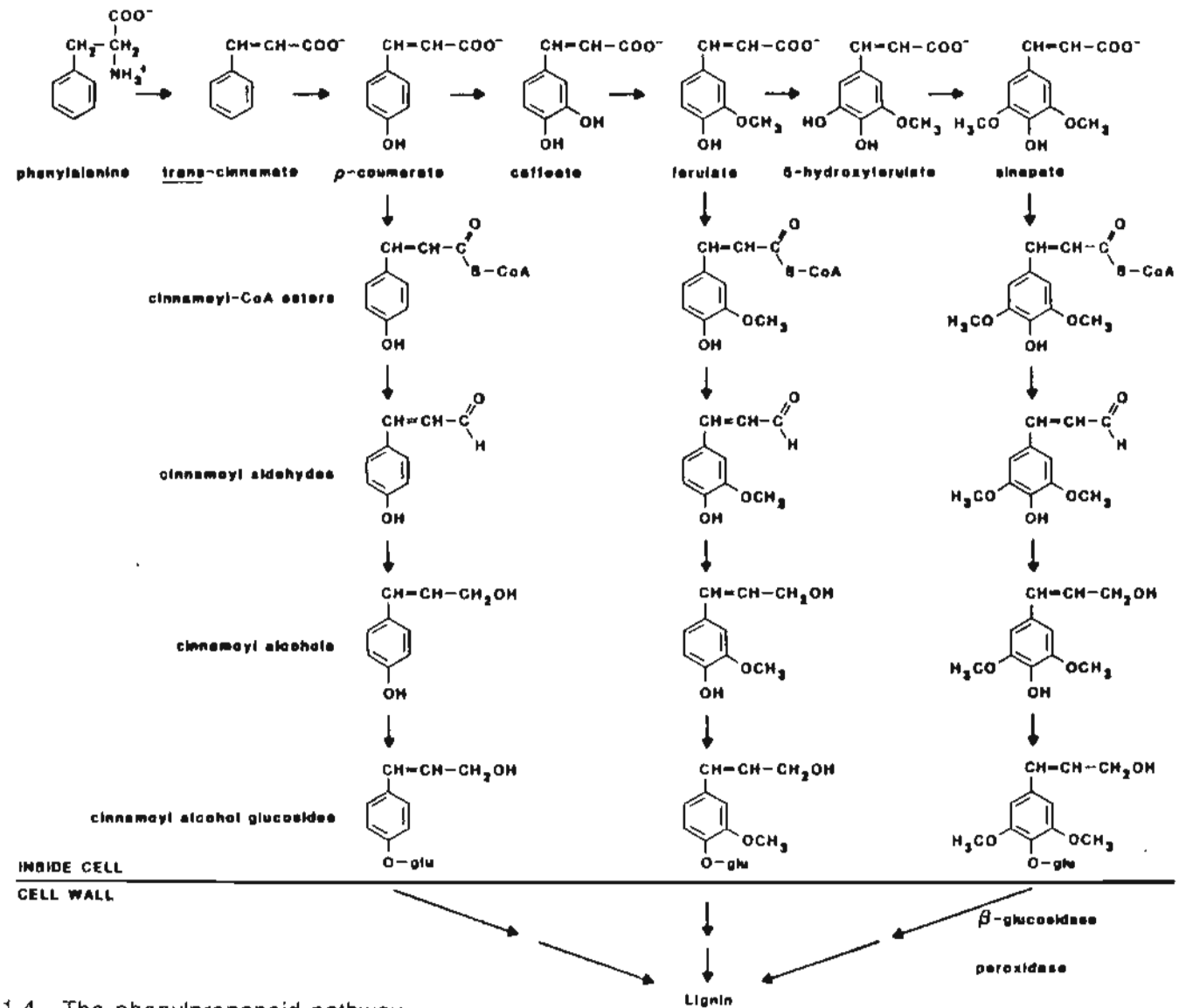


Figure 1.4 The phenylpropanoid pathway.

Multienzyme complexes (Stafford, 1981) may play a role in the control of lignin biosynthesis. Microsomal fractions from a *Quercus* species catalyze the three steps from phenylalanine to caffeic acid (Alibert *et al.*, 1972), and a microsomal fraction from cucumber cotyledons (Czichi and Kindl, 1977) catalyzes the conversion of phenylalanine to *p*- and *o*-coumaric acids. Wagner and Hrazdina (1984) noted that PAL was bound to the cytoplasmic surface of the endoplasmic reticulum in *Hippeastrum*. Cinnamic acid produced by the enzyme reaction could be carried in the membrane and sequentially hydroxylated by membrane-bound hydroxylases (Hanson and Havir, 1981; Butt and Lamb, 1981). The lignin precursors may be packaged in vesicles and transported to the wall by fusion with the plasma membrane.

The final steps in the synthesis of lignin monomeric units result in the reduction of cinnamic acids to cinnamyl alcohols (Higuchi and Brown, 1963; Mansell *et al.*, 1972; Ebel and Grisebach, 1973). First, the substituted cinnamic acids are activated to the corresponding coenzyme A esters. Cinnamic acid Co-A ligases from *Fortythia suspensa* (Gross and Zenk, 1974) and cell cultures of soybean (Knobloch and Hahlbrock, 1975) have been studied in detail. Reduction to the alcohols requires the two enzymes, cinnamoyl-Co-A oxidoreductase and cinnamyl alcohol dehydrogenase.

Freudenberg and Harkin (1963) noted that cambial tissue from actively growing spruce trees contained high concentrations of coniferin (ß-D-glucoside of coniferyl alcohol), but little free coniferyl alcohol or coniferaldehyde. Freudenberg (1965) proposed that cinnamyl alcohols may be transported as glucosides from the cambial zone to lignifying tissues where the glucose is removed prior to lignification. An enzyme that catalyzes the transfer of glucose from UDP-glucose to coniferyl alcohol

has been isolated from suspension cultures of "Paul's Scarlet" rose (Ibrahim and Grisebach, 1976) and from *Forsythia ornata* (Ibrahim, 1977). No correlation was found between the turnover of labeled coniferin and lignin biosynthesis in spruce seedings (Marchinowski and Grisebach, 1977). The relatively slow coniferin turnover and its small pool size suggest that only a part of lignin synthesis occurs by way of coniferin. Since coniferin and syringin are not ordinarily found in lignifying angiosperm tissues (Sarkanen and Ludwig, 1971; Terazawa and Miyake, 1984), glucosides are unlikely to be an important source of lignin precursors.

In 1933, Erdtman proposed the formation of lignin by dehydrogenative coupling of *p*-hydroxycinnamyl alcohols. Two enzymes, laccase and peroxidase, have been implicated in the polymerization of cinnamyl alcohols (Freudenberg and Neish, 1968; Sarkanen and Ludwig, 1971). Higuchi and Ito (1958) found that plant peroxidase, H_2O_2 and coniferyl alcohol produced a lignin-like dehydrogenation polymer with properties similar to that obtained using fungal laccase and O_2. Harkin and Obst (1973) showed that hydrogen peroxide was required for oxidation of syringaldazine in the zone of lignification for both gymnosperms and angiosperms. Substituted cinnamyl alcohols are polymerized by a free radical mechanism initiated by cell wall peroxidases. The first step in this process is the enzymatic removal of an electron and a proton from the phenolic hydroxyl group of an alcohol, producing an aroxyl free radical which can exist in a number of resonance forms (Figure 1.5). These radicals can then polymerize, resulting in the formation of lignin (Harkin, 1967).

Although several peroxidases isozymes have been found in plants (Stafford, 1974), not all of these peroxidases are involved in lignification. Three groups of peroxidase isozymes were found associated with cell walls in tobacco (Mäder,

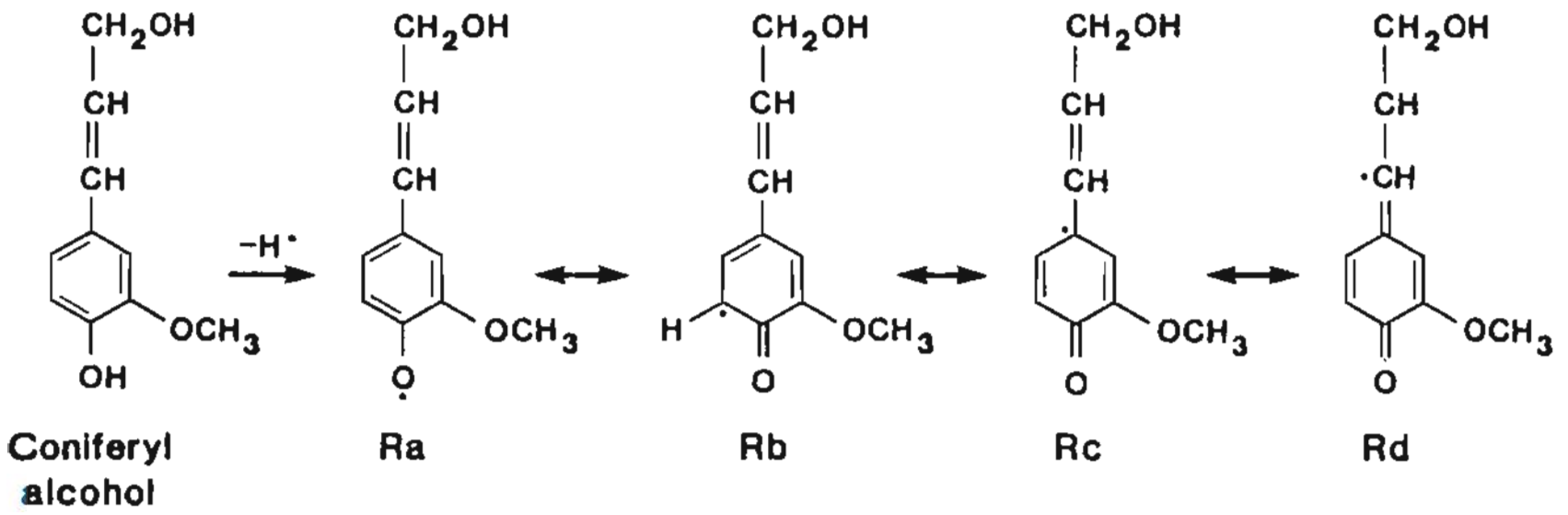


Figure 1.5 Free radicals of coniferyl alcohol.

1976). One group was loosely associated with the wall, while a second group was covalently bound, and a third group was bound ionically. *In vitro* polymerization studies using *p*-coumaryl and coniferyl alcohols showed that a lignin-like substance was formed predominately by the loosely associated and covalently bound peroxidase isoforms (Mäder *et al.*, 1977). Furthermore, Goldberg *et al.* (1986) found that two covalently bound, anionic isoperoxidases in mung bean hypocotyls increased in activity with decreasing cell wall plasticity. These results indicate that peroxidase isozymes specific for lignification are present in plant cell walls.

Regulation of lignin biosynthesis.

PAL, the first committed step in phenylpropanoid synthesis and most studied of the phenylpropanoid enzymes, has been found in lignifying xylem tissues of various plants (Camm and Towers, 1979). Multiple isomeric forms of PAL were isolated from bean tissues (Bolwell *et al.*, 1985). The synthesis or activities of various isozymes may be controlled by environmental or developmental stimuli such as light intensity or quality, hormones, fungal elicitors, and substrate or product concentrations (Hanson and Havir, 1981). Enzyme activity was relatively high in lignifying cells of various plant tissues (Higuchi, 1966; Rubery and Northcote, 1968), but considerably lower in young undifferentiated or highly lignified tissues.

Isozymes of *O*-methyltransferase (OMT) may play a role in directing phenylpropanoid units toward lignin, flavanoid or cinnamate ester synthesis (Hahlbrock and Grisebach 1979). The variations between gymnosperm and angiosperm lignins have been ascribed to the differential ability of these plants to synthesize the three lignin monomers. While investigating substrate specificity of OMTs extracted from gymnosperms and angiosperms, Shimada *et al.* (1970) found that the enzyme from

pine preferentially methylated caffeate, but was relatively inactive toward 5-hydroxyferulate. In contrast, the analogous enzyme from bamboo methylated both of these substrates at approximately equal rates (Shimada *et al.*, 1973). Grand (1984) has characterized a ferulic acid 5-hydroxylase in a microsomal fraction from poplar cells. Sclerenchyma cells containing syringyl lignin showed about twice the ferulate 5-hydroxylase activity of the guaiacyl-rich xylem tissue. These substrate specificities suggest that lignin composition may be controlled, at least in part, by ring hydroxylation (Grand, 1984) and may represent a biochemical explanation of differences between lignins from gymnosperms and angiosperms (Higuchi, 1977).

Mansell *et al.* (1972) isolated a cell-free system from *Salix* which reduced ferulic acid to coniferyl alcohol via coniferaldehyde. This reaction was greatly stimulated by coenzyme A. CoA ligases from several plants have been studied in detail (Gross *et al.*, 1975; McClure and Gross, 1975; Hahlbrock *et al.*, 1976). Multiple forms of cinnamyl CoA ligase were isolated from petunia and showed specificity for caffeate, ferulate or sinapate (Ranjeva *et al.*, 1976). Grand *et al.* (1983) found that CoA ligase extracted from syringyl-rich sclerenchyma tissues was sinapate specific, while that from xylem was not.

The final step in the formation of cinnamyl alcohols involves the conversion of cinnamaldehydes to their corresponding alcohols. Kutsucki *et al.* (1982) showed that gymnosperm cinnamyl alcohol dehydrogenase (CAD) was specific for coniferaldehyde, whereas CAD from angiosperms showed an equal or greater activity with sinapaldehyde. Although multiple forms of the enzyme have been found in a few cases (Mansell *et al.*, 1976; Wyrambik and Grisebach 1975), enzymes from most

plants appear to have broad specificities for the three cinnamaldehydes. Thus, CAD may not be an important enzyme in the control of lignin composition.

Transport of cell wall-forming materials may play a role in the regulation of lignin biosynthesis. In a pulse-chase experiment tracing the incorporation of tritiated cinnamic acid into cell walls of wheat plants, Pickett-Heaps (1968) found that label was concentrated in xylem cell walls. Electron microscopic observation of developing xylem cells showed that label was first localized in the endoplasmic reticulum and golgi bodies. The exact nature of the transported molecules were not investigated. From kinetic studies with spinach cells, Fry (1987) noted that feruloylation of polysaccharides occurred on newly forming arabinose dimers while they were still inside the plasmalemma. These intracellularly formed polysaccharides were secreted and incorporated into the cell wall matrix.

Based on the fact that cinnamyl alcohol glucosides are not found in many plants, Terazawa and Miyake (1984) have suggested that coniferin and the ß-glucosides of the other cinnamyl alcohols may be intercellular storage metabolites. Once the lignin precursors have been transported to the cell wall, polymerization could be controlled by the liberation of the free cinnamyl alcohols by glucosidases and/or by the availability of H_2O_2 or the peroxidase isozymes present (Imberty *et al.*, 1985).

Cell wall peroxidases are probably involved in both the oxidative polymerization of hydroxylated cinnamyl alcohols (Higuchi, 1957; Harkin and Obst, 1973; Mäder 1976), and in the biogenesis of the hydrogen peroxide needed in the final step of the lignification process. Goldberg and Catesson (1985) showed that wall bound peroxidases in poplar appear to be restricted to lignifying tissues. It was further noted by Goldberg *et al.* (1986) that lignification of mung bean hypocotyl was restricted to the walls of elongated cells. Moreover, specific fast-migrating anionic

isoperoxidase activity developed concurrently with the decrease in cell wall plasticity.

Since H_2O_2 is a required substrate in the peroxidative oxidation of cinnamyl alcohols, its availability in the cell wall may control peroxidase activity. Hydrogen peroxide has been detected in high concentrations in the xylem and bark of poplar (Sagisaka 1976). Experiments tracing the origin of H_2O_2 were carried out with horseradish cell walls (Elstner and Heupel 1976) and isolated cell suspensions from *Forsythia* xylem (Gross and Janse, 1977). These cell walls catalyzed the formation of H_2O_2 by a complex reaction requiring NADH and Mn^{2+}. A wall-bound peroxidase was found to be responsible for H_2O_2 formation. The NADH required for this reaction could be supplied by a tightly wall-bound malate dehydrogenase (Gross, 1977). Gross *et al.* (1977) formed a lignin-like dehydrogenation polymer of coniferyl alcohol with the addition of malate to isolated lignifying *Forsythia* cells that had been extensively washed. H_2O_2 was formed by this system. Gross *et al.* (1977) observed that H_2O_2 formation was stimulated by various monophenols, and that the strongest stimulation was obtained with 3×10^{-5} M coniferyl alcohol. This indicates that cinnamyl alcohols or other lignin precursors could be directly involved in regulating the H_2O_2 supply needed for polymerization. Higuchi (1957) suggested that a reducing system such as glutathione-ascorbic acid could be involved in controlling the dehydrogenative polymerization of coniferyl alcohol. As the cells age, the concentration of reducing compounds declines, leading to increased lignification. Various peroxidase isozymes have been isolated from a number of tissues that differ in their degree of lignification.

Growth, differentiation and lignification in plants are all affected by hormones (Galston and Davies, 1969). These growth regulators are active in very small

concentrations. Three hormones often identified with lignification are auxins, cytokinins and ethylene. Auxins such as indoleacetic acid (IAA) regulate cell elongation. They also function coordinately with cytokinins in the regulation of nucleic acid and protein metabolism and cell division. Ethylene is a gaseous compound that elicits cell maturation and senesence responses (Abeles, 1973). Its synthesis is strongly stimulated by auxin. In general, growth hormones have a wide action spectrum, and most cell growth processes appear to be governed by more than one type of hormone.

The central role of auxin as a limiting hormone in xylogenesis was established by Jacobs (1952) and Aloni and Jacobs (1977). After severing vessel strands in *Coleus* stems, Aloni (1976) showed that this wounding increased auxin levels in the stem above the wound and initiated cytodifferentiation of parenchyma cells. Jacobs (1952) and Clutter (1960) showed that the removal of the natural source of auxin (the leaves) could prevent the formation of xylem in wounded tissue. IAA and other nitrogen-containing compounds with mobile electronic systems have been shown to possess antioxidant activity (Pullman and Pullman, 1958). Siegel *et al.* (1959) found that IAA inhibited oxidation of eugenol by a peroxidase from celery vascular tissue. They suggested that, although IAA is essential to vascular differentiation, its antioxidant capacity temporarily suppresses peroxidase activity and lignin deposition, thus prolonging favorable conditions for growth. With the decline of IAA concentrations that accompanies maturity, lignification would increase.

It is generally believed that soluble phenolic compounds in plant tissues are important in the regulation of auxin levels (Grambow and Langenbeck-Schwich, 1983). Their results indicated that the oxidation of IAA was coupled to the oxidation of phenols via the formation of H_2O_2 as an intermediate. The presence of

monophenolic compounds generally increased the rate of the degradation of IAA. If the concentration of phenols was low, excess H_2O_2 was removed by oxidation of IAA to form a methyleneindolenine intermediate.

Although the presence of auxins is necessary for the initiation of lignification in most cases, kinetin and other cytokinins are known to increase the levels of lignification in plant cells. A correlation between the increase in PAL activity induced by hormones and the production of xylem elements has been established by several workers. Using cultured hypocotyl segments of *Helianthus anuus*, Aloni (1982) showed that cytokinins may be important only in the early stages of fiber differentiation. There was no fiber differentiation in the absence of kinetin or zeatin, but these cytokinins could not induce fiber differentiation without the presence of IAA. Shinninger and Torrey (1974) found that xylogenesis in pea stems required a higher concentration of cytokinin than was sufficient for optimal rates of cell division. When studying the relationship of auxin concentration and cytokinin metabolism in tobacco pith explants, Palni *et al.* (1988) noted that the stability of [^{3}H]zeatin riboside was inversely related to the α-naphthalene acetic acid (NAA) concentration in the incubation medium. The observed loss of cytokinin activity was attributed to the activity of a cytokinin oxidase.

Wounding generally promotes ethylene production (Abeles 1972, 1973), and ethylene has a stimulatory effect on lignin synthesis. In swede roots, Rhodes *et al.* (1976) observed that ethylene increased cell wall lignification and the activities of PAL, cinnamate-4-hydroxylase and cinnamate: CoA ligase. Miller *et al.* (1984) noted that lignified tracheary elements formed in *Lactuca* pith explants when an ethylene precursor or an ethylene releasing agent were added with auxin. These results suggest that both auxin and ethylene are required for xylem differentiation in

Lactuca. Additionally, ethylene may control lignification during xylogenesis by the induction of wall-bound peroxidase activity (Miller *et al.*, 1985).

Lignin production in culture

Lignin is associated with cell walls of cultured tissues grown in liquid (Yamada and Kuboi, 1976; Fukuda and Komamine, 1980, 1981) and on solid media (Higuchi and Barnoud, 1966; Venverloo, 1979; Fukuda, 1983). In tobacco suspension cultures, Kuboi and Yamada (1978) found that lignification was initiated between adjacent cells in cell aggregates. Using ^{13}C NMR methods, Nimz *et al.* (1975) showed that MWL prepared from cultured soybean cells was similar to that from hardwood lignin. Stafford (1962) and Romberger and Tabor (1975, 1976) studied the accumulation of water-insoluble phenolic material deposited beneath cultured *Phleum pratense* and *Picea abies* cells. The lignin-like character of these deposits was verified by ultraviolet spectroscopy and histochemical methods.

Tracheary element differentiation has been widely used as a model for plant cell differentiation (Dalessandro, 1973; Torrey, 1975; Roberts, 1975; Gross, 1979; Masuda *et al.*, 1983). This phenomenon is of interest because tracheids are highly lignified cells. Fosket and Torrey (1969) found an average of 5% but up to 20% of the cells in soybean callus could be induced to form tracheids. The experimental treatment producing the highest lignin content in cultured sycamore cells also showed the greatest number of differentiated xylem cells (Carceller *et al.*, 1971). Using a *Zinnia* suspension culture system, Fukuda and Komamine (1980) found evidence for cytodifferentiation of single cells into treacheary elements without intervening mitosis. They observed that approximately 60% of the tracheids that

differentiated from single cells proceeded without intervening cell division, while the remainder of the cells differentiated following mitosis.

The effect of growth hormones can be measured more precisely in cultured tissues than in intact plants. Venverloo (1969) noted that *P. nigra* tissues cultured on media containing IAA and kinetin produced larger numbers of tracheids and much higher lignin levels than those cultured on media containing (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D). The lowest lignin levels were found in tissues grown on 2,4-D-containing media. Generally a high concentration of kinetin inhibits growth and promotes differentiation and organization of cultured cells or tissues (Torrey and Fosket, 1970; Yamada and Kuboi, 1976). Wolter and Gordon (1975) found that callus tissues from *P. tremuloides* that were cultured on media containing 2,4-D or 2,4-D and the cytokinin [6-(3-methyl-2-butenyl)aminopurine] (2-iP) differed in growth rate and lignin content. They noted that auxin-treated tissues grew faster and lignified at a greater rate than those grown on cytokinin-containing media. By contrast, Carceller *et al.* (1971) found higher yields of lignin in sycamore cells on auxin-free media containing kinetin. Bergmann (1964) noted similar results in *Nicotiana*. Thus, although growth regulators can affect changes in lignin and the number of tracheids formed, these effects appear to depend on the combination of hormones used as well as the tissue being cultured.

The molecular bases for interactions between auxins and cytokinins in tissue culture can be studied by investigating changes in activities of those enzymes known to be involved in cell differentiation and lignification. Tracheid differentiation has been correlated with an increase in the activity of PAL. Bevan and Northcote (1979) investigated the dependence of the amount of induced PAL activity on the concentration of exogenous NAA or kinetin in bean suspension cultures. Timing of the

individual effects of each growth regulator on PAL induction was noted. Induction of PAL depended on the addition of NAA within a narrow concentration range, and its presence was required at least three days for enzyme induction. Even high concentrations of kinetin did not inhibit the induction of PAL, and this hormone was required only briefly for PAL induction. It is likely, then, that the two hormones regulate separate events leading to the increase of PAL activity.

Fukuda and Komamine (1982) and Masuda *et al.* (1983) investigated changes in activities of enzymes involved in lignin biosynthesis in suspension cultures of *Zinnia elegans*. Among the enzymes examined, PAL and two wall-bound peroxidases exhibited significant changes in their activities during tracheid differentiation. Yamada and Kuboi (1976) observed lignification in tobacco cell cultures with only kinetin present, but little or none after the addition of the auxins 2,4-D or indolebutyric acid. *O*-Methyltransferase activity in kinetin-treated cultures was considerably higher than in controls, and a rise in enzyme activity coincided with the onset of lignification. Lignin formation in other cultured tissues grown in the presence of kinetin was also paralleled by enhanced activity of PAL (Rubery and Fosket, 1969).

Several investigators have found that callus lignin resembles lignin found in the intact plant. Tracheids produced in callus cultures of *P. nigra* (Venverloo, 1971) gave a weak Mäule reaction (indicating the presence of syringyl residues), while other cells of the callus gave negative results. Furthermore, following oxidation, she found evidence of a higher proportion of syringyl groups in Mäule staining tissues as compared with the tissues not giving a positive Mäule stain. Wolter *et al.* (1974), however, found no evidence of syringyl residues in xylogenic cultures of *P. tremuloides*. Although these findings suggest species variation, they also show that

differences in cell wall components can occur in callus as well as in plants (Fergus and Goring, 1970a).

A number of workers have also noted ways in which lignin structure in cultured tissues differs from that found in wood of the same plant. Higuchi and Barnoud (1966) found that lignin in callus cultures of a number of woody angiosperms contained predominately guaiacyl residues, thus resembling gymnosperm lignin. From this, they concluded that the low frequency of sinapyl residues was due to the immaturity of the tissue and its lack of vascularization. Venverloo (1969) found that the cell walls from callus of *P. nigra* were much more resistant to degradation by oxidation than was the secondary xylem of wood. Fukuda *et al.* (1988), showed similar results from loblolly pine. They compared the sizes of oxidation products from MWL of wood with those from cultured tissues. Their results showed that the wood MWL fractions had a lower average molecular weight, suggesting more complete degradation of wood MWL. This difference was attributed to an increased number of carbon-carbon bonds between rings in callus lignin, leading to a more chemically resistant structure.

The amount of polyphenol in cell walls of cultured tissues can be regulated by the type and concentration of growth regulators present in the media. The relationship between content and composition of this phenolic polymer, however, is unknown. Studies described in the following chapters are designed to investigate the regulation of lignin biosynthesis in *Populus trichocarpa* using cultured tissues as a model system. Various methods of cell wall hydrolysis and chemical analysis were used to characterize the effects of growth regulators on the composition and structure of cell wall phenolics. Content and composition of phenolic polymers in cell walls of the intact plant were compared with those found in cultured tissues to provide a basis for

understanding the extent to which polyphenol in cultured cells represents true lignin.

CHAPTER 2

Materials and Methods

1. **Plant Material.** Stem cuttings of *Populus trichocarpa*, T. & G. were soaked in tap water for three days, rooted in soil and grown in large plastic pots in a greenhouse. Stem tissue used for lignin analysis was taken from the main trunk of a two-year-old tree. Callus cultures were initiated from young branches, disinfected with NaOCl (10 % chlorine bleach), and grown on Murashige and Skoog (1962) (MS) medium solidified with 1% (w/v) Bacto-agar (Difco) and supplemented with 5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma). Established cultures were maintained on basal medium supplemented with 2,4-D, benzylaminopurine (BAP) (Calbiochem), and/or α-naphthalene acetic acid (NAA) (Sigma) as indicated, and subcultured every 4-6 weeks. For experiments using hormones other than 2,4-D. stocks were first transferred to media without added hormones twice in succession for periods of 2 to 3 weeks each in order to remove residual 2,4-D. Subsequent transfers were made to media containing hormones as indicated in the individual experiments. Cultured tissues were grown at 27 °C under constant fluorescent lighting (7-9 W m^{-2} of photosynthetically active radiation).

2. **Growth analysis**. Growth of cultured tissues was expressed as $(W-W_o)/W_o$, where W and W_o are the final and initial fresh weights of the explants, respectively. The degree of cytokinin habituation was assayed after four weeks of growth by comparing the growth of explants on media containing10 μM NAA with that on media containing 10 μM NAA and 1 μM BAP (Meins and Binns, 1982).

3. **Cell counting**. The number of cells and tracheary elements was counted at 160 X magnification using a Zeiss research microscope after chromic acid maceration (Dodds and Roberts, 1982). Five randomly chosen fields were counted for each of 3 to 5 replicates per tissue sample.

4. **Cell wall staining for lignin and peroxidase**. Hand cut sections of poplar stem or pieces of callus were incubated in 1 N NaOH overnight at room temperature. Microscopic observations were made on tissues stained by the following methods: a) Safranin-O. An aqueous solution of 0.1 % safranin-O (Sigma) was added to tissues for about 2-3 hours and destained with several rinses of 1 N HCl (Dodds and Roberts, 1982). b) Phloroglucinol. A 1% solution of phloroglucinol (Sigma) in a 12% HCl-ethanol solution was added to tissue sections, and they were observed after about 3-5 minutes (Browning, 1967). c) Mäule. Solutions of 0.1% $KMnO_4$, 12% HCl and 2 N NH_4OH were consecutively added to tissue sections. Tissues were observed after 2-3 minutes (Browning, 1967). d) Sudan III. Tissues were stained in a saturated filtered Sudan III (Aldrich) solution in 70% ethanol (about 500 µl per 500 mg tissue) for 20 minutes and destained in 50% ethanol (Jensen, 1962). Photomicrographs were taken using a Nikon AFM adaptor with a Polaroid camera fitted to a Zeiss research microscope. e) Peroxidase stains. Transverse sections were hand cut from 1 year old greenhouse grown *P. trichocarpa* stems and from callus tissues that were firm enough to cut. Squash preparations were made from soft callus grown on media containing 2,4-D. Tissues were soaked in 10 mM phosphate buffer, pH 6.8, containing 0.16% (V/V) H_2O_2 for 1 minute, then placed on microscope slides and incubated for 1 to 2 minutes at room temperature with either 2% (W/V) syringaldazine (Aldrich) in dimethylsulfoxide or 0.6 mg/ml 4-

chloro-1-naphthol (Sigma) in PBS (10mM phosphate buffer, pH 6.8, 150 mM NaCl) and 5% methanol (V/V). Control experiments omitted only syringaldazine or 4-chloronaphthol. In some cases, sections were washed under running water or agitated in buffer before staining. Tissues were observed under a light microscope.

5. Cell wall purification. Plant tissue (2 g) was ground at high speed in 4 ml of methanol using a Brinkman Polytron homogenizer. The insoluble residue was collected by centrifugation at about 1500 X g, and extracted for 30 minutes with mixing using each of the following solvents in succession: methanol, acetone, ethylacetate, methanol-water (1:1, V/V) and distilled water (19:1, V/V). Cell walls in water were lyophylized and used for all subsequent analyses.

6. Synthesis of cinnamyl alcohols. *p*-Coumaryl, coniferyl and sinapyl alcohols were synthesized by the method of Allen and Beyers (1949) with several changes. *p*-Coumaric, ferulic and sinapic acids (Aldrich or Fluka) were used instead of the corresponding acetyl ethyl esters. Because of solubility problems with sinapic acid, tetrahydrofuran replaced ethyl ether in the synthesis of sinapyl alcohol. The reduction was carried out under nitrogen in a one liter three-neck flask fitted with a reflux condensor, drying tube, dropping funnel, thermometer and stir bar. The flask was sitting in an NaCl-ice bath that was maintained at about -20 °C during the reaction.

Initially, the flask contained 60 mmol lithium aluminum hydride in 20 ml dry ether or tertahydrofuran (THF). Thirty mmoles of the cinnamic acid and 165 ml ether or THF were dropped in slowly with stirring over a 2 hour period. The mixture was allowed to slowly come to room temperature overnight. The pale yellow

solid product was rinsed with dry ether and centrifuged or filtered. The solid was added to a solution of 60 mmol ammonium carbonate in 50 ml water per g of ammonium carbonate overlain with 100 ml ether. After CO_2 evolution, the ether layer was separated, and the yellow slurry was extracted twice more with 80-100 ml ether. Combined ethereal solutions were evaporated *in vacuo* to approximately 50 ml and added to about 700 ml dry petroleum ether (b.p. 35-55 °C) or hexanes. The container was sealed and placed in the freezer for several days until crystallization was complete. *p*-Coumaryl alcohol crystallized readily, but sinapyl alcohol was extremely difficult to crystallize, and better results were obtained using ethyl ether only. Sinapyl alcohol was also very sensitive to oxidation.

7. Synthesis of DHP. Dehydrogenation polymers (DHPs) were synthesized from each of the three substituted cinnamyl alcohols according to the method of Kirk *et al.* (1975). A 1:1:1 molar ratio mixed polymer was also synthesized from equimolar amounts of the three cinnamyl alcohols. The DHPs were synthesized as follows: Two hundred ml 0.01 M phosphate buffer (pH 6.5) and 190 units of horseradish peroxidase (HRP) (Sigma, type I) per mmole cinnamyl alcohol and 30 mg vanillyl alcohol were stirred in a 1 liter 3-neck flask. Over a 16-20 hour period, equal amounts of these solutions were dropped in from separatory funnels: A) 9.5 mmol cinnamyl alcohol and 800 units of HRP in 400 ml degassed buffer; B) 9.5 mmol H_2O_2 in 400 ml degassed buffer. After stirring 10 hours longer, the mixture was centrifuged, and the precipitate was washed twice with water. The precipitate was dissolved in dioxane and dripped slowly into ether. Ether-insoluble material was collected by filtration, giving 70-80% yields based on starting alcohol.

8. **Synthesis of 1-(3',4'-dimethoxyphenyl)-2-(2"-methoxyphenyl)-1-ethan-1-one-2-ol**. 1-bromo-3,4-dimethoxyacetophenone was synthesized by brominating 3,4-dimethoxyacetophenone in CCl4 according to the method of Kirk *et al.*, (1968). The ß-aryl ether was synthesized according to Adler *et al.* (1952) by refluxing 2 mmol guaiacol (Aldrich) and the bromo-acetophenone (2 mmol) in dry acetone with 2 mmol K_2CO_3. The product was purified by TLC (silica gel; benzene 4: ethyl acetate 1 as solvent system) and crystallized from 95% ethanol 4 times. See page 67 for the mass spectrum.

Samples of other ß-aryl ethers and ß-1 model dimers were obtained from Prof. Michael Gold of our department.

9. **Cell wall analysis**. Total polyphenol content was determined by absorbance at 280 nm after solublization of cell walls with acetyl bromide (Miller *et al.*, 1985). Coniferyl alcohol DHP was used as a standard for quantitation. Cellulose content was determined by the method of Updegraff (1969). Nitrogen was measured by a microkjeldahl procedure (Mitchell, 1972). The protein content was calculated by multiplying the nitrogen content by 6.25. Absorbances were measured on a Beckman Model 25 or a Shimadzu UV-260 spectrophotometer.

10. **Analysis of polyphenol composition**.

a) Diazomethane synthesis. Diazomethane was synthesized from p-toluene-sulfonylmethyl-nitrosamine (Diazald, Aldrich). Diazald (21 g) was dissolved in 200 ml ether and added dropwise into 35 ml of 30% ethanolic KOH (5 ethanol : 12 water). The diazomethane-ether solution was removed by distillation at 60 °C.

b) Oxidation. Purified cell walls were methylated with diazomethane-ether solution, taken to dryness under an N_2 stream, and then oxdized in alkaline $KMnO_4$ as modified from Erickson *et al.* (1973). To 30-50 mg of methylated walls was added in order: 3.75 ml *t*-butanol-water (3:1), 3.75 ml NaOH (0.5 N), 9.4 ml of $NaIO_4$ (0.06 M), and 3.75 ml of $KMnO_4$ (0.03 M). The mixture was incubated for 16 hours at 82 °C in a shaking water bath. The reaction was stopped by adding 1 ml ethanol, cleared by centrifugation, and the supernatant extracted twice with 10 ml of ether. The aqueous phase was brought to pH 7 with 2 M H_2SO_4, 1 ml of 30% H_2O_2 was added, and the mixture incubated at 50 °C for 10 minutes. MnO_2 (20 mg) was then added, the mixture was shaken until gas evolution ceased, and the precipitate was removed by centrifugation. The supernatant was brought to pH 2 with concentrated H_3PO_4 and extracted 3 times with ethyl acetate. The ethyl acetate fractions were pooled and dried *in vacuo*. The residue was taken up in ethyl acetate and silylated with N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) (Sigma or Pierce). Gas chromatography (GC) of silylated oxidation products was accomplished on a 1.7 m x 16 mm i.d. glass column packed with OV-101 using an FID detector. Carrier gas was N_2 (30 ml/min) and the oven temperature was 140 °C for the first two minutes and then increased at 15 °C per minute. Recoveries of oxidation products were estimated from parallel samples to which known amounts of *p*-hydroxybenzoic acid, vanillic acid and syringic acid were added prior to sample methylation. Conditions for GC-MS were the same except the carrier gas was He. Mass spectra of lignin oxidation products were obtained at 70 eV and compared with those of synthetic standards. Alternatively, oxidation products were quantitated by GC on a 15 m fused silica capillary column (J&W) in a Hewlet Packard gas chromatograph (5071A) using an FID detector and a 3390A Hewlet Packard

integrator. A known amount of standards (*p*-hydroxybenzoic, vanillic and syringic acids) were added to parallel samples prior to methylation of cell walls for purposes of quantitation. Yield was calculated from standard curves and verified by GC-MS. Analysis by GC/EIMS was carried out with a VG 7070E mass spectrometer connected to a 5790 Hewlet Packard GC fitted with a 25 m CP-Sil 8 capillary column having a 0.32 μm id and a 2 μm film thickness. Compounds eluting from the GC column were detected by EIMS at 70 ev. Mass spectra for recovered methylated benzoic acids after TMS derivitization (m/e, %) were: 4-methoxy-benzoic acid, 224 (25), 209 (83), 165 (53), 135 (100); 3,4-dimethoxybenzoic acid, 254 (70), 239 (81), 195 (75), 165 (100); 3,4,5-trimethoxybenzoic acid, 284 (100), 269 (95), 225 (87), 195 (97).

c) Alkaline copper hydrolysis. The copper hydrolysis method was revised from Morohoshi (1981): 30 to 50 mg purified cell walls were added to 0.63 ml NaOH (0.70 g/ml) and 2.2 ml $CuSO_4$ (0.23 g/ml) in a teflon lined acid digestion bomb (Parr, model 4746) at 180 °C for 5 hours. After air cooling, the contents were filtered, acidified to pH 2 with HCl, and extracted with ethyl acetate. The extract was dried under N_2 and used for permanganate oxidation as described above. Eight model aryl ether compounds were also treated with copper hydrolysis. See Chapter 4 for data.

d) Thioacidolysis. Thioacidolysis of cell walls was carried out as described by Lapierre *et al.* (1986). Ten mg of methylated or unmethylated cell walls or DHP were placed in a 15 ml ground glass-stoppered tube to which was added 5 ml of 9 : 1 dioxane : ethanethiol (Aldrich) containing 0.2 M BF_3 etherate (Aldrich). The tubes were purged with N_2 and incubated in a 100 °C water bath (in a fume hood) for 4 hours. Tubes were shaken 3 or 4 times during the reaction. After cooling on ice, 5

ml distilled water were added to each tube and the pH was adjusted to 3-4 with 0.4 M $NaHCO_3$. The aqueous solution was extracted 3 times with ethyl acetate, and the organic phase was evaporated *in vacuo*. Reaction products were verified by GC-MS and quantitated by integration of peak area. Mass spectroscopic data for reaction products from DHPs following TMS derivitization (m/e, %) were: *p*-coumaryl alcohol DHP (unmethylated), 388 (2), 327 (1), 239 (100), 205 (16), 75 (35), 73 (23); (methylated), 330 (3), 242 (3), 181 (100), 151 (4), 147 (16), 89 (6), 75 (21); coniferyl alcohol DHP (unmethylated), 418 (1), 403 (1), 269 (100), 75 (23), 73 (28); (methylated), 360 (2), 272 (2), 211 (100), 181 (2), 177(12), 151 (4), 89 (3), 75 (24); sinapyl alcohol DHP (unmethylated), 448 (5), 433 (4), 299 (100), 75 (25), 73 (33); (methylated), 390 (3), 241 (100), 207 (18), 89 (5), 75 (27), 59 (12).

d) Hydrolysis by base and ß-glucosidase. Purified cell wall samples were hydrolyzed in 1 N NaOH at 32 °C for 1 hour. The procedure was repeated a second time, and pooled supernatant was acidified and extracted into ethyl acetate. The solvent was evaporated *in vacuo*, residue dissolved in 0.5 ml ethyl acetate, silylated with 0.5 ml BSTFA, quantitated by GC (fused silica capillary column as described above for oxidation products), and verified by GC-MS. Recovery estimates were based on standard curves of verified substituted benzoic and cinnamic acids. The aqueous extracts were acidified with citric acid and hydrolyzed for 3 days at 32 °C with ß-glucosidase (Sigma) at 2 units per 10 ml sample. Base-treated cell wall residue was also hydrolyzed with ß-glucosidase, 2 units per 10 ml in 5 mmol phosphate buffer at pH 4.5 for 3 days at 32 °C. Hydrolysates were extracted into ethyl acetate and quantitated as for base hydrolyzed samples. Mass spectra for recovered benzoic and cinnamic acids after TMS derivitization (m/e, %) were:

4-hydroxybenzoic acid, 282 (43), 267 (100), 223 (54), 193 (45), 73 (63); vanillic acid, 312 (98), 297 (95), 282 (32), 267 (50), 223 (52), 193 (32), 73 (100); synapic acid, 342 (100), 327 (85), 312 (51), 283 (12), 253 (22), 223 (20), 141 (12), 73 (46); 4-hydroxycinnamic acid, 308 (74), 293 (78), 249 (29), 219 (73), 73 (100); ferulic acid, 338 (100), 323 (41), 308 (32), 249 (24), 73 (41); syringic acid, 368 (100), 353 (31), 338 (54), 249 (18), 73 (44).

11. Isolation of cloned cell lines. One to 2 grams of friable 2,4-D-grown callus was added to a flask containing 50 ml of liquid MS medium (Murashige and Skoog, 1962) supplemented with 5 μM 2,4-D and shaken at 120 rpm for 8 to 12 days. Protoplasts were made using the method of Dodds and Roberts (1982). Alternatively, 10 ml of the suspension was removed and filtered through 190 or 106 μm nylon mesh, and the filtrate concentrated by centrifugation at 200 X g for 2 minutes. An aliquot of supernatant and cells was added to warm (36° C) MS medium supplemented with 0.8% (w/v) agar and 5 μM 2,4-D (maintenance media) and the resulting mixture was plated onto a 100 X15 mm Petri dish containing 40 ml solidified MS media with 5 μM 2,4-D. The plates were sealed with Parafilm and incubated at 27° C in the light for about 3 weeks or until cloned colonies reached at least 1 mm diameter. Colonies were then transferred to vials containing 10 ml maintenance medium, and subcultured every 4 weeks. Approximately the same number of viable colonies were recovered from cultures filtered through 106 μm mesh and protoplast formation.

12. Shoot initiation. 20 to 30 mg callus explants were transferred from maintenance media to media containing 0.1, 1.0, 1.5 or 10 μM BAP. Shoot formation was tallied on each concentration over four or five 4-week transfer periods. Some lines formed shoots more readily when 1 or 3 μM NAA was added to the media. In some cases, shoots were placed on maintenance media for callus initiation or removed and assayed for polyphenol content.

For root generation, shoots were removed from the callus and placed on woody plant medium (WPM) (Lloyd and McCown, 1981) supplemented with 1μM NAA and 0.8% agar in plastic boxes (Magenta Plastics). Approximately 2 weeks later, the shoots were transferred to WPM media containing 1 nM NAA. After roots had begun to form, the shoots were transferred to WPM without hormone supplements. After roots became well developed, the small trees were planted in sterile soil and moved to the greenhouse.

13. Preparation of protein fractions. Two-week-old callus tissues and 1 year-old partially woody stems were ground (Brinkman Polytron) in 2 to 3 ml ice-cold buffer (5 mM Tris-HCl, pH 7.0) per gram tissue and 20% (W/W) insoluble polyvinylpyrrolidone (Sigma). The mixture was centrifuged at 500 X g at 4 °C for 5 minutes. Supernatant was recentrifuged at 18,000 X g for 20 minutes, and the pellet was washed 5 times with buffer. The combined supernatant fraction was used for soluble peroxidase. The pellet was incubated for 2 hours at room temperature with 0.2 M $CaCl_2$, centrifuged at 18,000 X g, and the pellet washed with 4 rinses of buffer. Pooled supernatants were used as a source of ionically bound peroxidase. The pellet comprised the insoluble wall fraction.

Liquids were concentrated under N_2 to 1 or 2 ml with a Swinex ultrafiltration cell (Millipore) (PSEUD, 10,000 MW cut-off filter) or Amicon concentrator (YM 10 Diaflo filter, 10,000 MW cutoff) and the filter was rinsed several times with column buffer (0.05 M potassium phosphate, pH 6.7) . Crude protein preparations were loaded onto a Sephadex G-100 column at 4 °C and eluted with column buffer. Fractions with syringaldazine activity were pooled and concentrated to about 800 µl and further purified by FPLC (Pharmacia) on a GF-450 XL column (DuPont). Syringaldazine positive fractions were concentrated to a small volume (25 to 50 µl) with a Centricon 10 microconcentrator (Amicon).

14. Enzyme assay procedures. Protein was measured according to Bradford (1976) using bovine serum albumin (Sigma) as a standard. The PAL activity was determined by a radiolabeled assay. ^{3}[H] Phenylalanine was purified by 2-dimensional TLC (cellulose, Baker) using the following solvent systems (V/V): butanol : acetic acid : water, 25:4:1 and pyridine : isoamyl alcohol : water, 7:7:6). The reaction mixture contained 0.88 µCi ^{3}[H] Phenylalanine, 20 µM unlabeled phenylalanine, 0.1 ml protein preparation (crude fraction), 0.4 ml of 5 mM Tris-HCl buffer at pH 7. A blank contained either the mixture described above with boiled protein or undenatured protein without phenylalanine. The control contained no protein. After one hour of reaction time at room temperature, the mixture was acidified to pH 2, extracted into ethyl acetate and counted in a scintillation counter (Beckman LS -3150).

Peroxidase was determined by procedures employing syringaldazine or gualacol (Aldrich). Syringaldazine assay (1 ml) contained protein preparation, 0.88 µM H_2O_2, 50 µl 0.1% syringaldazine in 80% methanol and 0.05 M potassium phosphate

buffer, pH 6.7. Oxidation of syringaldazine was estimated by monitoring absorbance at 530 nm. The guaiacol assay (1 ml) contained protein preparation, 1 µM H_2O_2, 50 µl 15 mg/ml guaiacol and 0.05 µM phosphate buffer, pH 6.7. Absorbance was determined at 470 nm. Activity for peroxidase preparations was calculated as absorbance per minute per µg protein.

15. Gel electrophoresis. Protein samples were resolved by isoelectric focusing on 0.35µm thick IEF 3-9 gels using the PhastSystem (Pharmacia). One µl of concentrated protein was added to each lane. Each half of the gel contained matching lanes, and gels were cut in half after separation so identical lanes could be stained with either 4-chloro-1-naphthol or syringaldazine; preparations were the same as those described for histochemical localization described above. The gels stained with chloronaphthol were soaked in reagent solution for 3 to 5 minutes. For syringaldazine staining, the gel was rinsed in the buffer-H_2O_2 solution, and the syringaldazine reagent (2% syringaldazine in 95% ethanol : 0.05M potassium phosphate buffer, pH 6.7, 5:1 (V/V) was sprayed onto the gels to prevent excessive diffusion of the stain. Gels were photographed and /or scanned with a densitometer (620 Video Densitomoter, Bio-Rad) immediately after staining, since the syringaldazine product faded rapidly. Isoelectric points were calculated using IEF standards (Sigma) following visualization by silver stain (Pharmacia).

CHAPTER 3

Characterization of Polyphenols in Cell Walls of Cultured *Populus trichicarpa* Tissues

Lignin is a heterogeneous, random and optically inactive polymer synthesized during secondary cell wall formation in vascular plants. Lignins can be classified according to the type of cinnamyl alcohol residues found in the polymer (Higuchi *et al.*, 1977). Fergus and Goring (1970) provided microspectrophotometric evidence that specific lignins are compartmentalized in birch wood, with guaiacyl lignin occurring primarily in xylem cells and middle lamellae, and with syringyl lignin restricted to the secondary walls of fiber and ray cells. It has been shown that variations in the levels of growth regulators added to culture media can affect the level of cell-wall polyphenol (Carceller *et al.*, 1971; Yamada and Kuboi, 1976) and the percent of cells that differentiate into tracheids (Fukuda and Komamine, 1980). Venverloo (1971) detected syringyl residues in polyphenol isolated from cultured *Populus nigra*, while Wolter *et al.* (1974) did not find evidence for syringyl residues in polyphenol isolated from similar cultures of *P. tremuloides*.

The purpose of this investigation was to study the effects of plant growth regulators on the composition and content of cell walls of cultured *P. trichocarpa* tissues. After varying the type and amount of growth regulator added to the culture medium, we examined the effects of these changes on polyphenol, comparing differences between treatments which produced either numerous tracheary elements or none. Our results show that the amount of syringyl residues in callus cell walls does not vary significantly in the two tissue types. However, the amount and composition of cell-wall polyphenol was under control of growth regulators.

Effects of plant growth regulators on growth and tracheid differentiation.

Tracheid differentiation in callus cultures of *P. trichocarpa* varied markedly depending on the amount and type of growth regulator supplied in the growth medium (Table 3.1). More rapid growth took place in callus grown on media containing higher concentrations of auxins, and the addition of BAP lead to slower growth. Tissues maintained on growth media containing 10 μM α-naphthaleneacetic acid (NAA) and 1 μM benzylaminopurine (BAP) produced large numbers of tracheid elements. When the NAA concentration was increased to 100 μM, tracheids still formed, but only in the absence of BAP supplements. No tracheid differentiation occurred in tissues grown with 5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) as the sole growth regulator in the medium. Tracheid formation was not correlated with growth, and occurred in the most rapidly growing as well as in the most slowly growing tissues (Table 3.1).

Partial composition of cell walls

Polyphenol concentration in cell walls was quantitated following solubilization in acetyl bromide and glacial acetic acid. Lignin preparations have an absorbance maximum near 280 nm. The extinction coefficient, $E_{1\%}$ (concentration as expressed in %, w/v), 1 cm, generally lies between 1200 and 2040 for various lignin preparations at 280 nm (Nord and DeStevens, 1958), whereas that of phenolic acids such as ferulic acid have an extinction coefficient two or three times higher in this region. Measurements of acetyl bromide digests of DHPs and cell wall material (Table 3.2) show that DHPs can vary widely in their $E_{1\%}$. Coniferyl alcohol DHP, which was used as a standard for cell wall polyphenol quantitation, has a very high extinction

Table 3.1

Effects of plant growth regulators on growth and xylem formation in *P. trichocarpa* callus grown for two weeks in culture.

Growth Regulator Treatment [a]	Growth $(W-W_o)/W_o$ [b]	Xylem Cells (Cells/g fr. wt.)
2,4-D (5 μM)	4.95 ± 0.67 (9) [c]	0
NAA (100 μM)	9.61 ± 1.3 (9)	1.35 X 105 ± 0.12 (5)
NAA (100 μM) + BAP (1 μM)	6.43 ± 1.5 (9)	0
NAA (10 μM) + BAP (1μM)	1.85 ± 0.20 (9)	6.49 X 105 ± 3.9 (5)

[a] Growth media were supplemented as indicated with 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP.

[b] Growth is expressed as $(W-W_o/W_o$, where W and W_o are the final and initial fresh weights of the explant, respectively.

[c] Mean values expressed ± s.e.m. (n).

Table 3.2

Absorbance at 280 nm of acetyl bromide digested DHP and cell wall material from *P. trichocarpa* tissues

	$E_{1\%}$ 280 nm [a]	% [b] Coniferyl Alcohol DHP
DHP		
p-Coumaryl Alcohol	1351	60
Coniferyl Alcohol	2233	100
Sinapyl Alcohol	2050	92
1:1:1 mixture	1924	86
Cell Wall Material		
Stem	496	
Callus [c]		
2,4-D	316	
NAA/BAP	453	
NH	446	
Amino acids		
Phenylalanine	0	
Tryptophan	1301	
Tyrosine	240	
Proteins		
BSA [d]	42	
HRP [e]	34	

[a] $E_{1\%}$ at 280 nm measured for each polymer sample as compared to that measured for coniferyl alcohol DHP.

[b] Concentration as expressed in %.

[c] Tissues cultured on media containing 5 μM 2,4-D, 10 μM NAA + 1 μM BAP or no added hormones.

[d] Bovine serum albumin.

[e] Horseradish peroxidase.

coefficient. Purified cell wall material had an $E_{1\%}$ of only14 to 20% of this amount at 280 mn. Because this measurement is affected by both quantity and composition, polyphenol content measured against coniferyl alcohol DHP as a standard may underestimate lignins containing a high proportion of *p*-coumaryl alcohol residues.

The composition of callus cell walls varied with growth regulator treatment. The polyphenol content of callus was correlated with the presence of BAP in the growth medium (Table 3.3). Cell walls from tissues grown with BAP supplements contained approximately 15% more phenolic polymer than cells grown without BAP, whereas polyphenol content appeared to be independent of the type and amount of auxin in the growth medium. Callus cell walls also contained less cellulose than stem wood (Table 3.3), but there was no significant difference in cellulose content with different growth regulator treatments.

The lesser amounts of polyphenol and cellulose in callus cell walls as compared with stem were due primarily to differences in protein, which accounted for about 30% of the callus cell walls but only 4 % of stem wood (Table 3.3). The protein content of cell walls from xylogenic tissues was less than that from non-xylogenic tissues, although some of the protein in callus walls might be attributable to contaminating cytoplasmic protein. Polyphenol, cellulose and protein together accounted for 70-80% of the cell wall, and the remainder is assumed to consist of hemicellulose and xylosans (Updegraff, 1971).

Since 25 to 32% of callus cell walls is composed of protein, it was important to find the contribution of protein to measurements of cell wall phenolics. When horseradish peroxidase (HRP), was treated with acetyl bromide (Table 3.2), the resulting $E_{1\%}$ was equal to approximately 2% of that obtained from coniferyl alcohol DHP. Bovine serum albumin (BSA) gave a similar result. The $E_{1\%}$ of cell wall

Table 3.3

Partial composition of cell walls of *P. trichocarpa* stem and callus tissues.

Tissue	Growth Regulator (treatment)	Tracheid Formation	(mg/g dry wt) Polyphenol	Cellulose	Protein
Stem	- -	+	196 ± 3 (6) [b]	505 ± 23 (3)	37 ± 2 (3)
Callus	2,4-D (5 μM) [a]	-	135 ± 3 (6)	307 ± 12 (3)	323 ± 11 (3)
Callus	NAA (100 μM)	+	134 ± 2 (6)	315 ± 9 (3)	225 ± 7 (3)
Callus	NAA (100 μM) + BAP (1 μM)	-	157 ± 3 (6)	335 ± 27 (3)	309 ± 5 (3)
Callus	NAA (10 μM) + BAP (1 μM)	+	156 ± 3 (6)	297 ± 17 (6)	276 ± 5 (3)

[a] Growth media were supplemented as indicated.

[b] Mean values expressed ± s.e.m (n).

material from callus grown on media containing 5 μM 2,4-D was approximately 316, while that of the proteins is about 10% of this amount. Since only about 30% of the cell wall of this callus tissue consists of protein, contribution of protein to the $E_{1\%}$ at 280 nm of the cell walls would be small. Hydroxyproline-rich glycoproteins (HRGPs) are present in cell walls. Between 1 and 10% of the wall matrix is composed of HRGPs (Fry, 1986). These proteins contain 40 to 60% carbohydrate content, and have a tyrosine content of approximately 6 to 10% (Showalter and Varner, 1988). If 10% of the cell wall protein consists of HRGPs and 40 to 60% od the HRGPs are protein, then a maximum of about 2mg/g of dry cell wall may be tyrosine. This small amount of tyrpsine is not likely to make a significant difference in cell wall polyphenol measurements.

Cell wall oxidation products

Since tracheid lignin is believed to lack syringyl residues, it was of interest to compare the phenolic constituents of polyphenol in xylogenic and non-xylogenic callus tissues. Gas chromatography and mass spectrometry of silylated permanganate oxidation products of methylated walls revealed *p*-hydroxybenzoic acid (**1**), *p*-methoxybenzoic acid (**2**), protocatechuic acid (**3**), vanillic acid (**4**), veratric acid (**5**), and 2,4,5-trimethoxybenzoic acid (**7**) (Table 3.4). Since cell walls were methylated prior to oxidation, and assuming that the methylation was complete, the presence of free phenols among the oxidation products indicates the occurrence of aromatic ether linkages in the phenolic polymer. Although the low yields of free phenols (**1**, **3**, **4**, and **6**) relative to the corresponding methoxy derivatives (**2**, **5** and **7**) (Table 3.4) suggests a relatively low abundance of aryl ether linkages, this result was more likely due to oxidation of free phenols by permanganate. This conclusion is

Table 3.4

Quantitation by gas chromatogaphy of silylated phenolics obtained from permanganate oxidation of methylated cell walls of *P. trichicarpa* stem and callus tissue.

Tissue Treatment [b]	Compound [a] (nmol/mg Polyphenol) 1	2	3	4	5	6	7	Ratio [c] S: V: P	Total [d] (mg/mg Polyphenol)
Stem	32 ± 5 [e]	196 ± 17	23 ± 2	189 ± 12	239 ± 13	nd [f]	73 ± 2	0.17: 1: 0.53	0.12
Callus									
5 μM 2,4-D	48 ± 5	47 ± 3	29 ± 2	76 ± 2	530 ± 15	nd	143 ± 12	0.24: 1: 0.16	0.15
100 μM NAA	82 ± 3	133 ± 12	29 ± 5	307 ± 16	395 ± 23	nd	203 ± 14	0.26: 1: 0.28	0.19
100 μM NAA + 1 μM BAP	19 ± 1	53 ± 2	43 ± 5	163 ± 14	504 ± 16	nd	119 ± 4	0.18: 1: 0.11	0.15
10 μM NAA + 1 μM BAP	34 ± 4	102 ± 4	29 ± 5	175 ± 14	318 ± 7	nd	130 ± 3	0.29: 1: 0.31	0.13

[a] Compounds are as follows: 1, *p*-hydroxybenzoic acid; 2, 4-methoxybenzoic acid (anisic acid); 3, 3,4-dihydroxybenzoic acid (protocatechuic acid); 4, 4-hydroxy-3-methoxybenzoic acid (vanillic acid); 5, 3,4-dimethoxybenzoic acid (veratric acid); 6, 3,5-dimethoxy-4-hydroxybenzoic acid (syringic acid); 7, 3,4,5-trimethoxybenzoic acid.

[b] Growth media were supplemented as indicated.

[c] Ratio of syringyl: S, (6 + 7); vanillyl: V, (4 + 5); *p*-coumaryl: P, (1 + 2) residues. Protocatechuic acid (3) was not included in calculation of ratios.

[d] M_rs of *p*-coumaryl alcohol (1 and 2), caffeoyl alcohol (3), coniferyl alcohol (4 and 5) and sinapyl alcohol (7) were used to calculate total phenolic recovered.

[e] Mean values expressed ± s.e.m. (n = 3); standard deviations were typically about 2 or 3% of the mean.

[f] Not detected.

supported by the fact that the phenolic monomers recovered accounted for only 12-19% of the monomers recovered by acetyl bromide (Table 3.4). Moreover, permanganate oxidation of cell walls without prior methylation gave low yields of phenolic monomers.

The identification of 3,4,5-trimethoxybenzoic acid (**7**) among the cell wall oxidation products indicated the presence of syringyl lingin. The yield of **7** was greater in callus than in stem, but there was no relationship between tracheid formation and the amount of **7** recovered from the callus (Table 3.4). On the other hand, xylogenic calli showed lower levels of coniferyl residues and higher levels of *p*-coumaryl residues than non-xylogenic calli, and in this respect, more closely resembled the phenolic constituents of stem wood (Table 3.4). These results do not support the work of Wolter *et al.* (1974), who failed to detect syringyl lignin in callus of *P. tremuloides*. This discrepancy may be due to species specific differences or, more likely, to differences in the methods employed. In the aforementioned work, oxidation products were methylated for gas chromatographic analysis. In our experience, methylation gave low yields owing to solubility problems and syringyl residues were not detected. Much higher yields were obtained with silylation, and substantial amounts of 3,4,5-trimethoxybenzoic acid were consistently found.

In all tissues, we routinely detected protocatechuic acid (**3**) which accounted for about 3% of the recovered phenolics (Table 3.4). This result was unexpected since it indicates that caffeic acid or caffeoyl alcohol is incorporated into lignin. The recovery of **3** may also indicate the presence of other phenolics in cell walls, or may be an artifact of our procedure.

Because tracheids represented a subpopulation of callus cells, our results showing the presence of syringyl residues in xylogenic callus does not prove that the phenolic

polymer in xylem cells contains sinapyl residues, and hence cannot be used to refute the hypothesis that syringyl lignin is compartmentalized. Tracheids accounted for up to 20% of the total cell number in the most xylogenic calli. Thus, most of the cell wall polyphenol of callus is not found in tracheids, and may also include other phenolic polymers such as suberin (Kolattukudy, 1981). We did find, however, that polyphenol in xylogenic callus contained elevated levels of *p*-coumaryl alcohol residues, typical of lignin in the Salicaceae (Venverloo, 1971). Thus, *p*-coumaryl alcohol may be a preferred substrate for xylem polyphenol in *P. trichocarpa*.

CHAPTER 4

Analysis of the Phenolic Polymer found in Cell Walls of *Populus trichocarpa* Tissues

We have shown that purified wall fractions from cultured *P. trichocarpa* cells contain significant amounts of polyphenol and that the composition of this polyphenol varies with hormone treatment (Chapter 3). Results obtained with permanganate oxidation of these walls gave low yields of C6 - C1 acids which possessed methoxylation patterns like those found in *p*-coumaryl, coniferyl and sinapyl alcohol residues. It is not known, however, whether the oxidation products were actually derived from C6 - C3 alcohols. In this chapter, we further characterize polyphenols in cultured *P. trichocarpa* tissues with several additional methods. These techniques include histological staining, a more efficient procedure for permanganate oxidation, thioacidolysis, and hydrolysis.

Histological Staining

A number of histological stains have been used to detect phenolic substances in woody plants. Safranin-O is a general stain that turns all lignified tissues red (Dodds and Roberts, 1982). Several other color reactions exhibit considerable selectivity. In 1900, Mäule observed that deciduous woods which were treated with 1% potassium permanganate followed by 12% hydrochloric acid and ammonium hydroxide developed an intense purple-red color. Brown or tan shades were noted in coniferous woods. Crocker (1921) suggested that manganese dioxide is produced which reacts with the acid to release chlorine which, in turn, displaces ring hydrogens and removes methyl

groups from syringyl residues. The color results from the reaction of ammonia with chlorinated 3,4,5-trihydroxyphenyl groups presumed to be derived from syringyl residues. Phloroglucinol-hydrochloride (Wiesner reagent) has also been used to detect lignin in plant materials. The reagent reacts with the aldehyde (Adler *et al.*, 1948), alcohol or methylene groups (Geiger and Fuggerer, 1979) on the side chains of coniferyl or *p*-coumaryl residues in the lignin molecule, resulting in a red-purple color (Figure 4.1). The intensity of the resulting color does not necessarily provide a measure of lignin content, since color depends on the number of reactive groups present (Spurny and Sladky, 1955), and those lignins possessing fewer reactive groups will give a less intense color.

Suberin is a lignin-like substance in which long chain fatty acids are attached to polymerized phenolic residues (Kolattukudy, 1981). If suberin is present in the cell walls, it may be stained with lignin-indicating reagents. To distinguish between the two phenolic-containing polymers, tissues were also treated with Sudan III, a lipid stain that does not detect phospholipids (Jensen, 1957).

Synthetic lignins (dehydrogenation polymerizates, DHPs) made from polymerized *p*-coumaryl, coniferyl or sinapyl alcohol or a 1:1:1 mixture of these alcohols were tested with phloroglucinol, Mäule reagents and Safranin-O. The information from these tests provided a basis for interpreting results obtained from cell walls (Table 4.1). The DHPs which contained polymerized *p*-coumaryl or coniferyl alcohols produced shades of red or purple with phloroglucinol-HCl, while those containing sinapyl alcohol derivatives gave a reddish color for the Mäule reaction. All four DHPs stained red with Safranin-O.

Cross sections of a one-year old tree stem were treated with each of the four stains as a control for identification of types of polyphenol found in callus tissues.

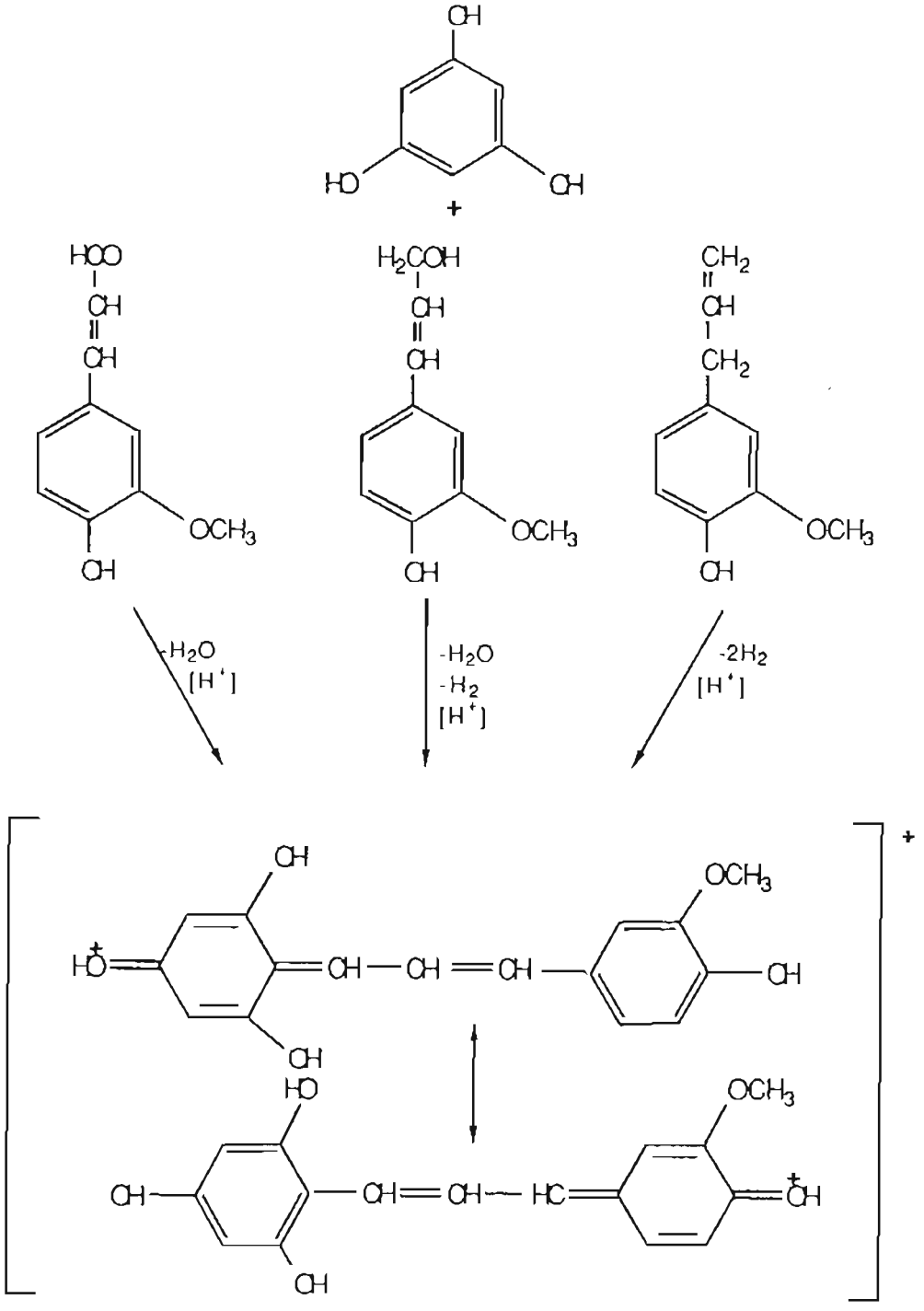


Figure 4.1 Suggested mechanism for the formation of the chromagen responsible for the red-purple color of the phloroglucinol reaction (from Geiger and Fuggerer, 1979).

Table 4.1

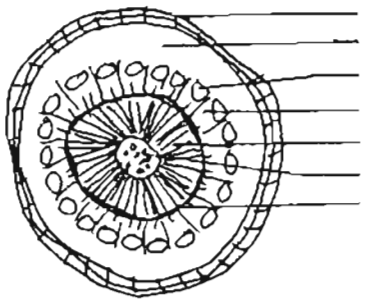
Differential color reactions of synthetic lignins (DHPs) with the lignin stains: phloroglucinol-HCl, Mäule reagents and Safranin-O

DHP [a]	Color of stain		
	Phloroglucinol	Mäule	Safranin-O
p-Coumaryl	red brown	tan	red
Coniferyl	red purple	brown	red
Sinapyl	tan	red purple	red
1:1:1 mixed polymer	violet	pink tan	red

[a] DHP polymer synthesized from the cinnamyl alcohol indicated.

Phloroglucinol and Mäule tests were used to distinguish between tissues containing coniferyl or syringyl lignins. Cork, primary phloem and xylem cells showed a positive test for lignin with Safranin-O (Figure 4.2). Since primary phloem and xylem cells gave a positive test with both phloroglucinol and Mäule, these tissues probably contain syringyl lignin. Secondary phloem and vascular cambium, were stained with phloroglucinol and Safranin-O, but not with Mäule. Pith cells stained weakly with Mäule reagents, and probably contain some syringyl residues. Only cork cells stained with Sudan III.

In general, callus cultures contained two distinct types of cells: undifferentiated parenchyma-like cells and tracheids. Some of the parenchyma cells were thin-walled and did not give any positive lignin test, while others were thicker-walled and tested positive for lignin with Safranin-O. Tracheids always stained shades of red with phloroglucinol, although not all tracheids stained strongly. With the exception of 2,4-dichlorophenoxyacetic acid (2,4-D)-grown cells, callus tissues were firm and compact (Table 4.2). Tissues maintained on 5 µM 2,4-D produced a fast-growing, friable callus. The majority of the cells in this callus were irregularly shaped and thin-walled (Figure 4.3 a). Although no cells showed signs of tracheid differentiation, those on the surface of the callus had thicker walls that stained light pink with Safranin-O or phloroglucinol. These cells may not be lignified, however, since they also stained strongly with Sudan III which indicates the presence of suberin (Figure 4.3 b). A few scattered cells on the internal portion of the callus also stained slightly with phloroglucinol, but the color appeared to be within the cells rather than in the cell walls. This may indicate storage of lignin-like materials within the vacuole. Phloroglucinol-staining material was also found by Carceller *et al.* (1971) inside cultured sycamore cells grown in the presence of 2,4-D.



	Safranin-O	Phloroglucinol	Mäule
Cork	+	±	-
Cortex	-	-	-
1° Phloem	++	±	++
2° Phloem	±	±	-
Xylem	++	++	+
Pith	±	-	±
Vascular Cambium	±	+	-

Figure 4.2 Differential Staining in Cross Section of a 1 Year Old Stem of *P. trichocarpa* . (++, Strong staining; +, moderate; ±, slight ; -, no detectable staining)

Table 4.2

Description of *P. trichocarpa* callus tissue growth and staining characteristics of callus tissues after treatment with Safranin-O, Phloroglucinol, Mäule reagents and Sudan III

Treatment [a]	% Tracheids	Callus Description	Safranin-O	Phloro-glucinol	Mäule	Sudan III
5 µm 2,4-D	0	Large thin walled Irregular shapes wet, friable	± [b]	±	-	+
Without Hormones	20	Large clumps tracheids Very slow-growing Small spherical masses	+	+	+	±
10 µm NAA +1µM BAP	13	Small cells Slow-growing and dense Larger spherical masses	+	+	±	±
10 µm NAA	4	Grows quickly Large, hard, dense Central cells elongated	+	+	-	+

[a] Callus maintained on media containing hormones described in the table.

[b] +, stained; ±, faint or indefinite staining; -, no staining.

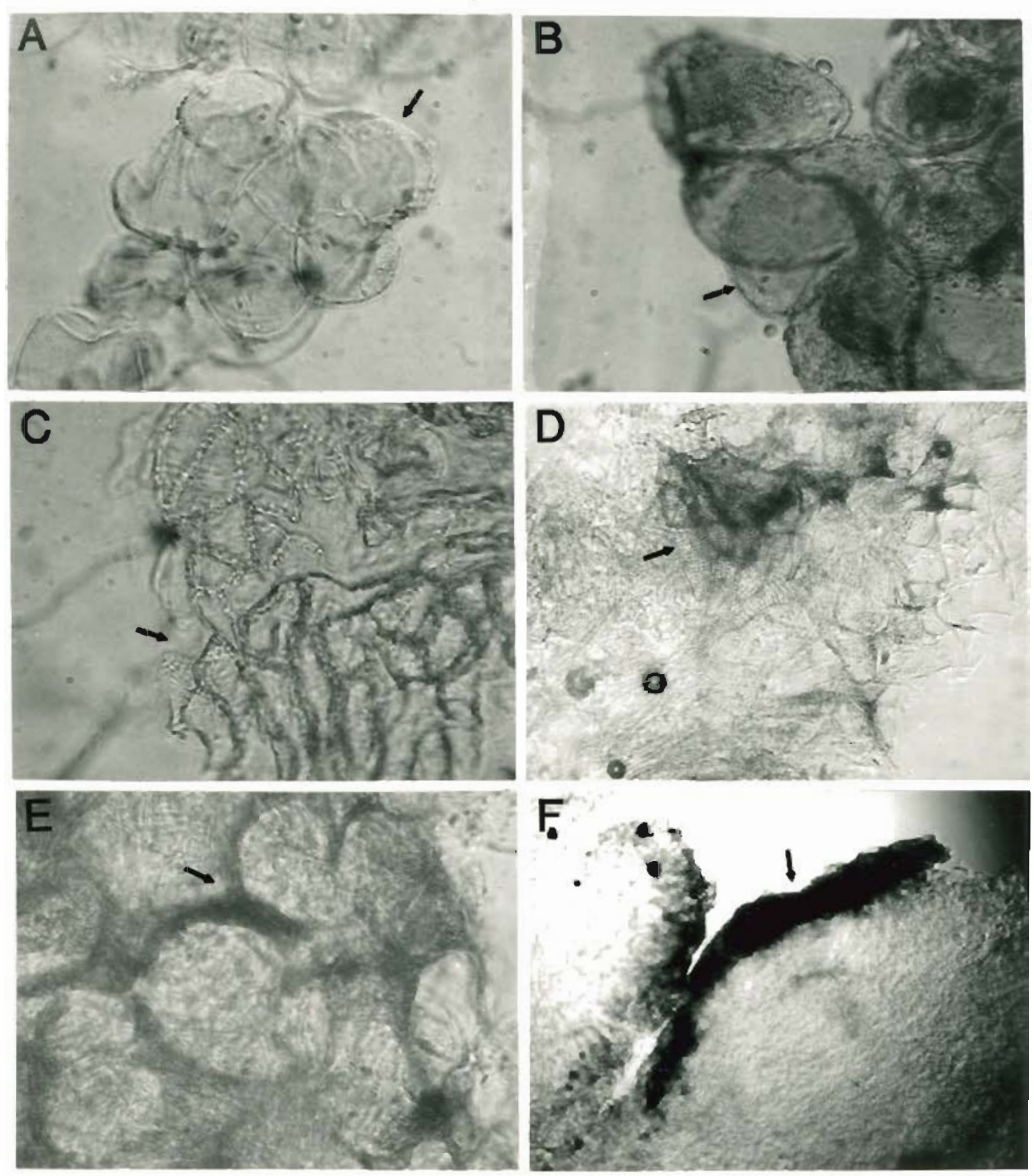


Figure 4.3 Micrographs of callus cells stained with lignin or suberin staining reagents. **A** 2,4-D callus walls, unstained, 400X; **B** 2,4-D callus surface cells, Sudan III, 400X; **C** NH callus, cluster of tracheids with birefringent walls, phloroglucinol-HCl, 400X; **D** NAA/BAP callus tracheid cluster, safranin-O, 160X; **E** NAA/BAP parenchyma, Mäule , 400X; **F** NAA callus cross section, Sudan III, 16X.

After callus maintained on 2,4-D was transferred to hormone-free medium for three successive subcultures, cells grew very slowly and formed small dense spherical masses. Approximately 20% of the cells differentiated into clusters of tracheids (Figure 4.3 c). Most of the tracheids in this callus had a reticulate or scalariform wall pattern and were thinner-walled than stem tracheids. Callus tracheids stained well with Safranin-O and phloroglucinol, but only weakly with the Mäule reagents. The callus growing on hormone-free medium also contained clusters of parenchyma-like cells which stained more strongly with Safranin-O and Mäule reagents than with phloroglucinol. Stained material was localized in the intercellular spaces.

Tissues maintained on media containing 10 μM α-naphthaleneacetic acid (NAA) and 1μM benzylaminopurine (BAP) grew slowly. Their growth habit was similar to that of 2,4-D-grown cells after several transfers onto hormone-free medium. Growth, however, was more rapid and fewer tracheids formed. Tracheids were arranged in many small nodules (Figure 4.3 d) throughout the tissue and were surrounded by groups of staining (Figure 4.3 e) and non-staining parenchyma cells.

Cells maintained on 10 μM NAA as the sole hormone supplement grew more slowly than cells cultured with 2,4-D, forming hard, spherical masses. The peripheryl cells were small and stained positively for suberin (Table 4.2, Figure 4.3 f). Immediately below the surface of the callus were several small groups of tracheids and a few clusters of lignin-staining parenchyma. Central cells in each mass were elongated to two or three times the length of those on the periphery and did not stain for lignin. Sudan III staining was observed on the surfaces of callus tissues maintained on media containing NAA or 2,4-D as the sole hormone supplement.

Analysis of Phenolic Composition of Cell Walls

Permanganate Oxidation.

A time course for permanganate oxidation of coniferyl alcohol DHP (Table 4.3) indicated that a 16-hour reaction period afforded a maximum yield of oxidation products. All subsequent permanganate oxidations were run for this length of time. Results from previous work showed that the polyphenol content of cultured *P. trichocarpa* cells ranged from about 12 to 20% of cell wall dry weight (Chapter 3). Only a very small portion (12-19%) of this polyphenol, however, could be recovered following permanganate oxidation. Venverloo (1971) also noted lower oxidation recoveries from cultured cells as compared to those from wood. She attributed this to the formation of "condensed" lignin in the cultured cell walls.

The major linkages between phenylpropanoid units in beech lignin (Nimz, 1974) include a number of ether bonds (Figure 4.4). Methylation of the cell walls before oxidation protects rings containing free hydroxyl groups from destruction, but phenolic groups that are freed during oxidation of ether bonds will be labile (Figure 4.4, rings 1, 2, 5, 6). Breaking bonds prior to methylation and oxidation should lead to a decrease in ring destruction and a corresponding increase in product yield. Pretreatment of cell wall preparations with NaOH and CuO (Erickson *et al.*, 1973) or NaOH and $CuSO_4$ (Morohoshi, 1981) was found to give approximately twice the yield of oxidation products.

Eight model compounds were subjected to alkaline copper sulfate treatment in an effort to determine which types of bonds found in lignin were broken by this method (Figure 4.5). Gas chromatographic traces for compounds A-E and G both before and

Table 4.3

Time course for recovery of vanillic acid after permanganate oxidation of coniferyl alcohol DHP

Experiment	Vanillic acid (µg/mg DHP)			
	Reaction time			
	0.5 h	2 hr	16 hr	21 hr
1	170	260	320	160
2	180	250	320	160

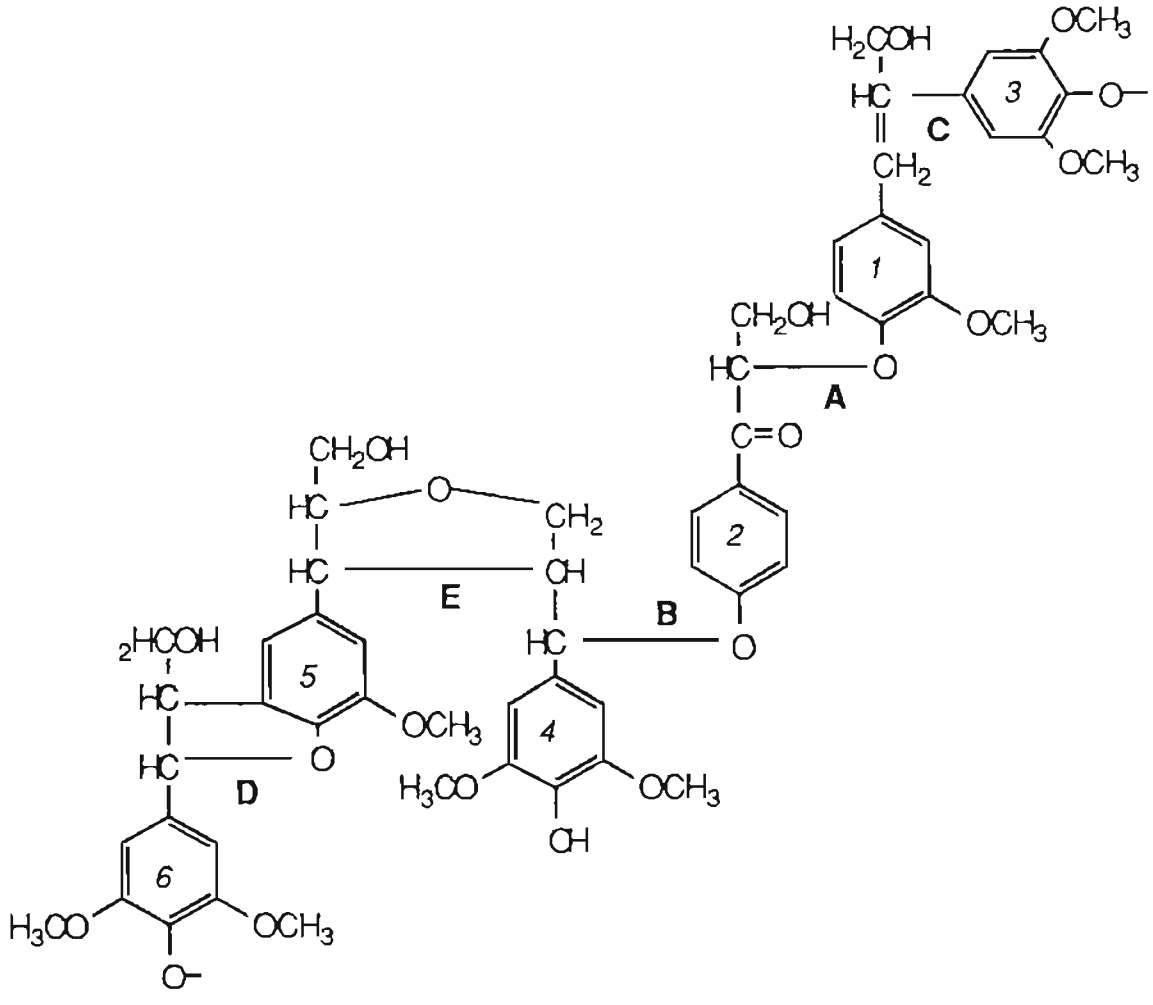


Figure 4.4 Major linkages between phenylpropanoid units in hardwood (beech) lignins (After Nimz, 1974). **A** and **B**: acyclic α and ß aryl ethers (65%); **C**: ß-1 bonds between 1,2-diaryl propane units (15%); **D**: ß-5 bonds between phenylcoumarin units (6%); **E**: ß-ß bonds (syringaresinol or pinoresinol units), (5%).

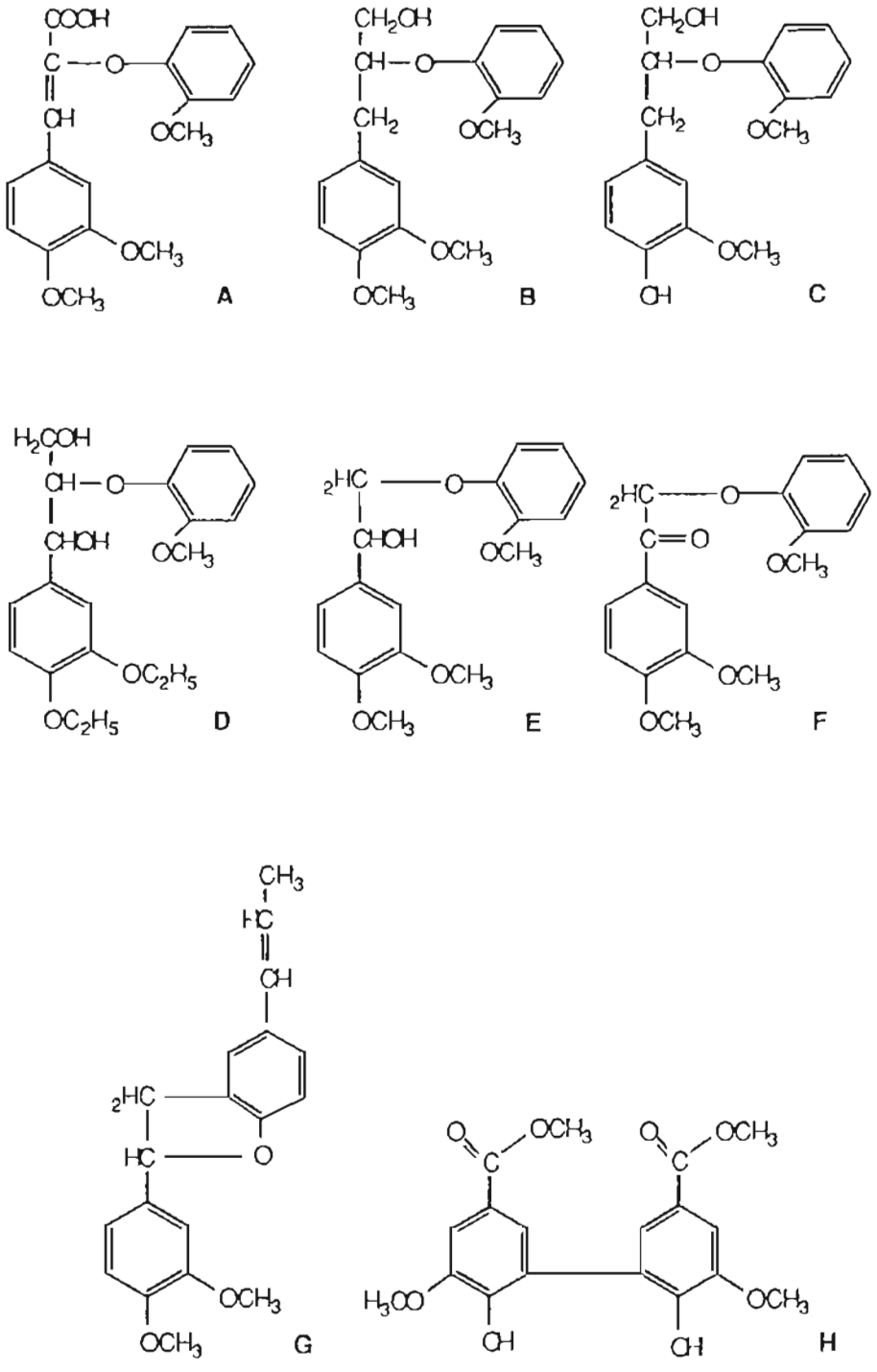


Figure 4.5 Structures of the model compounds used in the evaluation of bond cleavage by alkaline copper.

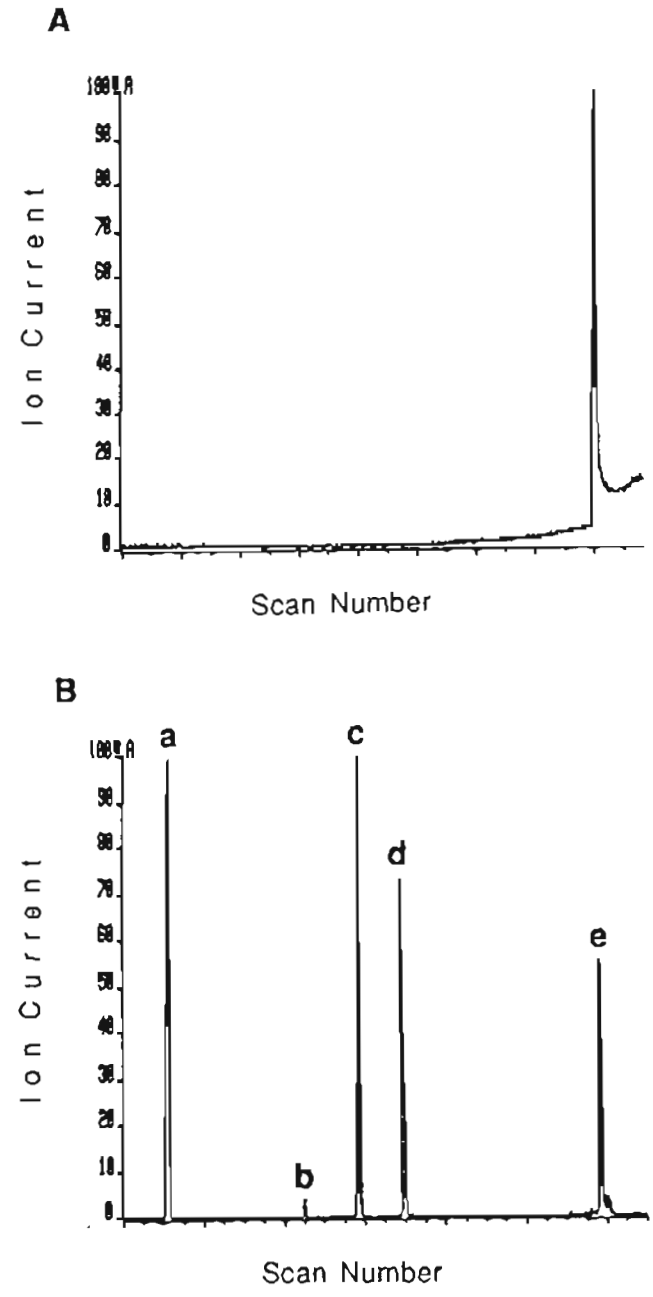


Figure 4.6 Ion current scans of compound F and alkaline copper reaction products. **A**: compound F, 1-(3',4'-dimethoxyphenyl)-1-oxo-2-(2"methoxyphenyl-1-ethane). **B**: **a**, guaiacol; **b**, acetophenone (contaminant); **c**, veratric acid; **d**, 2-hydroxy-1-(3',4'- dimethoxyphenyl)-1-oxo-ethane; **e**, starting material.

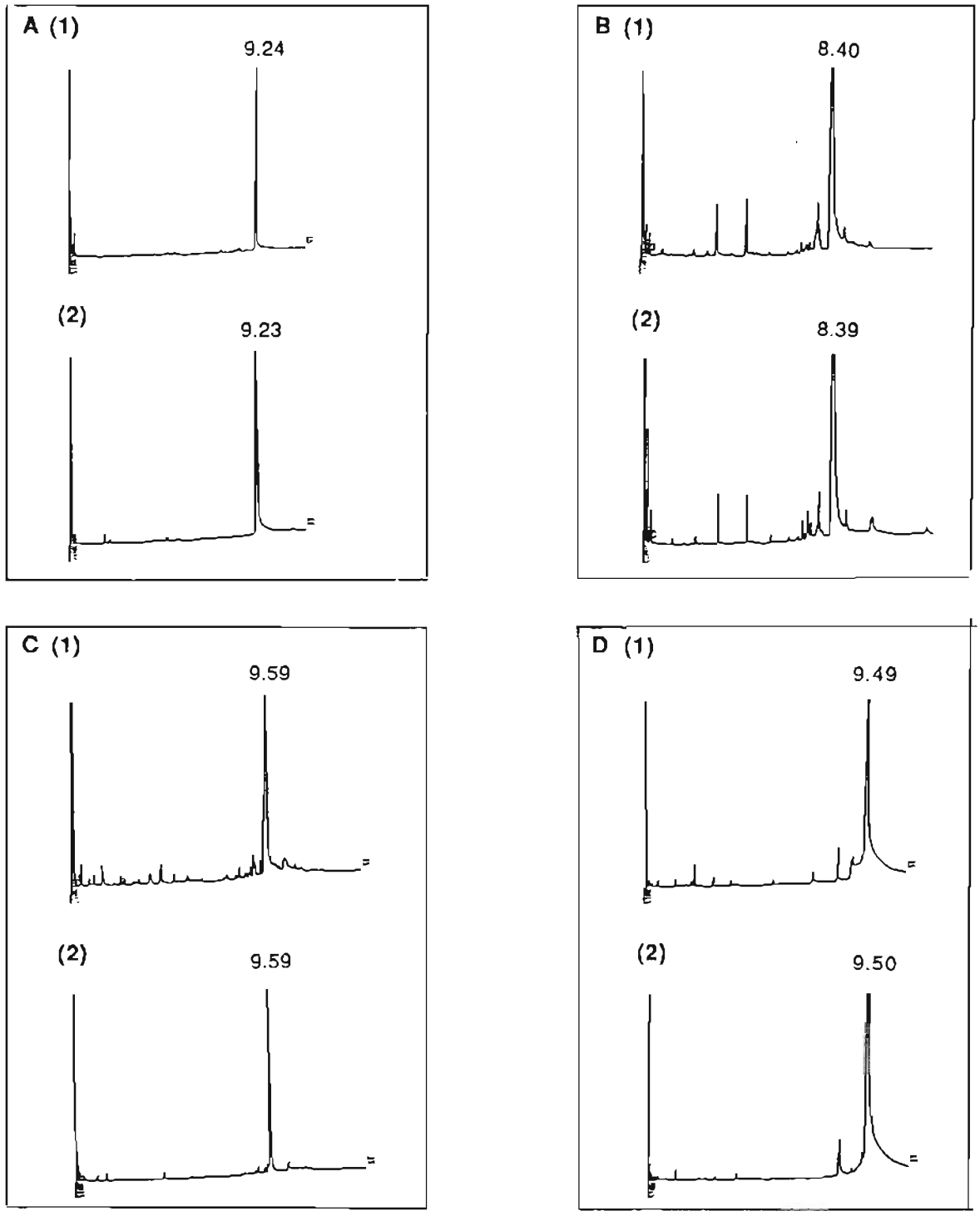
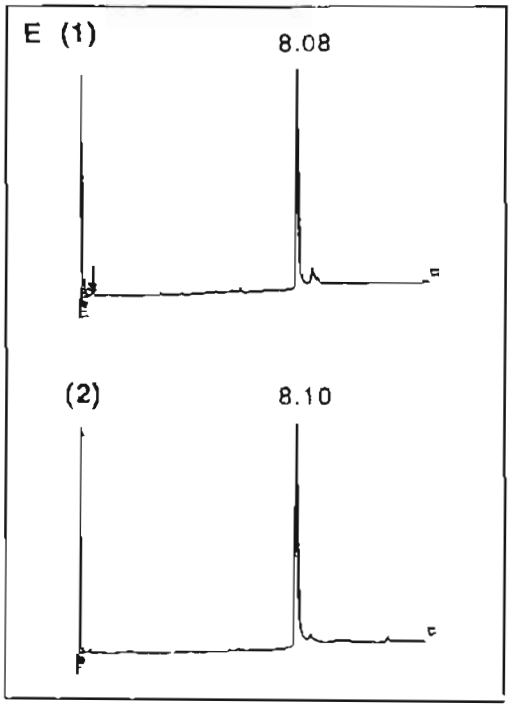
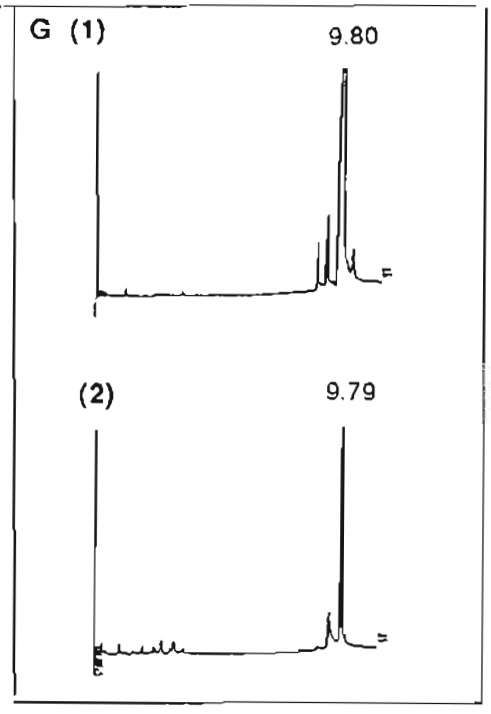


Figure 4.7 Gas chromatographic traces for compounds A-E and G,H before (1) and after (2) alkaline and copper hydrolysis. Retention times are indicated for the main peaks. Data for compound F is shown in Figure 4.6. Mass spectral data from compounds F and H is shown in Figures 4.9 and 4.11.





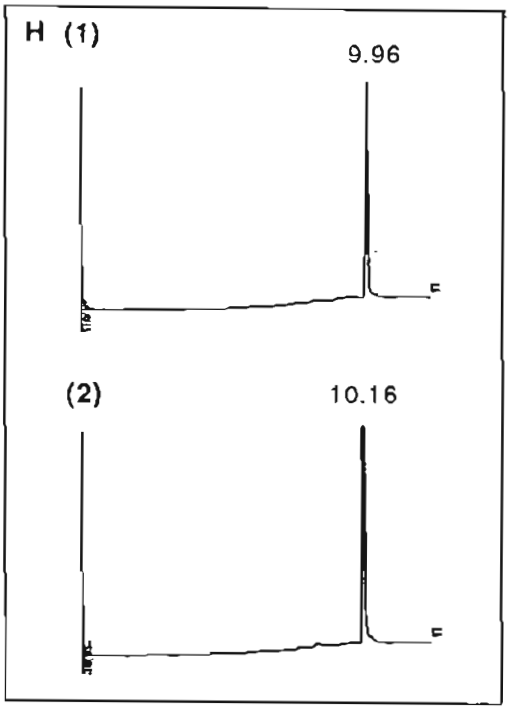


Figure 4.7 Continued.

after alkaline copper hydrolysis indicate that depolymerization did not take place place. Retention times for compounds F (Figure 4.6) and H (Figure 4.7) suggest that these compounds did react. The mass spectra and peak assignments for compound F are shown in Figure 4.8. Following the alkaline copper reaction, three major products were recovered (Figure 4.6). Mass spectra and peak assignments for the trimethylsilyl derivatives of these products are found in Figure 4.9. Guaiacol and 1-(3',4'-dimethoxyphenyl)-1-oxo-2-hydroxy-ethane were formed by cleavage of the ß-aryl ether bond. The mechanism is likely to involve a nucleophilic displacement reaction in which the copper (II) ion acts to stabilize the enolate intermediate, and this leads to a base catalyzed removal of ß-aryl ether groups (House *et al.*, 1973). Veratric acid may have been produced after the initial attack of an hydroxide on the α–carbon and followed by a C1-C2 bond cleavage. The anionic species formed through the hydroxide addition may have been stablized by the presence of Cu^{+2} ion. A proposed mechanism for these cleavages is shown in Figure 4.10. The reaction for compound H involves the removal of methyl ester groups (Figure 4.11) as would be expected in a strongly alkaline solution.

At least fourty aromatic compounds have been identified in permanganate oxidation reaction mixtures (Larsson and Miksche, 1969, 1971). The eleven compounds shown in Figure 4.12 are the most commonly detected oxidation products. In Chapter 3, it was noted that compounds 1, 2 and 3 (4-methoxybenzoic acid, (P); 3,4-dimethoxybenzoic acid, (V); and 3,4,5-trimethoxybenzoic acid, (S), respectively) and their unmethylated counterparts were recovered in appreciable amounts. The experiments described in this chapter were analyzed using a capillary GC column rather than a packed column. With the capillary column, only trace amounts of the unmethylated compounds were detected. The three methylated compounds (Figure

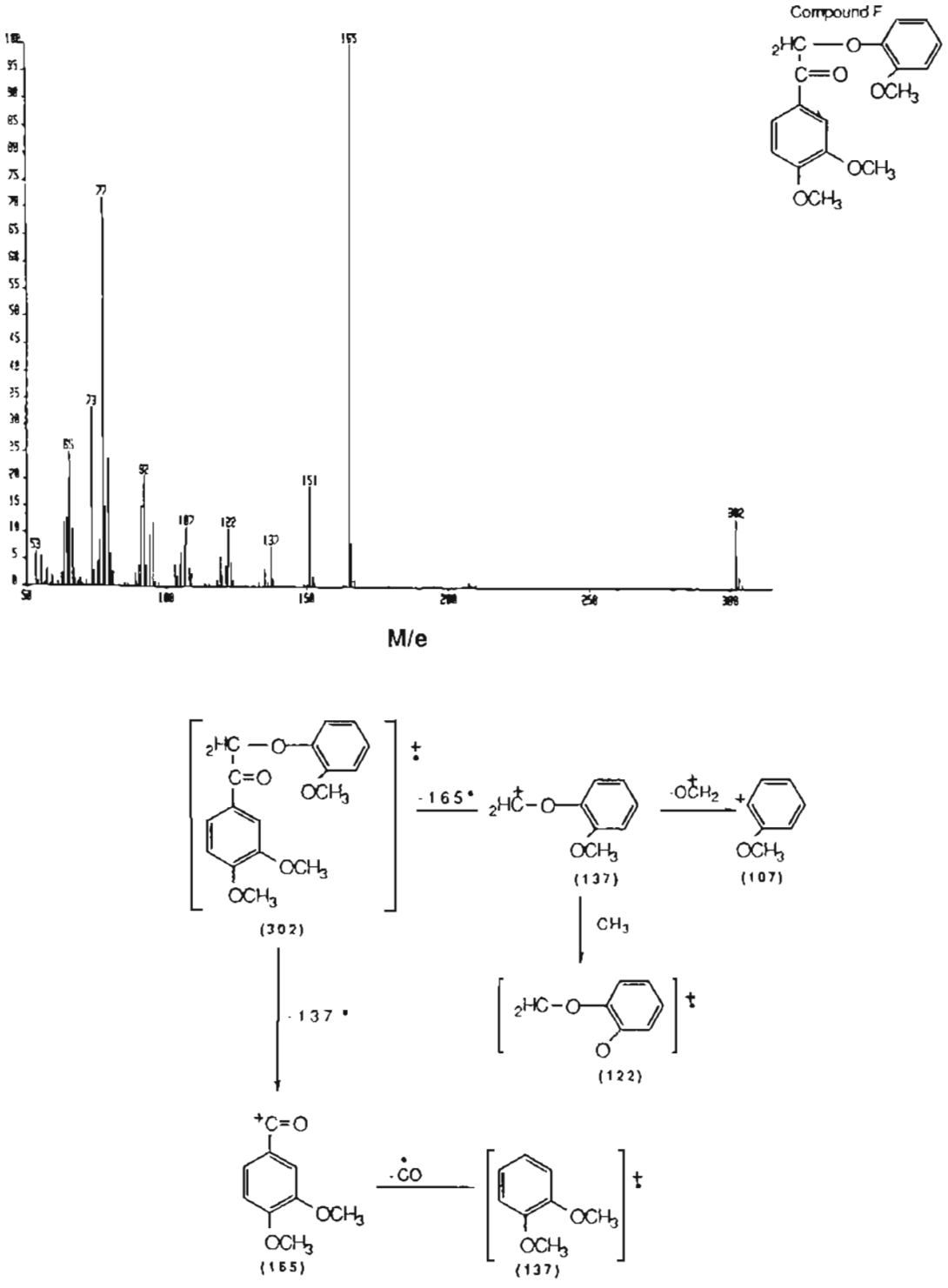


Figure 4.8 Mass spectral data and fragmentation for compound F, 1-(3',4'-dimethoxyphenyl)-2-(2"-methoxyphenyl)-ethan-1-one-2-ol.

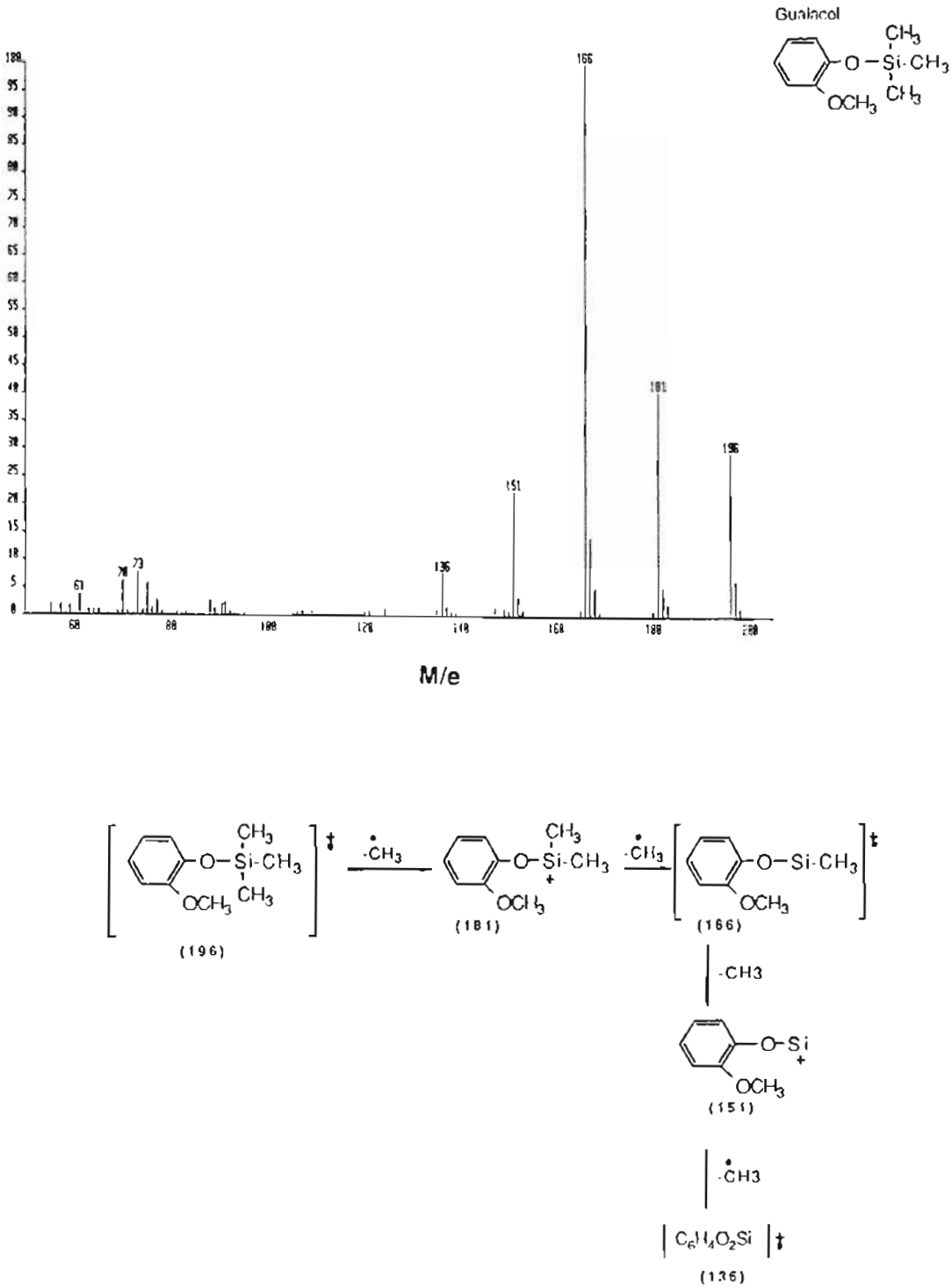


Figure 4.9 Mass spectral data and fragmentation for alkaline copper hydrolysis products from compound F. **a**, guaiacol; **b**, veratric acid; **c**, 1-(3',4'-dimethoxyphenyl)-1-oxo-2-hydroxy-ethane.

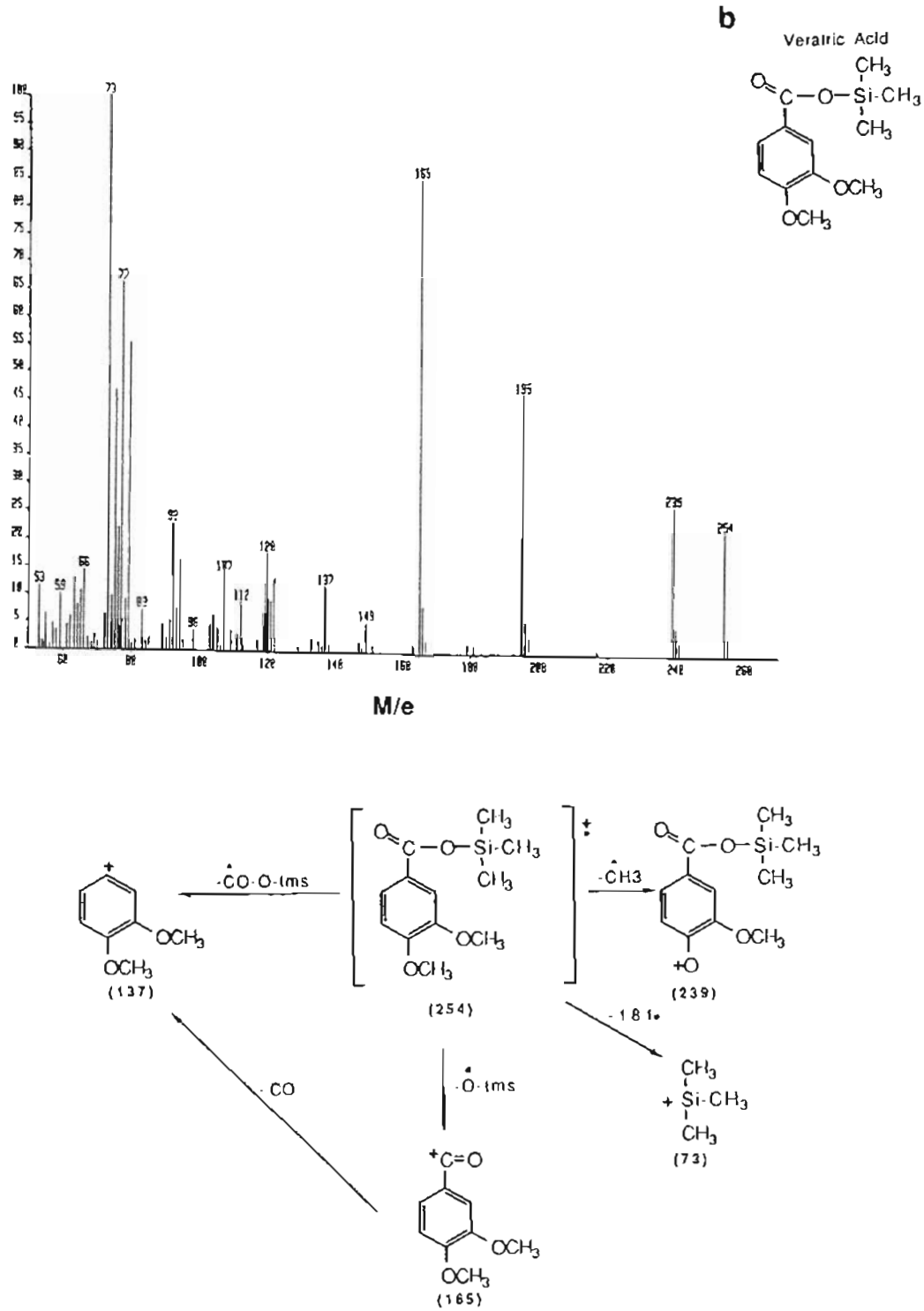


Figure 4.9 Continued. **b** , veratric acid.

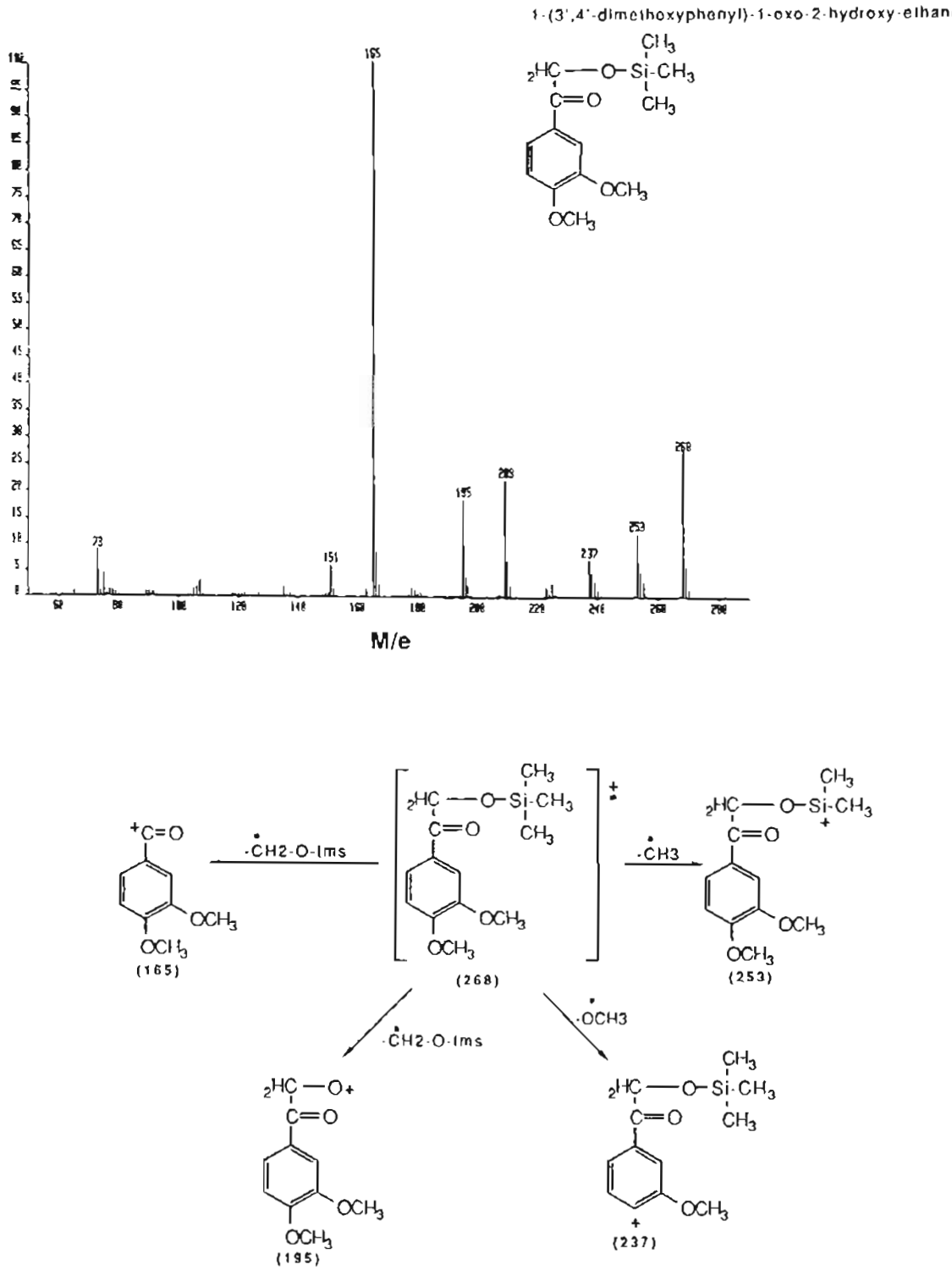


Figure 4.9 Continued. **c**, 1-(3',4'-dimethoxyphenyl)-1-oxo-2-hydroxy-ethane.

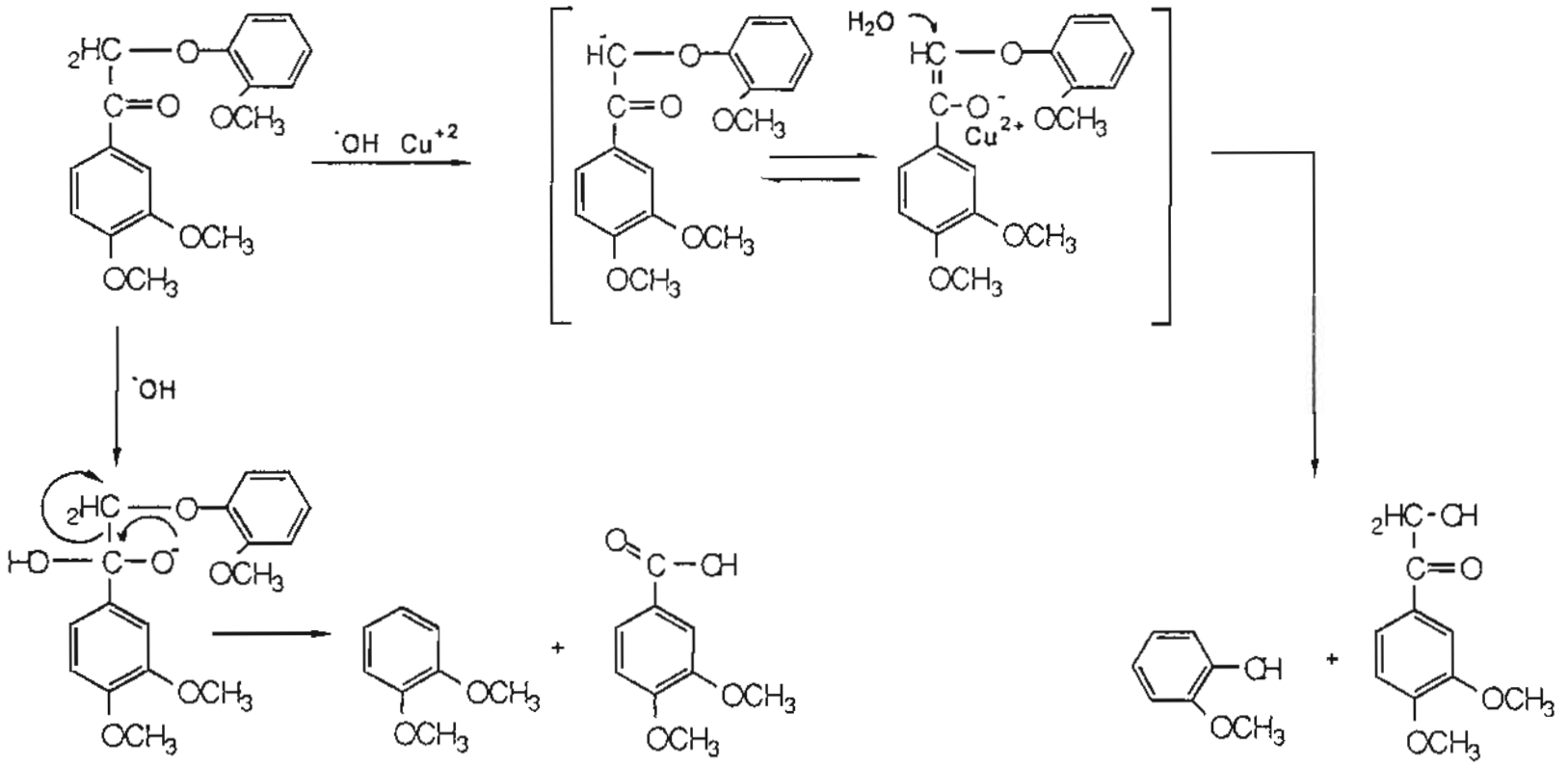


Figure 4.10 Possible mechanism for alkaline copper hydrolysis of lignin and lignin-like polymers.

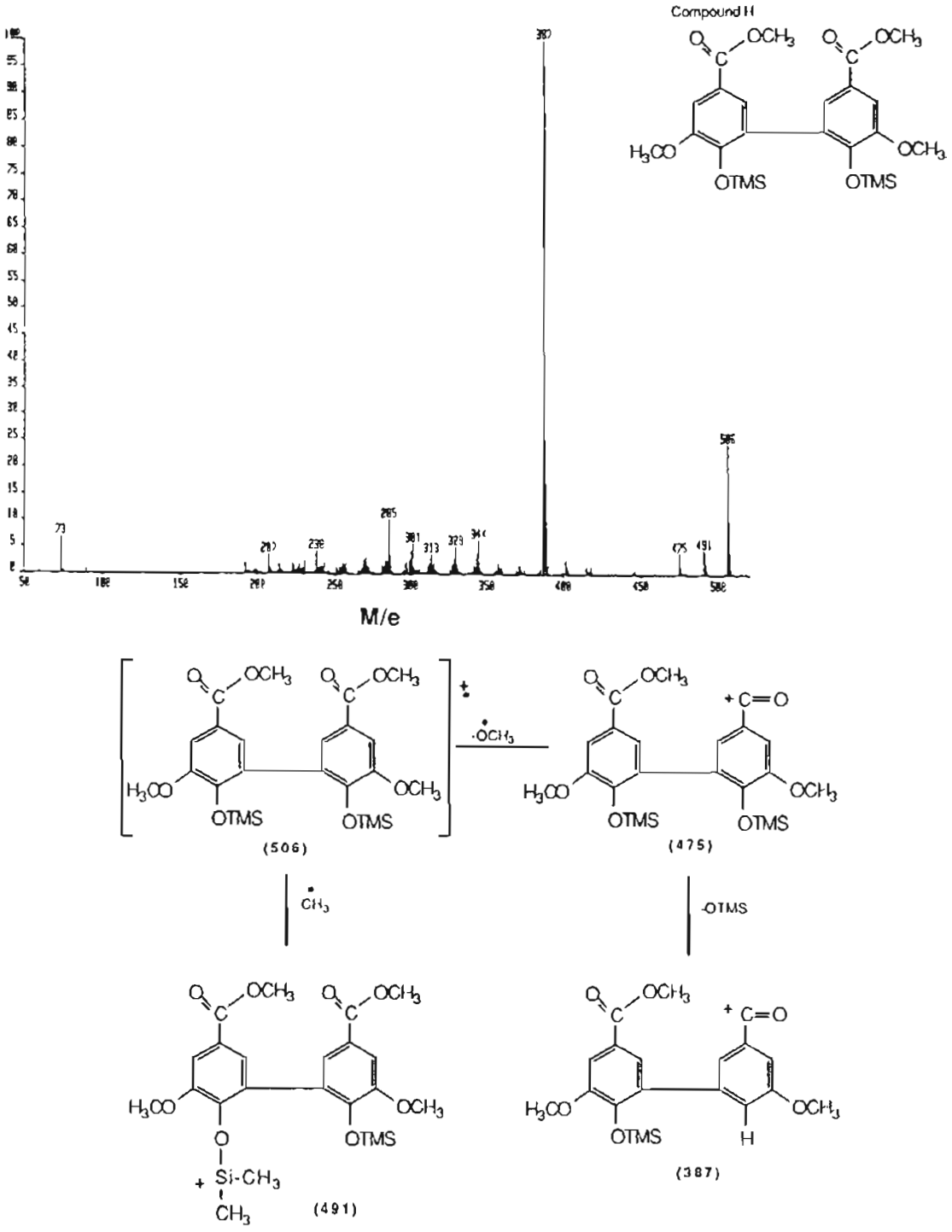


Figure 4.11 Mass spectral data and fragmentation for compound H: dimethyl 6,6'-dihydroxy-5,5'-dimethoxy-[1,1' biphenyl]-3,3'-dicarboxylate. **a**, Compound H; **b**, alkaline copper reaction product: 6,6'-dihydroxy-5,5'-dimethoxy-[1,1' biphenyl]-3,3'-dicarboxylate.

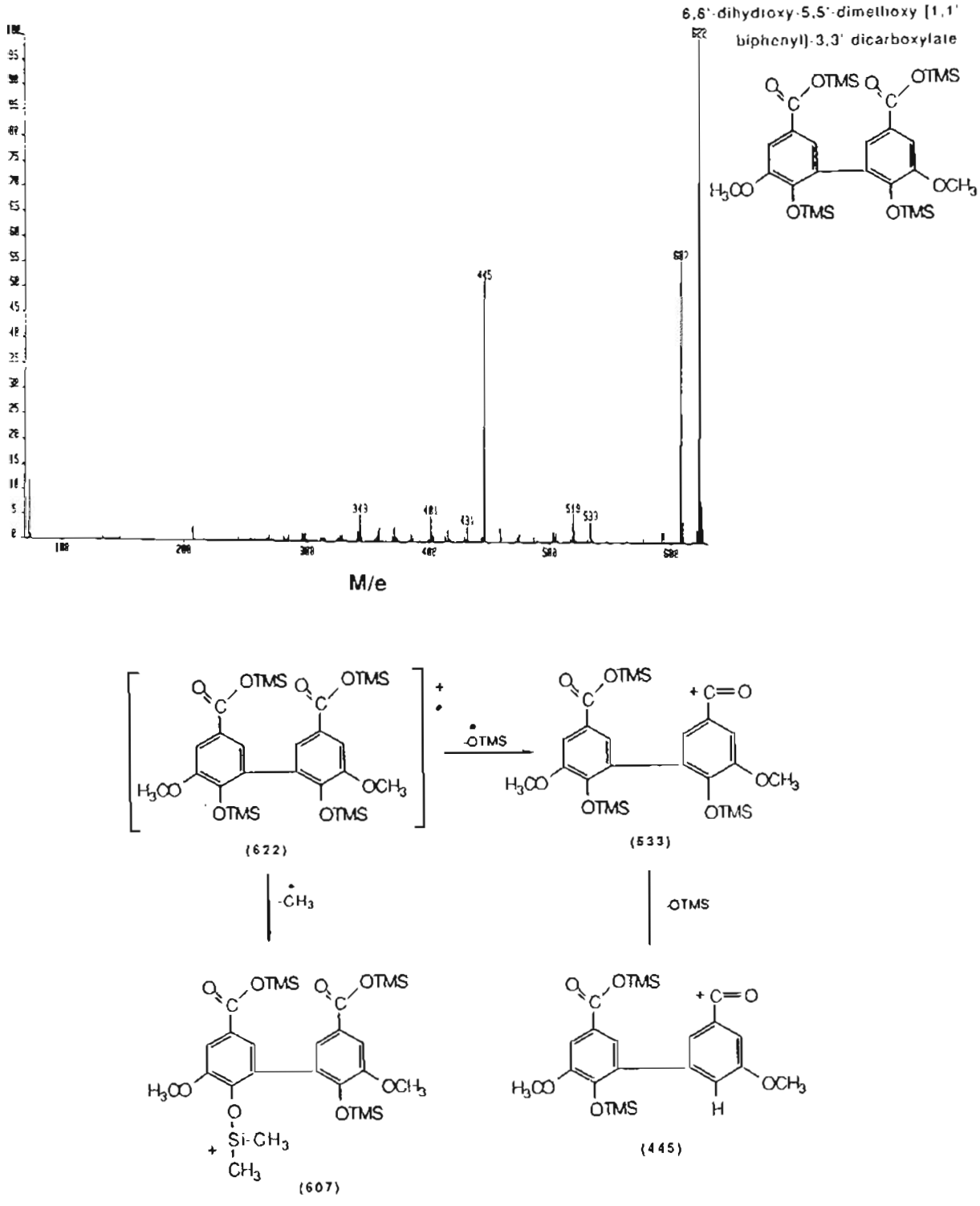
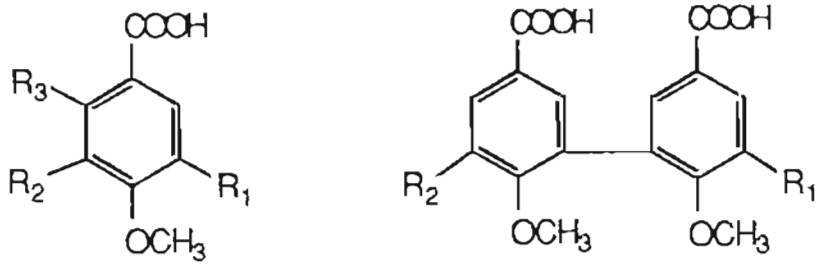


Figure 4.11 Continued. **b**: 6,6'-dihydroxy-5,5'-dimethoxy-[1,1' biphenyl]-3,3'-dicarboxylate.



*1. R1 = R2 = R3 = H
*2. R2 = R3 = H, R1 = OCH_3
*3. R3 = H, R1 = R2 = OCH_3
4. R3 = H, R1 = OCH_3, R2 = COOH
5. R2 = H, R1 = OCH_3, R3 = COOH
6. R1 = R2 = OCH_3, R3 = COOH
7. R1 = R3 = H, R2 = COOH
8. R1 = R2 = H, R3 = COOH

9. R1 = R2 = H
10. R1 = H, R2 = OCH_3
11. R1 = R2 = OCH_3

Figure 4.12 Structures for major reaction products from permanganate oxidation of methylated cell wall phenolic polymers. (The predominant products are marked with *.)

4.12, structures 1, 2, 3) were therefore monitored when identifying and quantitating oxidation products.

Alkaline copper hydrolysis of polyphenols before oxidation may lead to partial depolymerization of the phenolic polymers. Copper hydrolysis products were methylated and submitted to permanganate oxidation. A comparison of yields from oxidation without copper treatment to those treated with copper were used to provide an estimate of the abundance of susceptible bonds in these polyphenols.

Without copper pretreatment, oxidation yields for a mixed DHP (1:1:1 mole ratio of P, V, and S) were somewhat low (about 35% of the total polyphenol) for both replicates of the experiment (Table 4.4). On a micromolar basis, P and V gave similar yields, while S was somewhat greater. With copper treatment, DHPs produced about twice the total yield for each of the three acid products, and the molar ratios were similar for each of them. Since the increase in yield with copper pretreatment was similar for all three monolignols, approximately the same number of susceptible bonds must have been formed between each type of monomer during polymerization of mixed DHP.

Permanganate oxidation of stem cell walls (Table 4.5) without copper treatment yielded a molar ratio of benzoic acids of 32.5 : 49.0 : 18.5 (P: V: S, respectively). After copper treatment, however, syringic acid recovery was increased 5.6-fold and comprised approximately 50% of the total products. Little or no improvement in P was afforded by copper hydrolysis and permanganate oxidation. Total oxidation yields were again doubled by copper pretreatment, but increased yields were proportionally quite different from those of DHP.

Callus cell wall polyphenol showed very different patterns in recovery and overall

Table 4.4

The effect of alkaline copper hydrolysis pretreatment on the recovery of phenolic acids following $KMnO_4$ oxidation of a mixed DHP preparation [a]

	Recovery of oxidation products			
	No Copper Treatment		With Copper Treatment	
Acids [b]	µg/mg DHP	µmol/mg DHP	µg/mg DHP	µmol/mg DHP
P	83	0.60	172	1.25
	82	0.59	176	1.27
V	102	0.60	225	1.34
	100	0.59	231	1.37
S	133	0.67	246	1.24
	149	0.75	262	1.32
Total (Average)	325	1.90	656	3.90
% Recovery (average) [c]	34.7		70.3	

[a] DHP is made from *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (1:1:1).

[b] Benzoic acid oxidation products; 2 experiments, n = 2 for each; P = p-hydroxybenzoic acid; V = vanillic acid; S = syringic acid.

[c] % Recovery is based on molecular weights of related cinnamyl alcohols.

Table 4.5

The effect of alkaline copper hydrolysis pretreatment on the recovery of phenolic monomers following $KMnO_4$ oxidation of cell wall preparations from *P. trichocarpa* tissues

		Recovery of oxidation products (nmol/mg cell wall)		
Tissue	Compound	No Cu pretreatment	With Cu pretreatment	Ratio [a] (+Cu/-Cu)
Stem	P [b]	38.5	42.1	1.1
	V	57.7	92.6	1.6
	S	22.0	122	5.6
	Totals	118	257	
Callus 5μM 2,4-D	P	9.30	22.5	2.4
	V	80.9	88.5	1.1
	S	13.1	12.5	1.0
	Totals	103	123	
10μM NAA + 1 μM BAP	P	22.7	34.8	1.5
	V	71.6	84.4	1.2
	S	25.5	41.2	1.6
	Totals	119	160	
No Hormones	P	22.8	30.4	1.3
	V	69.6	87.9	1.3
	S	24.4	82.2	3.4
	Totals	117	201	

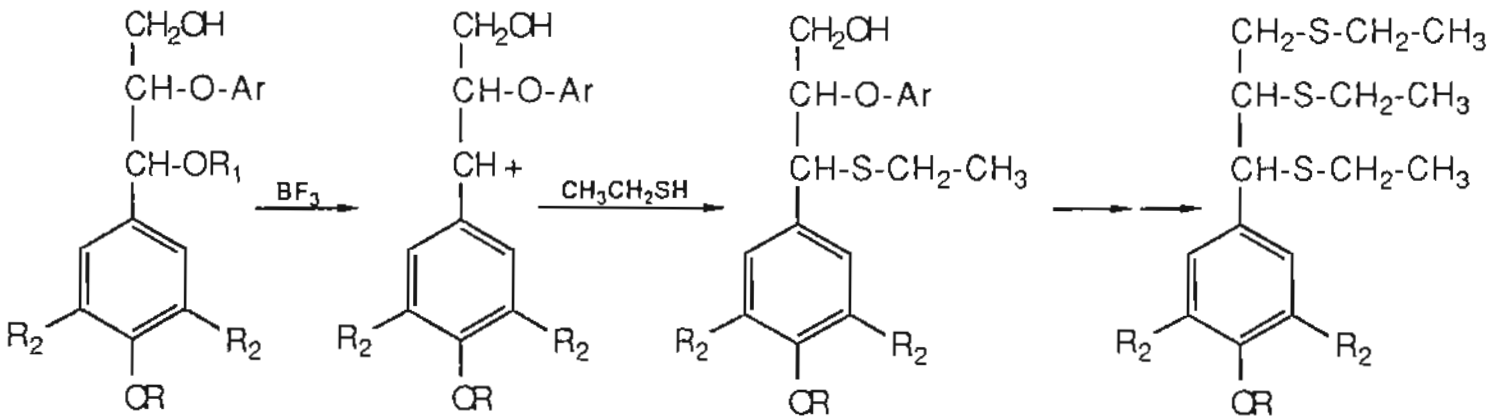
[a] Ratio of oxidation products.

[b] P = 4-methoxybenzoic acid, V = 3,4-dimethoxybenzoic acid, S = 3,4,5-trimethoxybenzoic acid, n=2 (Replicates gave similar recoveries).

yields when compared to stem. When compared to oxidation without copper pre-treatment, cell walls from tissues cultured on 2,4-D yielded an increase in P only. Those tissues grown in the presence of NAA and BAP showed small increases in both P and S, with lesser improvement in the V yield. Tissues cultured without additional hormones showed greatest increases in S. The overall oxidation product yields of cell walls from all three tissues were about equal before copper pretreatment. Following copper treatment, oxidation yields for the callus tissues were significantly lower than for stem. Cells grown on media supplemented with 2,4-D seemed to contain bound phenolics possessing few copper-susceptible linkages, and these appeared to be connecting P residues only. Tissues cultured on hormone-free media showed a 70% increase in overall oxidation product recovery after copper pretreatment (Table 4.5). Like the increase from stem, this was predominately in syringic acid. Cells maintained on media containing NAA and BAP showed an intermediate increase in recovered products, and this rise was found in both P and S acids. Based on copper and permanganate oxidation data, the polyphenol in stem is probably quite different from that found in callus cultured on 2,4-D, but more like that from tissues cultured on hormone-free media.

Thioacidolysis.

Since potassium permanganate oxidizes phenolic side chains, it was necessary to use another method of depolymerization to find out whether the phenolics were present as C6 - C3 or C6 - C1 units. Thioacidolysis hydrolyzes aryl ethers but does not cleave carbon-carbon bonds (Lapierre *et al.*, 1986). Both types of aryl glycerol-aryl ether bonds shown in Figure 4.4 (A and B) are likely to be broken by



R1 = Ar or H

R2 = H or OCH3

Ar = phenolic group

Figure 4.13 Degradation of lignin and lignin-like polymers by thioacidolysis (Lapierre *et al.*, 1985).

thioacidolysis, yielding monomers with free phenolic hydroxyl groups and thiolated sidechains (Figure 4.13). From premethylated polyphenols, thioacidolysis yields two classes of products: those with free hydroxyl groups, representing formerly ether linked phenols, and 4-methoxy substituted phenols, corresponding to originally free hydroxyl groups (Lapierre and Rolando, 1988). Because carbon-carbon bonds remain intact during thioacidolysis, this method can thus be used to verify the presence of C6 - C3 monolignols in the cell walls and to assess phenolic cross-linking.

Because standards were not available for unambiguous quantitation of thio-acidolysis products, recovery was measured as peak area per µg of dry material. Detector response was assumed to be equal for all products. Total yields from methylated and unmethylated 1:1:1 DHP (Table 4.6) indicates that about the same number of P and V units were released whether walls were methylated or not, but S yields were always lower. Of the recovered thioacidolysis products, approximately 70% of the P units were accessible to methylation, while much less than half of the S residues were accessible. Thus, the majority of the recovered P units have free hydroxyl groups, whereas most of the S residues are aryl-ether linked. Thio-acidolysis of methylated DHPs prepared from each of the cinnamyl alcohols gave results similar to those obtained with the mixed DHP prepared from all three cinnamyl alcohols (Table 4.7). This indicates that *p*-coumaryl, coniferyl and syringyl residues are linked in approximately the same way whether the DHP was formed from separate alcohols or by a mixed polymerization reaction.

By contrast with DHPs, thioacidolysis of cell wall polyphenols (Table 4.8) gave ambiguous results for P residues. If cell walls were not methylated, no P residues

Table 4.6

The effects of thioacidolysis on depolymerization of premethylated or unmethylated mixed DHP

Thioether	Thioethers, Peak Area/μg DHP						% OMe [a] (free OH)
	Unmethylated DHP			Methylated DHP			
	OH	OMe	Total	OH	OMe	Total	
P	157	ND [b]	157	45.1	101	146	69
V	162	ND	162	64.7	66.7	131	51
S	81.1	ND	81.1	48.0	20.2	68.2	30

[a] Percent of recovered products that were methylated. n=2

[b] ND = not detected.

Table 4.7

The effects of thioacidolysis on depolymerization of methylated or unmethylated DHPs made from *p*-Coumaryl, Coniferyl or Sinapyl alcohol

DHP	Thioether	Peak area/μg DHP					
		Unmethylated DHP			Methylated DHP		
		OH	OMe	Total	OH	OMe	Total
p-Coumaryl	P	427	ND [a]	427	152	266	418
Coniferyl	V	337	ND	337	172	180	352
Sinapyl	S	284	ND	284	187	74	261

[a] ND = not detected

Table 4.8

Recoveries of thioacidolysis products after depolymerization of premethylated and unmethylated cell wall material

		Thioethers, Peak area/μg cell wall						
		Unmethylated walls			Methylated walls			%OMe
Tissue	Thioether	OH	OMe	Total	OH	OMe	Total	(Free OH)
Stem	P [a]	ND [b]	ND	ND	3.89	ND	3.89	-
	V	59.6	ND	59.6	25.1	36.7	61.8	41
	S	114	ND	114	2.57	95.8	98.6	3
	Totals			174			164	
Callus 5 μM 2,4-D	P	ND	ND	ND	ND	ND	ND	-
	V	0.20	ND	0.20	0.14	0.06	0.20	71
	S	0.10	ND	0.10	0.09	0.02	0.11	83
	Totals			0.30			0.31	
10 μM NAA+ 1μM BAP	P	ND	ND	ND	0.08	ND	0.08	-
	V	0.61	ND	0.61	0.42	0.02	0.44	69
	S	0 45	ND	0.45	0.12	0.41	0.53	21
	Totals			1.06			1.05	
No Hormones	P	ND	ND	ND	2.78	ND	2.78	-
	V	11.3	ND	11.3	2.75	9.82	12.57	28
	S	2.07	ND	2.07	0.08	1.90	1 98	4
	Totals			13.3			17.33	

[a] P,V,S = ethylthiolated products

[b] ND = not detected

were detected, although unmethylated P groups were recovered from methylated walls. Taken alone, the methylated cell wall data suggests that a few P residues are ether linked to the polymer. If this were true, however, a similar number of P residues should have been detected in unmethylated walls. Stability of P thioethers is probably not the cause of this discrepancy, since these groups were recovered from the DHP experiment in good yield. Lapierre *et al.* (1988) found no P units in either methylated or unmethylated milled wood lignin from *P. euramericana*. The source of this discrepancy with P residue recovery is unknown, but its presence reduces the utility of thioacidolysis data for analysis of phenolic cross-linking.

In general, lower levels of all thioacidolysis products were recovered from callus tissues as compared to stem. Of the four cell wall preparations subjected to thioacidolysis, stem tissue contained the greatest number of differentiated xylem elements and afforded the highest recovery of thioacidolysis products. Approximately 20% of the cells in callus growing on media not supplemented with hormones formed tracheids, and with the exception of stem, this tissue gave the highest yield of thioethers. Tissues growing on media containing 2,4-D formed no tracheids, and only trace amounts of thioacidolysis products were recovered. Since thioacidolysis products are formed by breaking aryl ether bonds, only those without additional carbon-carbon bonds will be detected. The DHP polymers and stem cell wall material contain many C6 - C3 units that can be freed by thioacidolysis. The phenolic polymers in cell walls of the cultured tissues that formed tracheids resemble phenolics from stem cell walls most closely and are more likely to contain true lignin.

Hydrolysis

Absorbance studies with uv light showed that cell walls contained substantial quantities of phenolic material (Chapter 3). The presence of these phenolics was verified by permanganate oxidation. Thioacidolysis data from cell walls in cultured tissues suggests that most of this material is not a C6 - C3 polymer. Some of these phenolics may be acids that are linked to the cell walls. Phenolic acids can be bound to walls of both lignified and unlignified cells (Nakano *et al.*, 1961; Venverloo, 1969; Harris and Hartley, 1976). These acids may be esterified to wall-bound polysaccharides (Hartley *et al.*, 1976; Brokern *et al.*, 1987) or bound as glycosides (Harborne, 1979).

Purified cell walls of tree stem and callus tissues were subjected to a series of hydrolyses designed to release cell wall esters and glucosides (Figure 4.14). Cell walls were treated first with NaOH at 32 °C to remove wall-bound esters (Figure 4.14, product A). After extraction with ethyl acetate, the remaining aqueous fraction was digested with ß-glucosidase to release any phenolics present as water soluble glucosides (Figure 4.14, product B). Cell walls remaining after base hydrolysis were also treated with ß-glucosidase to remove wall bound glucosides (Figure 4.14, product C).

p-Hydroxybenzoic, vanillic and syringic acids comprised the major portion of all cell wall esters and glucosides that were recovered. In every case, *p*-hydroxybenzoic acid was most abundant. Even though enzyme efficiency with these substrates is difficult to quantify, this indicates that both acids and glucosides are present in cell walls.

Using these extraction methods, cell walls from tissue grown on 2,4-D were found to contain only about 30% as many acids as stem or callus grown on media containing

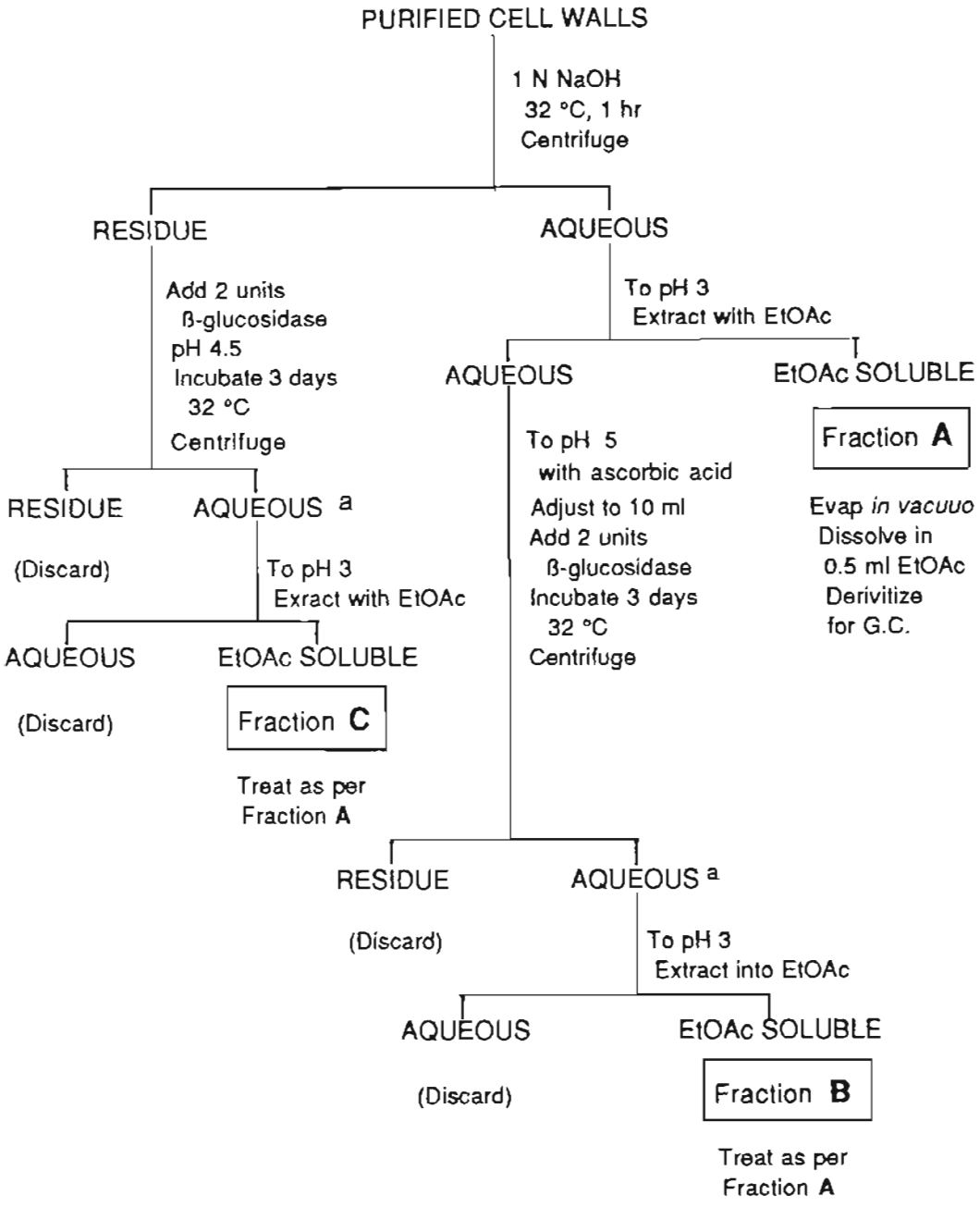


Figure 4.14 Scheme for the extraction of phenolic acids from purified cell walls.

[a] As a control, a portion of the aqueous fraction was incubated without ß-glucosidase but otherwise treated as the experimental fraction. Phenolic acids were not detected in control fractions.

Table 4.9

Total phenolic acids recovered from *P. trichocarpa* cell walls by base and enzymatic hydrolysis

Tissue	Compound	Recovered acids, pmol/mg cell wall: Hydrolysis Fraction A[a] n = 6	Hydrolysis Fraction B n = 4	Hydrolysis Fraction C n = 4	Total acids
Stem	Benzoic acids [b]				
	P	323.0±50.1	238.3±10.3	61.6±3.0	622.9
	V	74.2±7.0	ND[d]	19.2±4.2	93.4
	S	37.7±7.1	12.6±7.6	ND	50.3
	Cinnamic acids [c]				
	P	74.0±9.2	14.8±7.3	8.5±1.0	97.3
	V	28.8±1.5	7.6±0.8	ND	36.4
	S	2.4±1.6	ND	ND	2.4
Callus [e] 2,4-D	Benzoic acids				
	P	112.0±8.9	23.9±8.9	29.9±24.1	165.8
	V	20.2±6.6	ND	9.9±4.5	30.1
	S	50 8±3.5	11.0±6.5	41.4±6.1	103.9
	Cinnamic acids				
	P	55.2±8.1	0.8±0.8	24.9±8.5	80.9
	V	ND	ND	ND	ND
	S	ND	ND	ND	ND
NAA/BAP	Benzoic acids				
	P	474.0±6.1	320.1±16.7	66.9±9.6	861.0
	V	22 6±1.8	ND	4.7±0 8	27.3
	S	98.1±9.6	6.4±3.3	10.8±5.1	115.3
	Cinnamic acids				
	P	104.1±6.1	16.6±2.5	2.6±0.5	123.3
	V	35.1±5.8	ND	ND	35.1
	S	ND	ND	ND	ND

[a] See Figure 4.14 for explanation.

[b] Substituted benzoic acids : P = *p*-hydroxybenzoic, V = vanillic, S = syringic.

[c] Substituted cinnamic acids : P = *p*-coumaric, V = ferulic, S = sinapic.

[d] Not detected.

[e] Callus grown on media containing 5 μM 2,4-D or 10 μM NAA + 1 μM BAP.

NAA and BAP. Yields from 2,4-D-grown callus showed lower percentages of benzoic acids, and especially of *p*-hydroxybenzoic acid (Table 4.9). The recovery of acids was similar for stem and callus grown on NAA and BAP. In both tissues, about 85% of all recovered acids were benzoic acid derivatives, and the majority were *p*-hydroxybenzoic acid. Phenolic acids appear to be minor components of stem and callus cell walls, comprising only about 2 to 3% of the benzoic acids freed by permanganate oxidation. Thus, the presence of hydrolyzable acids does not explain the differences between copper-permanganate oxidation and thioacidolysis yields.

Discussion

Large amounts of phenolics remain in the cell walls after extraction with water and organic solvents. Approximately 14 to 21% of cell wall preparation from *P. trichocarpa* tissues was composed of uv absorbing material as compared with coniferyl alcohol DHP (Chapter 3). Cell wall staining techniques have shown that primary phloem and xylem tissues in young stem wood possess a high degree of lignification (Figure 4.2), while cultured tissues contained fewer lignified elements. Slowly growing tissues cultured on media containing NAA and BAP or without added hormones contained differentiated tracheids. These tissues stained more strongly for lignin than did the faster growing 2,4-D tissues (Table 4.2).

The question arises as to whether the polyphenol found in cultured tissues is actually lignin. Structures shown in Figure 4.4 were typically found in lignin. These C6 - C3 units were linked by ether and carbon-carbon bonds. Phenolics esterified to cell walls cannot be considered lignin. Attempts at releasing acids from cell walls by treatment with alkali or ß-glucosidase showed that both benzoic and cinnamic acids were bound to cell walls of stem wood as well as callus tissues, but their con-

centrations were too small to account for more than a few percent of the estimated polyphenol (Table 4.9). Although cell walls of cultured tissues contained a high concentration of protein, the presence of aromatic amino acid residues would not account for more than a small percentage of the total phenolic estimate (Chapter 3). Proteins or carbohydrates in the cell walls may be cross-linked by ferulic acid or tyrosine dimers. These residues have been detected at low levels in cell walls of cultured potato, tomato or sycamore cells at low levels (Fry, 1982; Epstein and Lamport, 1984). Neither ferulic acid nor tyrosine dimers, however, would have been detected after cell wall depolymerization. Thus, phenolics detected in cell walls of cultured tissues have not been accounted for by the above methods.

Trace amounts of thioethers were recovered from 2,4-D cell walls. A ten-fold increase in total yield was noted in tissues grown on media containing NAA and BAP, while a 100-fold increase was recovered from tissues grown on media without added hormones (Table 4.8). A similar pattern has also been seen in the number of tracheids produced by callus tissues (Table 4.2) and the increase of total yields from oxidation following copper treatment (Table 4.5). These correlations indicate a relationship between type of polyphenol formed and level of tracheid differentiation in the tissues. Tissues cultured on media containing 2,4-D formed no tracheids and showed little increase in recovery of acid products after copper hydrolysis and oxidation as compared to oxidation without copper treatment. They also showed little indication of the presence of aryl ether linked C6 - C3 units. On the other hand, approximately 20% of the cells in tissues growing on media without added hormones differentiated to form tracheids. Permanganate oxidation of polyphenol from these cells showed a large increase in recovery of S residues following copper pre-treatment, and the total number of aryl ether linked C6 - C3 units was much higher.

The phenolic polymers in wood from birch (Fergus and Goring, 1970; Hardell *et al.*, 1980) and *P. tremuloides* (Wolter *et al.*, 1974) exhibit at least some compartmentation. The secondary walls of fibers and ray cells contain a lignin that is rich in syringyl units, while middle lamella and tracheid lignin is rich in coniferyl residues. Histological staining of cross sections of *P. trichocarpa* stems has shown similar results (Figure 4.2). In xylogenic callus tissues, staining also shows that coniferyl and syringyl residue-containing polyphenol may be localized in specific cells. Tracheids were stained best with phloroglucinol, while some parenchyma-like cells stained with Mäule reagents. Thus, a type of compartmentation may also exist in cultured tissues.

The phenolics in callus tracheids may be similar to those found in stem wood. Increased tracheid formation in cultured tissues paralleled a rise in coniferyl and syringyl alcohol residues and in total thioacidolysis yields (Table 4.8). This indicates that a more condensed polyphenol formed in high tracheid cell walls, and the phenolic polymer showed an increased lignin-like character. Furthermore, larger amounts of syringic acid were recovered from cultured tissues that showed increased tracheid differentiation (Table 4.5). Perhaps the increased yields of syringic acid released by oxidation came about because of a simultaneous increase in the incorporation of S units into the walls of the parenchyma-like cells (Table 4.2).

The C6 - C3 units recovered after thioacidolysis were detected only in trace amounts in 2,4-D-treated callus, and they were not found in substantial amounts in either of the other cultured tissues. This does not necessarily mean that C6 - C3 units are not present in these tissues. Cinnamyl alcohols that are polymerized by producing carbon-carbon bonds at any point on a residue would not be recovered as monomers after thioacidolysis. Phenolic residues that were polymerized with

carbon-carbon bonds connecting only the side chain portion but not the ring may be freed after copper hydrolysis and permanganate oxidation but not thioacidolysis. These types of linkages may be much more abundant in callus polyphenol than in stem, and this may help explain some of the differences between callus and stem phenolic polymers. Furthermore, if a greater number of carbon-carbon bonds are formed between rings in callus polyphenols, the recovery of products from both thioacidolysis and oxidation will be lower than for stem. Although our results show that callus phenolic polymer is different from that in wood, they suggest that cultured tissues constitute a good system for *in vitro* study of lignification. Under growth conditions that lead to increased numbers of tracheids, we begin to find appearance of a lignin-like polymer in the cell walls. Thus, by controlling the conditions for growth and differentiation of cells, one may study the biochemical events that take place during the induction and biosynthesis of lignin.

CHAPTER 5

Auxin and Cytokinin Regulation of Polyphenol Biosynthesis in Cultured *Populus trichocarpa* Tissues

Studies of polyphenol composition have shown that the relative proportion of the three phenylpropanoid alcoholic subunits vary greatly between species (Gross, 1981). There is even variation within tissues of a single plant. Such variations in polyphenol composition were associated with age and stage of plant development (Sarkanen and Hergert, 1971). Increased age and maturation were correlated with a greater methoxyl content of lignin, indicating an increased syringylpropane content (Higuchi 1957). We have shown that phenolics in the cell walls of cultured tissues vary in amount and composition, depending on the hormones added to the growth medium. In this chapter, the effect of hormone concentration is examined in more detail.

Reversibility of tracheid differentiation and polyphenol content

The data described in the two preceeding chapters showed that growth, tracheid formation and polyphenol biosynthesis are regulated by the hormones added to the medium. In order to test the effect of different hormone treatments on an individual tissue line, it was important to know whether effects induced by hormone (2,4-D) in stock cultures were reversible, and, if so, how long effects of this maintenance hormone persisted in the tissues after its removal from the medium. The experiment described in Figure 5.1 shows changes in both polyphenol content and the percent of cells that differentiate into tracheids are reversible.

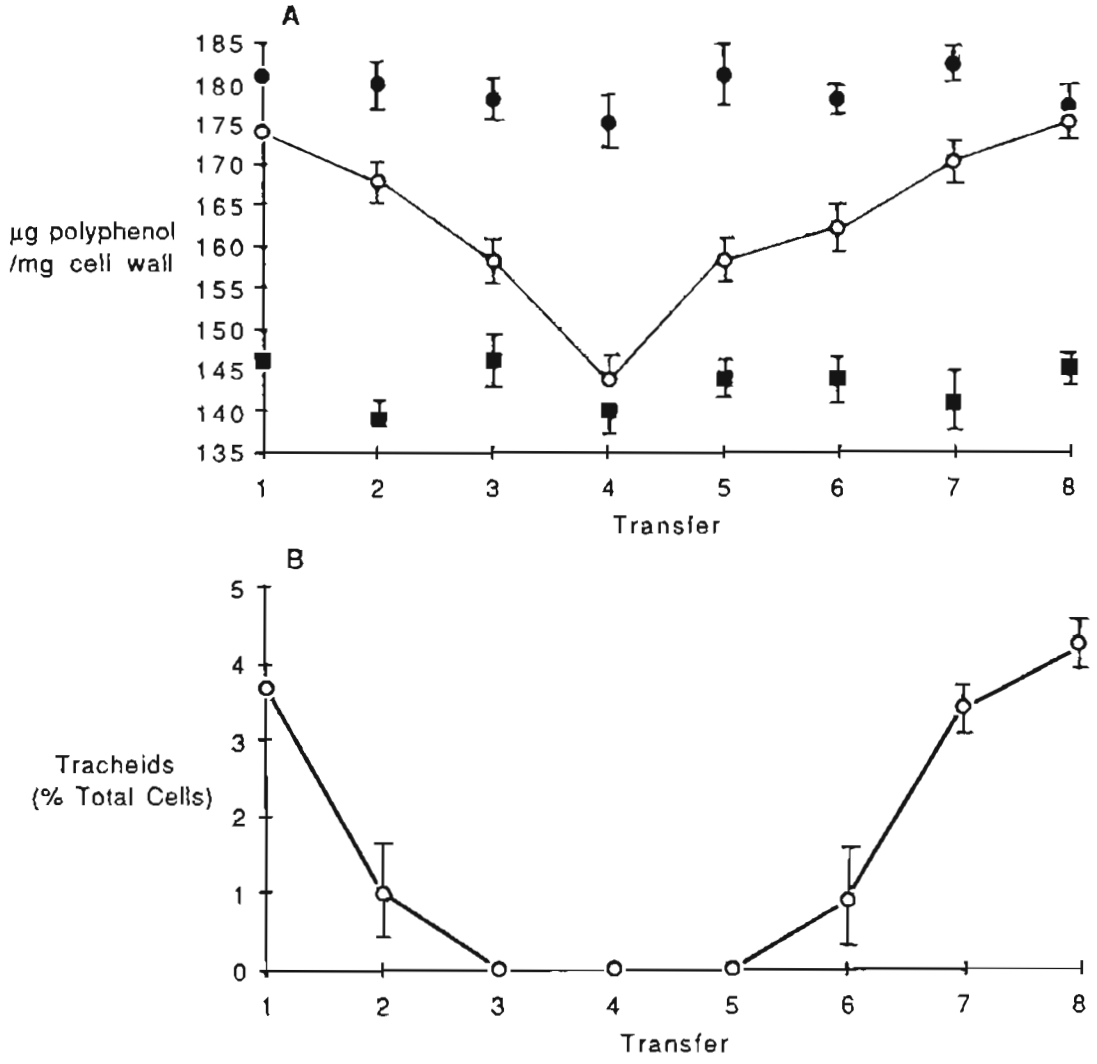


Figure 5.1 **A** Change in polyphenol content of cell walls is reversible during 8 consecutive 4-week transfers onto media containing different growth regulators . Tissues maintained on media containing 10 µM NAA and 1µM BAP for 4 or more transfers showed consistently high levels of polyphenol (·●-), while those maintained on 5 µM 2,4-D contained polyphenol levels that were consistently low (·■-). **B** Variation in the proportion of cells that differentiated into tracheids (·o-). Transfers 1,5,6,7,8 were cultured on media containing 10 µM NAA and 1 µM BAP; transfers 2,3,4 grown on media containing 5 µM 2,4-D. Data in A and B were taken from the same experiment, n = 3 for each.

Polyphenol content in tissues maintained on media containing either 5 µM 2,4-D or 10 µM NAA plus 1 µM BAP did not show any significant change during eight months of culture. If tissues were transferred from NAA plus BAP to 2,4-D, then the level of polyphenol (Figure 5.1A) and the number of tracheids (Figure 5.1B) decreased. By the third transfer on 2,4-D, the amount of polyphenol in the cells was identical to that normally found in 2,4-D-grown tissues, and no tracheids had differentiated. After three transfers back onto medium containing NAA and BAP, the polyphenol and tracheid levels returned to their original condition. A gradual increase in polyphenol level and tracheid differentiation was observed. The experiment was monitored for at least one more cycle with similar results. This experiment shows that neither 2,4-D nor NAA and BAP induced lasting changes in the callus cells. Since the tissues maintained on 2,4-D grew very well, stock cultures were maintained on this growth regulator, but at least 2 or 3 transfers onto other media were necessary to remove the influence of 2,4-D.

The effects of hormone concentration on tracheid differentiation and polyphenol content

Tissues that had been maintained on 2,4-D were placed on hormone-free media for two transfers before they were placed on test media that was supplemented with 10 or 100 µM NAA and varying concentrations of BAP. Time courses of tracheid development (Figure 5.2) and polyphenol formation (Figure 5.3) were monitored for an 8 week period of time. Those tissues that were cultured on media containing 10 µM NAA with BAP showed a lag of 3-4 weeks before tracheids appeared in the callus (Figure 5.2 A), whereas tracheids appeared after 2-3 weeks with 100 µM NAA (Figure 5.2 B). On the other hand, tissues cultured with the lower NAA dose

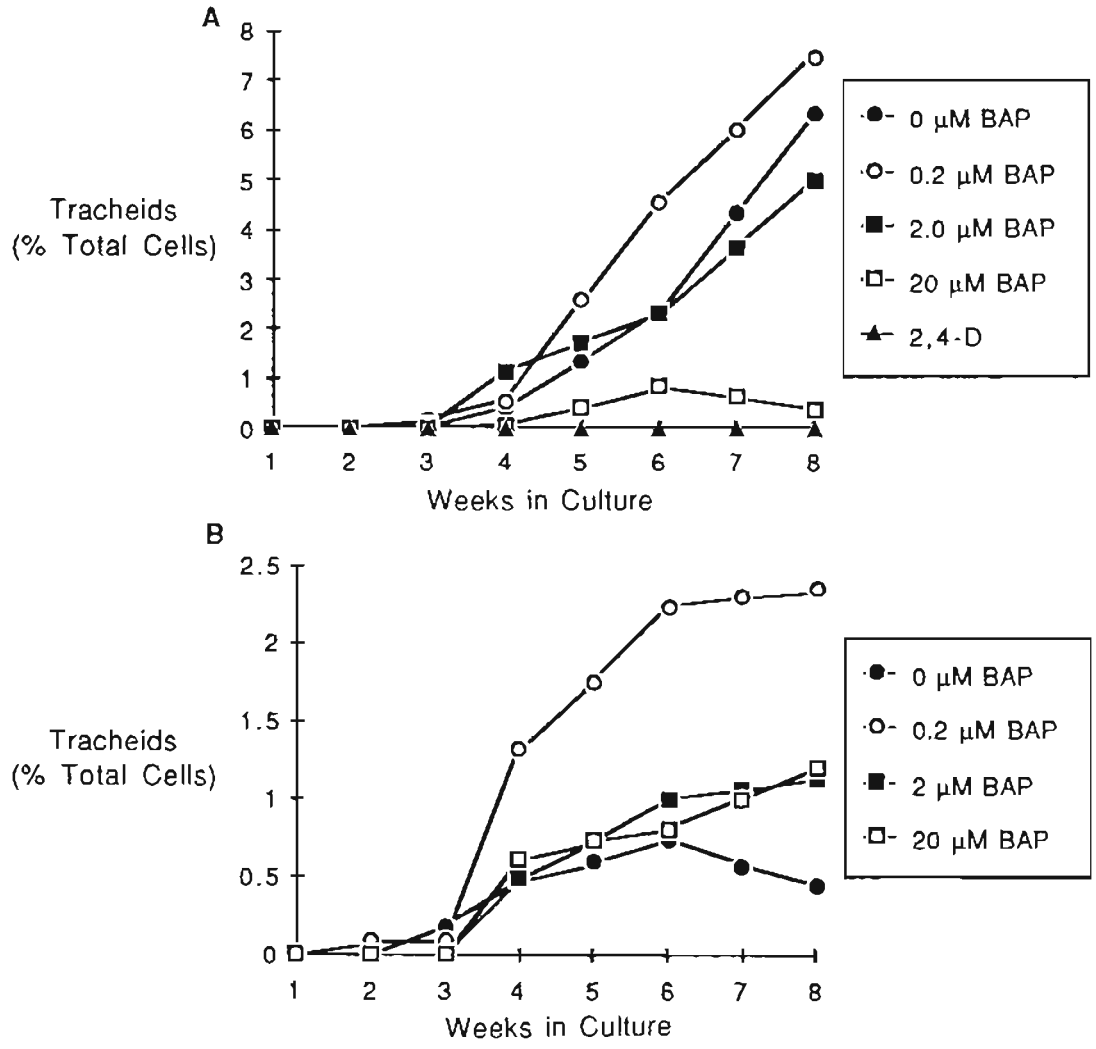


Figure 5.2 Time course of tracheid differentiation. Tissues were maintained on media containing 5 µM 2,4-D, then transferred to media without added hormones for two 2-week transfers, after which they were transferred to media containing hormones as indicated. **A** Tissues were transferred to media containing 10 µM NAA and BAP concentrations as shown, or 5 µM 2,4-D. **B** Tissues transferred to media supplemented with 100 µM NAA and BAP as shown n = 5.

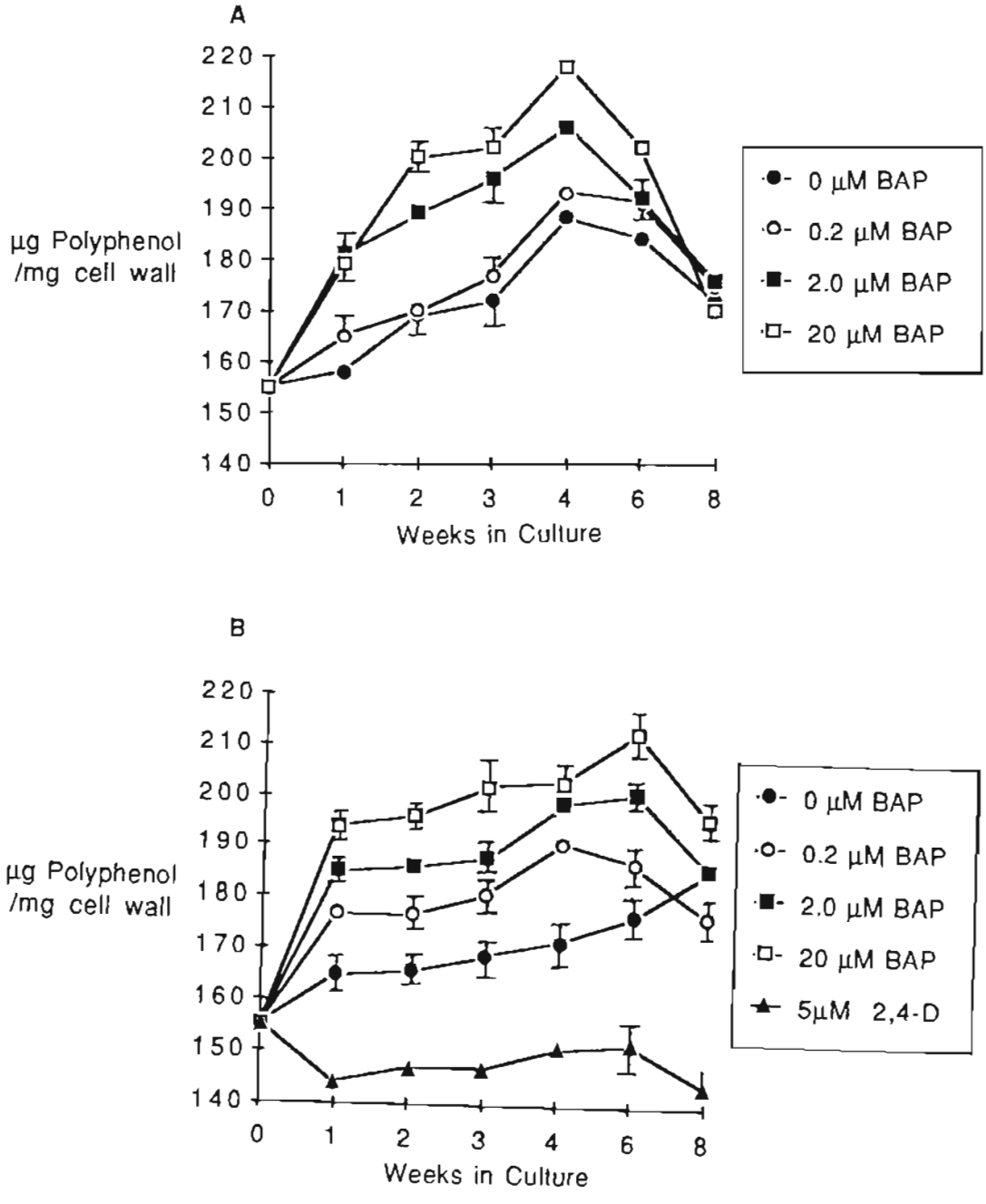


Figure 5.3 Time course for polyphenol synthesis in *P. trichocarpa* tissues cultured on **A** 10 µM NAA with BAP concentrations as indicated; **B** 5 µM 2,4-D or 100 µM NAA with BAP concentrations as indicated n = 3.

produced more tracheids than tissues grown with the higher dose. Tracheid formation in response to a range of BAP concentrations was biphasic. At both NAA doses, the greatest number of tracheids formed at 0.2 μM BAP. Higher concentrations of BAP proved to be inhibitory to tracheid formation.

Figure 5.3 summarizes the results of polyphenol measurement in the same tissues. Polyphenol content increased progressively with increasing BAP concentrations at both 10 μM and 100 μM NAA. Comparable amounts of polyphenol were produced at the two NAA doses, although the rate of polyphenol accumulation was more rapid at the higher NAA concentration.

The effect of hormone concentration on polyphenol composition

The effect of various hormone concentrations on polyphenol composition are shown in Table 5.1. As seen in Chapter 4 , cells cultured without hormones produced the greatest number of tracheids and showed a polyphenol composition which most closely resembled that found in stem. On the other hand, cells cultured on 2,4-D did not form tracheids and showed a polyphenol composition quite different from stem. This difference is characterized by a relatively high proportion of P and low proportion of S. With increasing 2,4-D concentration, there was a small but progressive increase in polyphenol content. This was accompanied by a striking increase in P which reached 40% of the recovered benzoic acids at 100 μM 2,4-D. On the other hand, there was little or no change in S with increasing 2,4-D dose. Addition of 2,4-D together with 1 μM BAP or BAP plus 10 μM NAA resulted in an increase in S over that obtained with 2,4-D alone. However, the proportion of S in these tissues was considerably less than that found in tissues treated with NAA and

Table 5.1

Cell growth and the content and composition of polyphenols in cell walls of *P. trichocarpa* stem and callus tissues cultured on media containing various growth hormones after 4 weeks of growth

Tissue	Treatment	Growth [a] $[W-W_o/W_o]$	Polyphenol mg/g wall	Tracheids [b]	Phenolic Composition [c] nmol/mg cell wall				Mole %			% [e]
					P	V	S	Total	P	V	S	Recovery
Stem	None	- -	216±2	NC [d]	120	430	562	1102	10	39	51	52
Callus	No Hormones	0.79 ± 0.1	221±5	20.1	49	397	365	811	6	49	45	41
	2,4-D 0.1µM	22.9 ± 1.6	147±1	0	116	377	46	539	21	70	9	18
	" 1.0µM	32.5 ± 16.3	151±1	0	130	566	69	765	17	74	9	20
	" 10µM	45.5 ± 6.9	156±3	0	201	469	75	745	27	63	10	19
	" 100µM	7.30 ± 2.7	164±4	0	308	385	77	770	40	50	10	21
	2,4-D 5 µM + 0 BAP	29.2 ± 12.2	149±4	0	149	467	60	676	22	69	9	20
	" + 1 µM BAP	ND [f]	181±3	0	158	435	108	701	23	62	15	23
	" + 1 µM BAP + 10 µM NAA	ND	191±2	0	161	409	130	700	23	58	19	25
	NAA 10 µM + 0 BAP	1.50 ± 0.9	193±0.3	4.2	165	463	157	785	21	59	20	24
	" + 0.1µM BAP	3.30 ± 1.4	202±1	8.5	167	416	174	757	22	55	23	23
	" + 1.0µM BAP	2.10 ± 0.3	208±2	10.2	176	431	192	799	22	54	24	29
	" + 10 µMBAP	1.50 ± 0.2	213±0.7	12.8	164	419	238	821	20	51	29	30
	" + 100µMBAP	0.80 ± 0.1	221±3	4.1	159	411	285	855	19	49	34	32

(Continued)

Table 5.1, Continued

Tissue	Treatment	Growth [a] [W-W_o/W_o]	Polyphenol mg/g wall	Tracheids [b]	Phenolic Composition [c] nmol/mg cell wall P	V	S	Total	Mole % P	V	S	% [e] Recovery
10 μM NAA + 0.1μM Kinetin		1.50 ± 0.9	201±3	4.7	178	427	171	776	23	55	22	28
"	+ 1.0μM Kinetin	2.90 ± 1.2	209±2	7.7	159	420	214	793	20	53	27	29
"	+ 10μM Kinetin	0.90 ± 0.2	217±3	3.9	146	407	260	813	18	50	32	29
"	+100μM Kinetin	0.50 ± 0.1	222±2	0.9	143	395	302	840	17	47	36	31
NAA 100 μM + 0 BAP		19.9 ± 3.0	185±3	0.3	142	449	157	748	19	60	21	27
NAA + 0.1μM BAP		20.1 ± 1.4	189±1	6.7	160	472	168	800	20	59	21	29
"	+ 1.0μM BAP	10.7 ± 1.1	195±1	8.0	158	459	174	791	20	58	22	28
"	+ 10 μM BAP	5.10 ± 0.4	199±2	12.1	156	442	221	819	19	54	27	29
"	+100μM BAP	2.20 ± 0.2	206±1	10.6	157	422	248	827	19	51	30	30
"	+ 0.1μM Kinetin	27.0 ± 1.4	192±2	2.2	156	460	164	780	20	59	21	28
"	+ 1.0μM Kinetin	30.0 ± 3.0	197±2	6.7	154	470	186	800	19	58	23	29
"	+ 10μM Kinetin	8.60 ± 1.2	202±0.7	11.9	148	444	230	822	18	54	28	29
"	+100μM Kinetin	2.50 ± 0.4	210±3	8.4	154	429	274	857	18	50	32	33

[a] Growth expressed as mean ± SEM, n = 19 (W and W_o = final and initial weights of explant, respectively)

[b] Tracheid counts expressed as % total cells, n = 5.

[c] Phenolic acids: P = *p*-hydroxybenzoic acid; V = vanillic acid; S = syringic acid. Determined after alkaline copper hydrolysis and permanganate oxidation; n = 3.

[d] NC = not counted; stem wood contains numerous xylem and vessel elements.

[e] Percent of estimated phenolic content that was recovered as benzoic acid oxidation products.

[f] ND = not determined.

BAP alone. These results indicate that 2,4-D both promotes the accumulation of P and suppresses the accumulation of S.

At both 10 µM and 100 µM NAA, increasing concentrations of BAP or kinetin caused an increase in polyphenol content. This was accompanied by a progressive increase in the proportion of S in the recovered oxidation products. On the other hand, increasing cytokinin concentrations resulted in no change, or perhaps a slight decrease in the proportion of P. Thus, auxin (2,4-D) and cytokinin (BAP or kinetin) appear to have opposite and antagonistic effects on the incorporation of P and S into cell walls.

The response of tissues to increasing hormone concentrations with respect to growth and tracheid formation was biphasic, whereas both polyphenol concent and composition changed monotonically throughout the concentration range. Thus, there was no direct relationship between wall polyphenol and the growth and differentiation of the cells.

Discussion

When cells were cultured on 2,4-D, they contained a relatively low polyphenol level and did not produce tracheids. However, if they were transferred to growth media containing no hormones or NAA plus BAP, polyphenol content increased, and some cells differentiated into tracheids. Since these changes were reversible (Figure 5.1), stock lines maintained on 2,4-D could be used in experiments with other hormones, provided several transfers intervened so the residual 2,4-D could be removed from the tissues. Changes in growth rate, polyphenol content and tracheid differentiation were all modulated by the growth regulators.

Auxins and cytokinins play important roles in the control of xylem differentiation (Roberts 1976). In culture, these hormones have also been shown to affect polyphenol formation. Cytokinins, in particular, led to increased polyphenol content. Since cells become lignified as they differentiate, one might expect that an increase in polyphenol content would be closely related to formation of tracheary elements. Data presented in Table 5.1, however, show that, in cultured *P. trichocarpa* cells, polyphenol levels could be increased without the formation of tracheids (Table 5.1). Cell growth and tracheid formation exhibited biphasic responses to the addition of cytokinin, while polyphenol levels increased progressively with increased cytokinin concentration. Thus, although tissues cultured on BAP always contained a higher level of polyphenol, there was no direct correlation between the polyphenol content of purified cell walls and tracheid formation in the callus.

Total phenolic composition gradually changed when greater concentrations of cytokinin were added to media containing NAA. None of the combinations of auxin or cytokinin was able to duplicate results obtained from stem or from callus cultured on hormone free medium (Table 5.1). Increased concentrations of cytokinin in the medium led to the recovery of more syringic acid after permanganate oxidation of purified cell walls. The levels of S were directly proportional to cytokinin concentration but not to growth or tracheid differentiation.

When tissues were cultured on media containing BAP, the concentrations of NAA had little effect on the polyphenol content. Compared to treatment on media without hormones, auxin treatment led to accumulation of P and suppression of S, whereas cytokinins promoted the accumulation of S and suppression of P. NAA did not block the effect of cytokinin on S accumulation, but the magnitude of S accumulation was

greater at the lower NAA dose. The 2,4-D was more effective at suppressing S than is NAA. Thus, auxin and cytokinin have quite different effects on polyphenol composition.

CHAPTER 6

Clonal Analysis of Cytokinin Autonomy and Polyphenol Biosynthesis In Cultured *Populus trichocarpa* Cells

With many plant species, it is possible to grow callus and regenerate fertile planta from single, cultured cells (Murashige, 1974). Analysis of cloned cell lines has revealed striking variation in a number of traits (Larkin and Scowcroft, 1981). This clonal variation can thus be used to obtain cell lines and plants with new properties. In this chapter, we examine clonal variation in cell-wall polyphenol content and composition. Stable cell lines with abnormal polyphenol deposition would be useful systems for studying the regulation of polyphenol synthesis and would provide the first step in isolating woody plants with an altered lignin content.

One example of clonal variation is in the requirement for exogenous hormones. While cultured cells and tissues normally require auxin and cytokinin supplements for growth, cultured cells may spontaneously loose the requirement for one or both of these hormones, a phenomenon known as habituation (Meins, 1982). Clonal analysis of tobacco cells habituated for cytokinin showed that cells may vary considerably in their cytokinin requirement (Meins and Binns, 1977). We have shown that cytokinin treatment affects both polyphenol content and composition (Chapter 5), and here we compare clonal variation in cytokinin habituation with polyphenol production.

Single cell cloning of *Populus trichocarpa*

Populus trichocarpa tissues cultured on 5 µM 2,4-D for one to two years were transferred to liquid medium and cultured with shaking for various periods of time.

Single cells were selected and plated on solid medium. Two methods were used for obtaining single cell clones. In the first method, protoplasts were made from the cells grown in liquid culture. This ensured that clones originated from single cells. In the second method, cells were filtered through nylon mesh with 106 or 190 micron pores. The majority of cells that passed through these filters were single. Ten to 20% of the cells passing through the 106 μ filter were in clusters of 5 or 6 cells, while some of those going through the 190 μ filter (at least 10%) were in clusters of 15 or 20 cells. Thus, some of the colonies formed from filtered cells were probably derived from cell clusters rather than single cells.

Cloning efficiency is the measure of the plated cells that actually divided to form colonies. The effects of cell density and length of time in liquid culture on the plating efficiency are summarized in Table 6.1. No colonies were obtained if the plating density was below approximately 3 X 10^3 cells per plate; whereas, with greater than 10^4 cells per plate, growth was too dense to distinguish separate colonies. Increased time in liquid culture for up to 15 days raised cloning efficiency. The highest cloning efficiency obtained was 4%. For comparison, Sticklen *et al.* (1986) cloned elm with an efficiency of 5%.

Clonal variation

More than 700 clones were isolated from *P. trichocarpa* stem callus. These cloned calluses exhibited a number of phenotypic variations (Figure 6.1A) such as growth rate, friability and amount of chlorophyll. Through the first 4 to 5 transfers, slower growing clones tended to have higher polyphenol levels, but after 5 or 6 transfer periods, these tissues tended to grow faster, and the various cell lines tended to look

Table 6.1

The effect of cell density, cloning method and time in liquid culture on the plating efficiency of *Populus trichocarpa* [a] cells after three weeks of culture time on semisolid medium containing 5 µM 2,4-D. Cells were isolated from callus that had been in culture for one or two years.

	Protoplasts[b]			Filter Pore Size[c]					
				106 µ			190 µ		
Days in Suspension Culture	Plating Density[d] (Cells/plate)	Colonies /plate	% Efficiency	Plating Density (Cells/plate)	Colonies /plate	% Efficiency	Plating Density (Cells/plate)	Colonies /plate	% Efficiency
3	1.0 X 10^4	5±3[e]	0.05	3.3 X 10^3	1±1	0.03	3.2 x 10^3	20±7	0.63
7	1.0 X 10^4	7±3	0.07	3.6 X 10^3	6±1	0.17	6.0 X 10^3	28±10	0.47
10	ND	ND	ND	4.2 X 10^3	12±3	0.29	7.7 X 10^3	50±10	0.65
13	1.0 X 10^4	180±12	1.80	5.6 X 10^3	39±7	0.70	8.7 X 10^3	320±24	3.70
15	1.0 X 10^4	300±40	3.00	5.6 X 10^3	140±20	2.50	1.0 X 10^4	350±30	3.40

[a] The original callus used in these cloning experiments was uncloned.

[b] Protoplasts were prepared as described in Chapter 2; n = 3.

[c] Cells were filtered through nylon filters of designated pore size. These protoplasts were not made from these filtered cells. The quality of clones obtained from both protoplasted and filtered cells was comparable.

[d] Cells were dispensed onto 100 X 15 mm plastic petri plates containing 40 ml MS medium supplemented with 5 µM 2,4-D. Plating densities higher than 1.0 X 10^4 resulted in colonies too dense to count.

[e] Colonies per plate ± SEM. For protoplasts, n = 3; for filtered cells, n = 6.

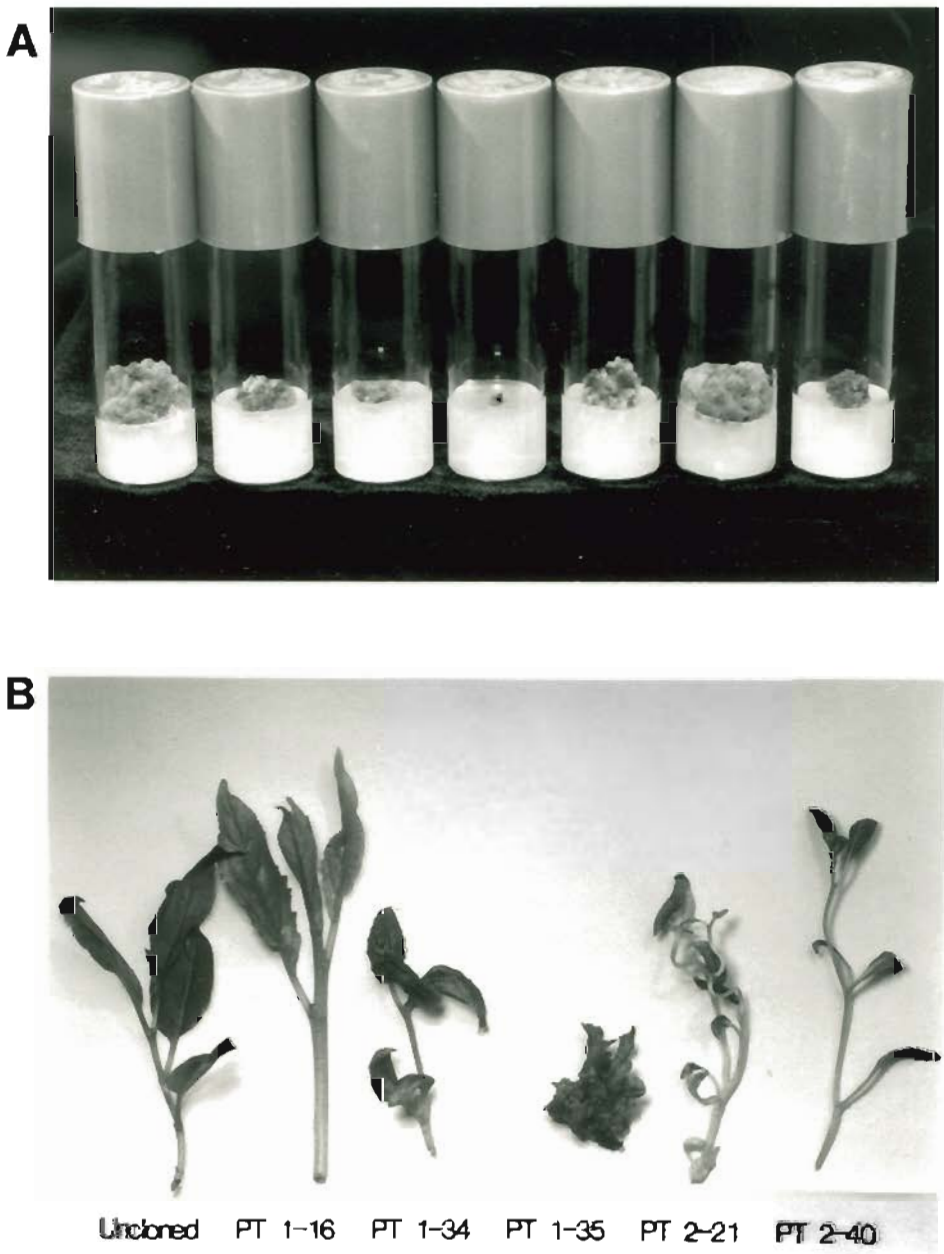


Figure 6.1 Clonal variation in *Populus trichocarpa* tissues. **A** Variation in the morphology of callus growing on media containing 5 µm 2,4-D **B** Variation in the morphology of regenerated shoots.

and grow similarly. A number of clones, however, did retain variations, many of which have remained stable for more than four years in culture.

Polyphenol content was measured in cell walls of the clones during six 4-week transfer periods on media containing 5 μM 2,4-D. There was considerable variation in polyphenol levels among individuals in the clone population from transfer to transfer (Figure 6.2). The variability within each population approximated a normal frequency distribution.

The polyphenol content in walls of uncloned tissues cultured on 5 μM 2,4-D was estimated at 14 or 15% of the wall fraction, and this level remained stable from transfer to transfer. Analysis of 720 clones showed that polyphenol levels can range from 10 to 25% of the cell wall dry weight when grown on 2,4-D media.

Several cloned lines were tested for their ability to regenerate shoots. Tissues were transferred from 2,4-D to media containing BAP only. Shoots formed on explants from approximately 60% of the clones (Table 6.2). These shoots formed most readily on clones with lower levels of cell-wall polyphenol in 2,4-D-grown callus. The results indicate that a narrow range of between 1.0 and 1.5 μM BAP was optimal for shoot formation in most clones. Kinetin was less effective in shoot induction.

Besides variation in shoot initiation, shoot phenotypes were also quite variable (Figure 6.1 B). Some shoots appeared to resemble those of greenhouse grown trees (Uncloned and PT 1-16), while others exhibited variation in leaf structure (PT 1-34, PT 2-21, PT 2-40) or lacked petioles (PT 1-35). Two of the cloned lines shown in Figure 6.1 B were continuously cultured on hormone-free medium for 36 months (PT 1-34 and PT 1-35). Shoots from PT 1-34 and 1-16 were readily rooted and were grown on soil in the greenhouse, while shoots from PT 1-35 never

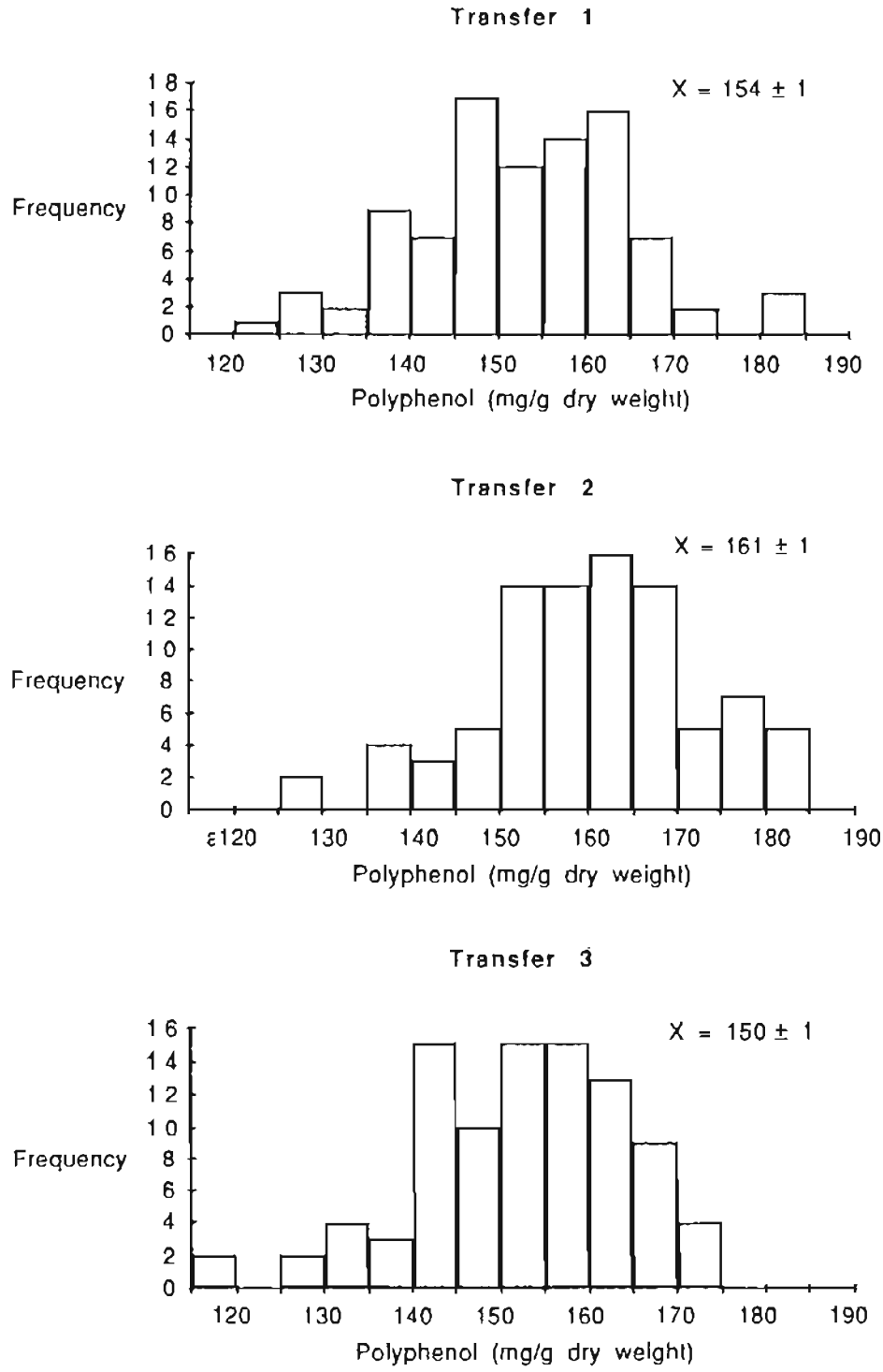


Figure 6.2 Frequency distribution of individuals in a set of 92 clones (PT-2) during the first three 4-week transfers on media containing 5 µM 2,4-D.

Table 6.2

The effects of BAP concentration on the regeneration of *P. trichocarpa* clones.[a]

Tissue	BAP:	% Explants with Shoots 0.5 μM	1.0	1.5	2.0
Below Average Clones					
PT 1-16		0	75	63	0
PT 1-21		0	0	0	0
PT 1-28		0	0	13	13
PT 1-35		0	0	0	0
PT 1-43		0	38	50	0
PT 2-40		0	13	0	0
	Mean:	0	21	21	6.5
Average Clones					
PT 1-13		0	0	0	0
PT 1-37		0	13	0	0
PT 1-38		0	13	0	0
PT 2-8		0	0	25	0
PT 2-20		0	38	25	0
	Mean:	0	11	8	0
Above Average Clones					
PT 1-3		0	0	0	0
PT 1-4		0	0	0	0
PT 1-15		0	13	0	0
PT 1-34		0	0	0	0
PT 2-1		13	0	13	0
	Mean:	2	2	2	0
Uncloned Callus (PT-0)		0	38	38	0

a Values are totals of the second through the fifth transfer onto media containing BAP; n = 8 explants per tissue per transfer.

produced roots. With increasing length of time in culture, it became easier to generate shoots on some clones, while others lost the capacity to regenerate.

Since many of the clones exhibited variation in polyphenol content, it was of interest to find out whether they also varied in polyphenol composition. Permanganate oxidation of purified walls from cells of several clones (Table 6.3) shows that variation also exists in polyphenol composition, whether tissues are cultured on media containing 5 µM 2,4-D, 10 µM NAA or NAA plus 1 µM BAP. When grown on 2,4-D, the recovered *p*-hydroxybenzoic acid ranged from 18-25 µg/mg cell wall, whereas vanillic acid varied from 91-101 µg/mg cell wall and syringic acid ranged from 9-15 µg/mg. The differences between highest and lowest levels of P, V or S in tissues cultured on NAA were similar to those from 2,4-D. Tissues cultured on NAA plus BAP showed a wider range of variation: P (13-31 µg/mg), V (72-87 µg/mg) and S (23-38 µg/mg). There does not, however appear to be any relationship between polyphenol content and composition.

Stability of polyphenol content in cloned cell lines

Although there was considerable variation in growth, polyphenol content, composition and shoot regeneration among many of the clones, a number of the lines maintained these traits through at least six 4-week transfers. These clones were considered to be "stable". Several of the stable cloned cell lines were subcloned in an effort to obtain clones with more extreme levels of phenolic polymer (Table 6.4). Subclone populations from PT 1-43 (a clone with lower polyphenol content) and PT 1-15 (a clone with higher polyphenol content) had ranges similar to that of the original cloned population. As measured by repeated subculturing, each population of clones that was studied showed a different percent of stable individuals. Table 6.5

Table 6.3

Clonal variation in polyphenol composition of 8 cloned cell lines cultured on 5 μM 2,4-D, 10 μM NAA or 10 μM NAA + 1 μM BAP

	μg Polyphenol/mg cell wall									
		2,4-D [a]			NAA			NAA + BAP		
Clone line	Polyphenol	P [b]	V	S	P	V	S	P	V	S
PT 1-16	133±1	17.9 [c]	95.1	13.7	24.2	80.3	22.9	19.3	82.9	35.9
PT 1-35	131±4	25.0	95.9	10.5	19.7	87.8	23.6	13.3	86.7	33.4
PT 1-38	115±2	24.6	92.1	13.0	23.0	87.8	24.3	27.5	82.4	27.5
PT 1-43	150±1	20.6	101.4	15.1	23.3	81.5	24.6	27 5	76.0	27.5
PT 2-07	158±2	25.2	91.9	8.8	25.6	79.5	23.1	31.7	78.0	22.5
PT 2-28	147±1	22.7	97.6	13.4	24.8	83.4	22.2	29.8	78.6	27.1
PT 2-71	166±2	23.1	91.1	14.1	23.8	84.7	23.8	27.3	80.4	28.6
PT 2-85	155±2	22.4	96.1	13.2	22.8	77.3	31.1	24.3	72.4	38.0

[a] Media contained 5 μM 2,4-D or 10 μM NAA or 10μM NAA + 1 μM BAP following 2 transfers on media without hormones; harvested after 3 weeks of growth.

[b] Permanganate oxidation products after copper pretreatment: P = *p*-hydroxybenzoic acid, V = vanillic acid, S = syringic acid.

[c] An average of measurements from two oxidation samples.

Table 6.4

Population stability of polyphenol content in cell walls of cloned *P. trichocarpa* tissues through six consecutive transfers

	Parent Tissue Cell line					
	PT-0 [b]		PT 1-43 [b]		PT 1-15 [b]	
Transfer [a]	Mean[c]	Range	Mean	Range	Mean	Range
1	131±2	97-161	156±2	123-185	188±2	142-238
2	158±2	132-179	147±1	113-179	177±1	148-218
3	172±2	141-200	162±2	129-199	167±1	136-197
4	157±2	121-179	167±1	142-196	172±1	143-196
5	156±2	143-193	159±2	132-190	169±1	140-200
6	159±2	129-181	149±1	127-169	164±1	141-199

[a] Tissues cultured on media containing 5 µM 2,4-D, subcultured every 4 weeks.

[b] Parent tissue that was subcloned: "PT-0" (uncloned), original polyphenol level = 148±3, n = 3
"PT 1-43", original polyphenol level = 142±2, n = 3
"PT 1-15", original polyphenol level = 169±3, n = 3.

[c] Number of clones generated from each parent: "Uncloned", 48 clones (which include PT 1-15 and PT 1-43); "PT 1-43", 86 subclones; PT 1-15, 98 subclones. Mean value is ± SEM for all clones or subclones in each set.

Table 6.5

Variation in cell wall polyphenol among individual *Populus trichocarpa* clones through six consecutive transfers [a]

Clone Number [b]	Below Mean	Mean [c] ± SD	Above Mean
PT 1-15-1	1 5	2 3	4 6
-2		1 2 3	4 5 6
-3	1 2 5 6	3 4	
-4	1 2 5 6		3 4
-5	4	5 6	1 2 3
-6		1 2 5 6	3 4
-7 *			1 2 3 4 5 6
-8	4	5 6	1 2 3
-9 *			1 2 3 4 5 6
-10	3		1 2 4 5 6
-11	1 3	2	4 5 6
-12	1 3	2 5	4 6
-13	1 2 3	4 5 6	
-14 *	1 2 3 4 5 6		
-15	1 5 6	2 3 4	
-16	1 3		2 4 5 6
-17 *			1 2 3 4 5 6
-18 *			1 2 3 4 5 6
-19	1 3	4 5 6	2
-20	1 5		2 3 4 6
-21	1 5 6	2 3 4	
-22	3 4 6		1 2 5
-23	3 5 6	4	1 2
-24		2 3 4	1 5 6
-25	1 3	4 5 6	2
-26	1	3 4	2 5 6
-27 *	1 2 3 4 5 6		
-28 *	1 2 3 4 5 6		
-29 *	1 2 3 4 5 6		
-30	1 2 3	4 5 6	

a Tissues cultured on media containing 5 µM 2,4-D; transfer period is 4 weeks. Polyphenol level for the original PT-1-15 clone = 169±2. Polyphenol is measured as µg/mg cell wall as compared with coniferyl alcohol DHP (Chapter 2); n = 3, ± SEM

b Subclones 1-30 from clone PT-1-15; * refers to subclones that are consistently above or below the mean through all 6 transfers.

c Means for the 6 transfers: 1 = 182±11; 2 = 177±5; 3 = 167±5; 4 = 172±5; 5 = 179±5; 6 = 169±5. (±SD) Numbers refer to the transfer number. Their positions in "below mean", "mean" or "above mean" columns indicate the relative levels of polyphenol measured for the subclone.

shows the variation in cell wall polyphenol for the first 30 subclones from PT 1-15. Throughout the duration of the six transfers, seven individual subclones consistently had polyphenol concentrations at least one standard deviation higher or lower than the population mean. Although some stable individuals in the subclone populations did contain more extreme polyphenol contents in one or two transfers, no expectionally high or low stable individuals were obtained.

When stable clones were transferred from 5 µM 2,4-D to media containing 10 µM NAA or 10 µM NAA plus 1 µM BAP, the level of cell wall polyphenol and the range of variation increased. Nevertheless, the relative differences in polyphenol content between clones were maintained. Clones that contained lower levels of polyphenol on 2,4-D generally produced less polyphenol when cultured on NAA or NAA/BAP medium (Figure 6.3). The polyphenol content in 30 stable clones grown on 2,4-D was compared with their polyphenol levels when cultured on NAA or NAA with BAP. The correlation coefficients for the linear regression analyses were 0.93329 and 0.95472, respectively. Thus, the amounts of polyphenol in the walls of cells cultured on NAA or NAA plus BAP always remained similar, although they were somewhat higher on NAA plus BAP.

As a further measure of polyphenol stability in clones, polyphenol content was measured in 2,4-D-grown callus, regenerated shoots or roots, and 2,4-D-grown callus derived from these regenerated structures (Table 6.6). For each cloned line tested, polyphenol levels in callus, regenerated shoots or roots and derived callus was essentially the same irrespective of the type of tissue used to generate the callus. Thus, polyphenol levels appeared to be stable through the cycle of regeneration.

Polyphenol content in PT 1-16 callus cultured on 2,4-D was lower than that for the tissue from which it was cloned. Polyphenol levels in stem or leaf from a 2-year

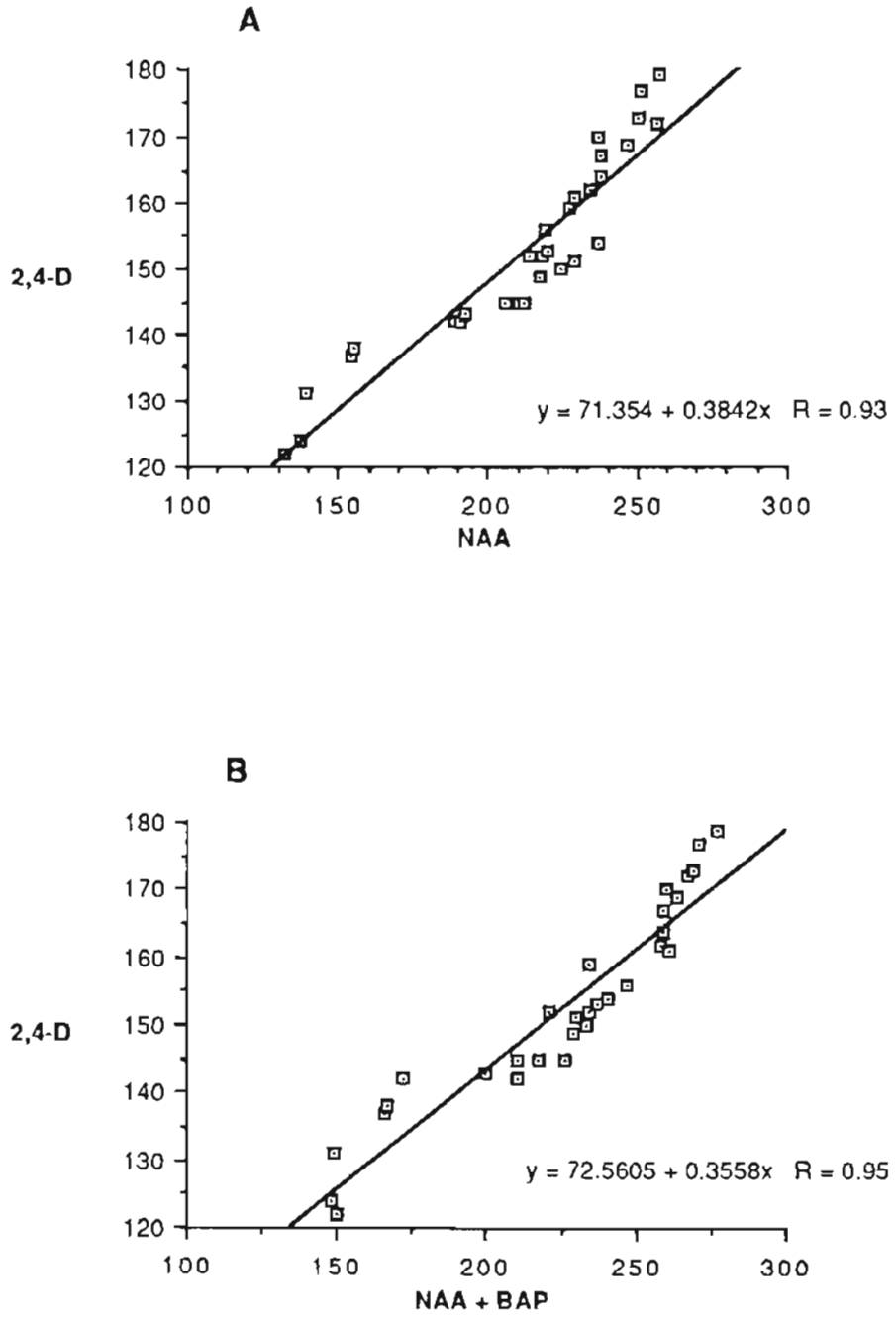


Figure 6.3 Correlation of polyphenol content in 30 stable clones of *P. trichocarpa* cultured on media containing **A** 10 µM NAA or **B** 10 µM NAA + 1 µM BAP.

Table 6.6

Comparison of polyphenol content in cloned *P. trichocarpa* tissues with differentiated structures and callus generated from them

Clone number	Polyphenol (µg/mg cell walls)				
	Callus [a]	Regenerated Shoot [b]	Callus from regenerated plant [c]		
			Shoot	Root	Stem
PT 1-15	169±2	160±3			159±3
PT 1-16	132±1	138±4			139±2
PT 1-21	132±2	138±3			140±3
PT 1-34	157±3	156±2			156±2
PT 1-35	129±5	127±3			121±1
PT 1-38	100±1	112±2	99±2	95±2	107±2
PT 1-43	149±0.3	146±3			142±1
PT 2-08	132±4	136±2	138±3	134±3	140±4
PT 2-09	146±4	147±4			152±2
PT 2-20	169±3	182±4			179±2
PT 2-36	178±3	180±2			175±2
PT 2-69	163±4	159±4			159±1
PT 2-85	155±2	151±1			161±2
PT 2-86	161±1	162±2			161±3

[a] Callus cultured on medium containing 5 µM 2,4-D

[b] Shoots were generated on calluses cultured on medium containing 1 µM BAP as sole hormone. The shoots formed after 3 to 5 transfers following removal from 2,4-D-containing media. Leaves were removed from shoots for "stem" measurements. Roots formed on callus growing on BAP-containing media after the second or third transfer from media containing 2,4-D.

[c] Callus was formed from shoot, root or stem tissue of regenerated structures Polyphenol was measured on the second transfer onto media containing 5 µM 2,4-D.

old plant regenerated from PT 1-16 also showed reduced polyphenol content (Table 6.7). Both two year-old woody stem and leaf, as well as 2,4-D callus from PT 1-16 contained approximately 90% of the amount measured in similar tissue from the original uncloned line. Thus, it is possible to select for a tree with lower lignin content by screening 2,4-D-grown variant callus for tissues containing lower than average levels of polyphenol.

Clonal analysis of cytokinin habituation

Since exogenous cytokinins affect polyphenol amount and composition, it was of interest to study how polyphenol content was related to the cytokinin requirements of the cells. Studies in cultured tobacco have shown that cytokinin habituation may occur to varying degrees (Binns and Meins, 1977). Hormonal requirements of cultured plant cells may be estimated by comparing their growth rates on media containing or lacking the hormone to be studied. Since plant tissues show a biphasic dose response to cytokinins (Chapter 5), the ratio (R) of growth obtained without (-C) or with (+C) cytokinin supplements (i. e. R = -C/ +C) can be used to provide a measure of hormone autonomy (Binns and Meins, 1973). Tissue which grew better without cytokinin than with cytokinin supplements (i.e., $R > 1$) are considered to be highly habituated. On the other hand, tissues which grew better with cytokinin supplements ($R < 1$) are considered to be habituated to a lesser degree.

Several stable clone lines were grown on media containing 10 µM NAA (-C) or 10 µM NAA and 1 µM BAP (+C) or 5 µM 2,4-D. Their growth ratios (R values) were calculated and compared with polyphenol levels. As with tobacco cells (Binns and Meins, 1977), *P. trichocarpa* tissues exhibited varying degrees of cytokinin habituation when grown in culture. In the sixty cell lines studied, R values ranged

Table 6.7

Polyphenol concentration in PT 1-16 and uncloned *P. trichocarpa* wood and callus

Treatment	Tissue	
	Uncloned	PT 1-16
Callus		
5 μM 2,4-D	149±3 [a]	133±2
10 μM NAA + 1 μM BAP	181±3	138±4
Plant		
2 year old Stem	219±2	199±2
Leaf	147±3	137±3
Regenerated Stem callus [c]		
(5 μM 2,4-D)	ND [b]	135±2

[a] n = 3, ± SEM.

[b] Not determined.

[c] Callus regenerated on 5 μM 2,4-D from 2 year old stem tissue, measured on the third transfer.

from 0.1 to 2.2. Table 6.8 shows data for several of these cell lines. When polyphenol levels for each hormone treatment were plotted as a function of R value, correlation coefficients from the linear regression line of these plots did not reveal any relationship between polyphenol content and habituation. These coefficients were -0.15661, -0.08958 and -0.00747 for 2,4-D, NAA, and NAA plus BAP, respectively. Thus, variation in polyphenol content did not appear to result from cytokinin habituation.

Although degree of habituation was not correlated with polyphenol content, it may be related to polyphenol composition. Purified cell walls from four highly habituated and five less-habituated clones were subjected to copper hydrolysis and permanganate oxidation. Polyphenol composition in these cloned lines (Table 6.9) shows that approximately 20 to 30 µg of *p*-coumaric and syringic acids were recovered from each of the tissues, while three to four times this amount of vanillic acid was found.

There do not appear to be any systematic differences between composition of polyphenol in more or less habituated clones when they are grown on 2,4-D media. If one compares polyphenol composition in highly habituated tissues grown on NAA with those grown on NAA plus BAP, one finds an increase of S and a small decrease in P when cytokinin is present in the media. With less habituated clones, on the other hand, there is only a slight increase in S and an increase in P. Although these differences are small, they are consistent for all the tissues that were tested (Table 6.9). The ratio of the mole% of (-C/ +C) for the three oxidation products make the differences in change for P, V and S easier to see. The ratio for P was greater than one for more habituated cell lines, and less than one for those that were less habituated (correlation coefficient = 0.94761, see note, Table 6.9). Ratios for V did

Table 6.8

Clonal variation in growth and cytokinin autonomy of cloned *P. trichocarpa* tissues.

Cell Line [a]	Treatment [b]	Growth [c] ($W-W_o/W_o$)	R [d]	Polyphenol [e] (µg/mg cell wall)
PT-1-35S	2,4-D	ND		141±2
	NAA	8.70±1.87	2.20	159±2
	NAA + BAP	3.95±0.71		170±2
PT-LN	2,4-D	ND		132±2
	NAA	48.86±6.36	2.02	155±5
	NAA + BAP	24.69±2.56		193±2
PT-HN	2,4-D	52.57±2.25		152±3
	NAA	23.68±5.20	1.74	174±2
	NAA + BAP	13.16±2.17		210±6
PT 1-16-24-18	2,4-D	ND		172±1
	NAA	10.74±0.50	1.67	194±2
	NAA + BAP	6.45±0.66		232±3
PT 1-43	2,4-D	ND		153±1
	NAA	10.06±0.75	1.10	201±2
	NAA + BAP	9.13±0.56		223±2
PT 1-43L	2,4-D	ND		149±1
	NAA	6.03±0.33	1 07	208±3
	NAA + BAP	5.64±0.37		234±2
PT 2-71	2,4-D	37.24±1.48		169±3
	NAA	6.03±0.58	0.68	201±8
	NAA + BAP	8.82±0.83		228±2
PT-D	2,4-D	21.29±1.10		150±6
	NAA	4.29±0.48	0.42	162±2
	NAA + BAP	10.14±0.76		185±3
PT 2-85	2,4-D	19.0±1.65		171±4
	NAA	3.51±0.43	0.42	245±2
	NAA + BAP	8.40±0.82		275±11
PT 1-38	2,4-D	ND		127±1
	NAA	6.54±0.78	0.39	147±2
	NAA + BAP	16.62±2.80		166±1

(Continued)

Table 6.8, Continued

Cell Line [a]	Treatment [b]	Growth [c] ($W-W_0/W_0$)	R [d]	Polyphenol [e] (µg/mg cell wall)
PT 1-43-44-15	2,4-D	ND		127±3
	NAA	1.13±0.18	0.33	132±8
	NAA + BAP	3.42±0.38		163±5
PT 2-28	2,4-D	9 45±1.43		148±1
	NAA	2.11±0.28	0.32	214±3
	NAA + BAP	6.62±1.09		239±4
PT 1-38L	2,4-D	ND		124±1
	NAA	1.16±0.43	0.24	152±3
	NAA + BAP	4.90±0.24		161±1

Linear regressions for polyphenol plotted as a function of R for the 3 Treatments and for the ratio of polyphenol NAA / NAA + BAP:

2,4-D R = -3.30 (polyphenol) + 154.9 Correlation coefficient = -0.15661
NAA R = -14.2 (polyphenol) + 197 2 Correlation coefficient = -0.08958
NAA + BAP R = -0.38 (polyphenol) + 225.1 Correlation coefficient = -0.00747

NAA / BAA + BAP R = -0.02 (polyphenol) + 0.892 Correlation coefficient = 0.24791

[a] Cell lines PT-LN, -HN, -D are uncloned. PT 1-35S has a teratoma-like growth habit. It grows and forms shoots without added hormones. Cell lines followed by "L" are derived from shoot leaves.

[b] Tissues cultured on media containing 5 µM 2,4-D or 10 µM NAA or 10 µM NAA and 1 µM BAP.

[c] Growth and polyphenol were measured following the third transfer onto treatment media. W = final weight of explant, W_0 = initial weight; n = 18, ±SEM; ND = not determined.

[d] R = ratio of $W-W_0/W_0$ for tissues grown on media containing NAA ÷ $W-W_0/W_0$ for tissues grown on media containing NAA and BAP. If R is greater than 0.8, tissues are considered to be cytokinin habituated.

[e] Polyphenol measured in the same tissues as were measured for growth; Polyphenol level as compared to coniferyl alcohol DHP.

Table 6.9
Clonal variation in cell wall polyphenol composition in highly habituated and less habituated clones of *P. trichocarpa*

Cell Line [a]	R [b]	Treatment [c]	Polyphenol µg/mg cell wall P	V	S	Polyphenol mole % P	V	S	Phenolic Ratio (-C / +C) [d] P	V	S
PT 1-35S	2.20	NAA	20.6	91.8	24.7	15	67	18	1.50	1.03	0.72
		NAA + BAP	13.9	90.4	34.8	10	65	25			
PT-LN	2.02	NAA	24.3	81.1	29.7	18	60	22	1.20	1.05	0.79
		NAA + BAP	20.6	78.3	38.5	15	57	28			
PT-HN	1.74	NAA	27.1	88.4	23.7	19.5	63.5	17	1.22	1.04	0.74
		NAA + BAP	22.7	86.6	32.7	16	61	23			
PT 1-16-24-18	1.67	NAA	25.3	83.8	23.9	19	63	18	1.36	1.05	0.69
		NAA + BAP	18.7	80.2	34.7	14	60	26			
PT 2-71	0.68	NAA	23.0	87.8	24.3	17	65	18	0.85	1.08	0.90
		NAA + BAP	27.3	81.8	27.3	20	60	20			
PT-D	0.42	NAA	25.7	83.7	25.7	19	62	19	0.86	1.07	0.95
		NAA + BAP	29.6	78.1	26.9	22	58	20			
PT 2-85	0.42	NAA	24.7	86.3	26.0	18	63	19	0.86	1.09	0.90
		NAA + BAP	28.5	78.8	28.5	21	58	21			
PT 1-43-44-15	0.33	NAA	25.7	86.7	23.0	19	64	17	0.86	1.10	0.85
		NAA + BAP	30.4	80.0	27.6	22	58	20			
PT 2-28	0.32	NAA	27.2	84.3	24.5	10	62	18	0.87	105	0.95
		NAA + BAP	33.1	91.0	24 8	23	59	18			

Table 6.9, Continued

Linear regression for ratios of mole % of P (-C/+C) and S (-C/+C) plotted as a function of R for each cell line:

P: R = 0.310 (ratio for P) + 0.722 Correlation coefficient = 0.94761 (significant to 99.9%)
S: R = -0.111 (ratio for S) + 0.953 Correlation coefficient = 0.87946 (significant to 99.9%).

[a] See footnote a, Table 6.6.

[b] See footnote d, Table 6.6.

[c] Tissues grown on media containing 10 μM NAA or 10 μM NAA plus 1 μM BAP.

[d] Tissues were cultured on media containing hormones indicated. Purified cell walls were hydrolyzed with copper and oxidized with permanganate Oxidation products products were P: 4- methoxybenzoic acid; V: 3,4-dimethoxybenzoic acid; S: 3,4,5-trimethoxybenzoic acid. Mole % of P, V, S were calculated, and the (-C/+C) ratios are for P or V or S in tissues grown without cytokinin (-C) / with cytokinin (+C).

not vary with degree of habituation. More habituated clones had lower ratios for S (correlation coefficient = 0.87946). Both correlations are significant to greater than 99% confidence.

A number of shoots and roots formed on the callus explants during the habituation experiments (Table 6.10). Shoots formed only on highly habituated clones, and these grew on explants cultured on both NAA and NAA plus BAP. The largest number of shoots grew on the two cell lines that were most highly habituated. While it is unlikely that shoots are required for cytokinin habituation to take place, habituated tissues tend to form shoots more readily. As expected, roots tended to form on calli that were cultured on media containing auxin. Although a few roots appeared when cytokinin was added to the medium, addition of BAP was usually inhibitory to root formation.

There was much variation between cell lines in the degree of tracheid formation when callus was grown on NAA or NAA plus BAP (Table 6.8). In all cell lines that formed tracheids, the number of tracheids that differentiated increased when cytokinin was added to the medium. Tissues that formed shoots had a higher frequency of tracheid differentiation.

Discussion

The clones obtained from cultured *P. trichocarpa* cells revealed a wide range of variation with respect to polyphenol, growth rates and regeneration. Of particular interest were those clones which displayed high or low polyphenol content that remained stable after repeated transfers. After several attempts to obtain clones with extremely high or low levels of polyphenol, it was evident that the content of polyphenol in the cells ranged between about 110 and 180 µg/mg cell wall.

Table 6.10

Clonal variation in tracheid formation, and regeneration of roots and shoots from highly habituated and less habituated *P. trichocarpa* cell lines.

Cell Line [a]	Treatment [b]	R [c]	Percent of Explants with [d] Roots	Shoots	% [e] Tracheids
PT 1-35S	NAA	2.20	0	57	14.29
	NAA + BAP		0	67	20.00
PT-LN	NAA	2.02	0	83	7.07
	NAA + BAP		0	72	14.29
PT-HN	NAA	1.74	11	0	0.22
	NAA + BAP		6	6	1.96
PT 1-16-24-18	NAA	1.67	78	6	5.72
	NAA + BAP		11	17	9.32
PT 2-71	NAA	0.68	33	0	1.78
	NAA + BAP		0	0	3.02
PT-D	NAA	0.42	31	0	0.00
	NAA + BAP		0	0	0.00
PT 2-85	NAA	0.42	6	0	4.76
	NAA + BAP		0	0	6.75
PT 1-43-44-15	NAA	0.33	78	0	1.19
	NAA + BAP		0	0	2.71
PT 2-28	NAA	0.32	50	0	0.53
	NAA + BAP		0	0	2.38

[a] See footnote a, Table 6.6.

[b] Tissues grown on media containing 10 μM NAA or 10 μM NAA plus 1 μM BAP.

[c] See footnote d, Table 6.6.

[d] Percent of explants that formed shoots or roots on NAA or NAA + BAP treatments; n = 18; no explants had both roots and shoots.

[e] Percent of total cells that differentiated to form tracheids; (only callus cells were counted, not roots or shoots).

Several methods were used to test for clonal stability of polyphenol content. Polyphenol levels were measured in the clones for six consecutive transfers on 2,4-D media. If polyphenol content remained above or below the mean on all six transfers, the level of polyphenol was considered stable. In another measurement for stability, clones were cultured on three different hormone treatments. A cell line that exhibited polyphenol stability on 2,4-D was also stable on media containing NAA or NAA plus BAP. Furthermore, regenerated shoots and callus initiated from these shoots also maintained relatively constant polyphenol levels. The characteristic differences between higher or lower clones were therefore maintained. This indicates that polyphenol content is an inherited trait, and that each clone that was stable with respect to polyphenol content on one growth hormone was stable on others as well.

Polyphenol levels did not show any correlation with the degree of habituation (Table 6.8). The degree of cytokinin habituation, however, was positively correlated with differences in polyphenol composition (Table 6.9). Permanganate oxidation of polyphenol from highly habituated clones yielded a higher amount of syringic acid and lower levels of *p*-coumaric acid when grown on media containing cytokinin. In relatively unhabituated clones, P levels rose when the cells were cultured on cytokinin-containing medium. Thus, although exogenous cytokinin increased polyphenol content in all tissues, those cells that were most highly habituated for cytokinin responded differently to this hormone.

The stable clones with lower polyphenol content described in this chapter may be used for regenerating trees containing decreased levels of lignin. The polyphenol levels remained stable in the cloned 2,4-D callus, in the regenerated shoots and in 2,4-D callus derived from these shoots (Table 6.6). Wood from the stem of a two-year old plant regenerated in culture from a clone with lower levels of polyphenol

showed an 8 to 10% reduction in lignin as compared with lignin from the stem the origina tree (Table 6.4). This suggests that tissue culture may be useful in the selection of traits that exhibit themselves in both cultured tissues and plant. Further work, however, is needed to verify whether this trend would continue for other variants. Since it is likely that lignin levels in regenerated plants grown in the field might vary depending on environmental conditions, it is unclear how regenerated variant plants would behave in the wild. Selection of low lignin trees by means of tissue culture, however, remains a distinct possibility.

CHAPTER 7

Isolation and Partial Characterization of Phenylalanine Ammonia Lyase and Peroxidase Isozymes in Cultured *Populus trichocarpa* Tissues

Variation in the phenolic content of cell walls may result in part from hormonal regulation of the activities of phenylalanine ammonia lyase (PAL) and peroxidases. Phenylalanine ammonia lyase is a well characterized enzyme that catalyzes the first reaction specific to the phenylpropanoid pathway. It is important in the control of carbon flow to polyphenols and other phenolic compounds (Camm and Towers, 1973). Peroxidase activity in both herbaceous and woody plants is known to be affected by growth hormones (Lee, 1972) and appears to be closely related to growth, differentiation, and lignification (Obst and Harkin, 1973; Wolter and Gordon, 1975; Masuda *et al.*, 1983; Goldberg *et al.*, 1986). We have studied changes in PAL and peroxidase activities under different hormone regimes which affect polyphenol synthesis, tracheid differentiation and tissue morphology.

Protein extraction

Protein was extracted from cells of *P. trichocarpa* stem and from callus tissue grown on media containing 5 µM 2,4-D, 10 µM NAA plus 1 µM BAP, or without added hormones. Tissues cultured on hormone-free media contained more than twice the amount of protein per gram fresh weight as found in two-year-old stem (Table 7.1), while those cultured on media containing added hormones produced more than three times that amount. Since stem tissue was woody and the majority

Table 7.1

Protein content in the growth medium and in soluble and ionic fractions from *P. trichocarpa* stem and cultured tissues

Tissue	Cell Wall [a] Polyphenol (µg/mg cell wall)	Crude Protein Fractions		
		Fraction [b]	µg Protein/ g fresh wt	% [d]
Stem	217 ± 2	Soluble	75.90 [c]	
			85.82	91
		Ionic	7.83	
			7.66	9
Callus:				
5 µM 2,4-D	148 ± 2	Soluble	245.65	
			212.23	56
		Ionic	74.26	
			79.27	19
		Medium	108.62	
			95.75	25
10 µM NAA +1 µM BAP	201 ± 3	Soluble	190.86	
			152.14	60
		Ionic	111.27	
			116.25	39
		Medium	2.13	
			3.67	1
No hormones added	215 ± 2	Soluble	120.00	
			128.53	61
		Ionic	68.21	
			72.33	35
		Medium	8.15	
			8.08	4

(continued)

Table 7.1, Continued

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[a] Expressed as µg polyphenol/mg cell wall. No differences were seen between polyphenol content in walls of callus cultured on liquid or solidified media; ± S.E.M., n = 2.

[b] Soluble = soluble in buffer; ionic = extracted into $CaCl_2$; medium = liquid culture medium.

[c] Data from 2 experiments, n = 2 for each.

[d] Percent of protein in the Soluble, Ionic or Medium fractions (The value is the average for both experiments).

of the cells were no longer living, they were expected to contain less protein than callus tissues. For each tissue, at least half of the total extracted protein per gram fresh weight was found in the soluble fraction. Cultured tissues with higher polyphenol levels contained a lesser amount of soluble protein.

Peroxidases in cultured tissues are often secreted into the medium (Schloss *et al.*, 1987). Since proteins secreted into solidified media are difficult to quantify, callus tissues were grown on polyester fabric pads soaked with liquid media, so that protein in the medium could be more readily isolated. Tissues cultured in the presence of 2,4-D grew much faster and secreted significantly more protein than those cultured on media containing NAA and BAP or with no hormones. There was no difference between cell wall polyphenol content in tissues cultured on liquid and solidified media.

Measurement of PAL activity

Xylem formation in cultured tissues has been correlated with increased levels of PAL activity (Haddon and Northcote, 1976; Fukuda and Northcore, 1982). Tissues cultured on media which promoted greater tracheid differentiation contained higher PAL activity when calculated on the basis of either protein concentration or fresh weight (Table 7.2). Stem, which contained many tracheids, was used as a control. When compared with 2,4-D-grown tissues, protein fractions from tissues cultured on NAA plus BAP or without hormones contained a higher specific activity for PAL. These tissues also had greater polyphenol content and a higher degree of tracheid differentiation. Thus, PAL activity was related to the presence of tracheids and elevated polyphenol content.

Table 7.2

Phenylalanine ammonia lyase activity in crude protein extracts from *P. trichocarpa* stem and cultured tissues

Tissue	Hormone Treatment	Cinnamate µmol/hr /µg Protein	Cinnamate µmol/hr /mg fresh wt
Stem [a]	None	64.1 [b]	5.5
		68.5	5.2
Callus:	5 µM 2,4-D	14.6	3.1
		13.4	3.3
	10 µM NAA + 1 µM BAP	22.0	3.8
		25.0	4.2
	No Hormones	35.0	4.3
		35.0	4.3

a Tissue from 2 year old stem, harvested in the spring.

b Data is from two experiments, n = 2 for each.

Histochemical localization of peroxidases

Hand-cut sections of young *Populus trichocarpa* stems or two-week-old callus cultured with or without hormones were stained using hydrogen peroxide and two different hydrogen donors. 4-Chloro-1-naphthol was oxidized by a number of isoperoxidases, and stained tissues and gels very well. On the other hand, syringaldazine was more specific for the isozymes involved in lignification (Harkin and Obst, 1973), but produced water-soluble products that tended to diffuse into surrounding tissue, making localization difficult. Thus, chloronaphthol was useful in localizing peroxidases, while syringaldazine was used to test for the presence of peroxidases that were more specific for polyphenol formation.

Table 7.3 shows the relative intensity of staining with chloronaphthol or syringaldazine when viewed through a light microscope. When chloronaphthol was used as a hydrogen donor, most of the staining took place inside the cell, but cell walls remained clear. When syringaldazine was used as a substrate, cytoplasm remained clear, but walls were stained. Cut surfaces stained with both chloronaphthol and syringaldazine. Xylem tissue in stem stained very strongly with both reagents. Cell walls of tracheids in cultured tissues also stained for peroxidases, as did the walls of superficial cells from all the cultured tissues.

Several problems were encountered during peroxidase localization in tissues. Photography of syringaldazine-stained material was difficult because of poor color contrast. This problem had also been noted by Harkin and Obst (1973) and Goldberg *et al.* (1983). When H_2O_2 was omitted from the staining assays, cells stained very slowly with both chloronaphthol and syringaldazine. This may indicate the presence of low levels of endogenous H_2O_2. Since syringaldazine is also a substrate for laccase (Leonowicz and Grzynowicz, 1981), the presence of laccase

Table 7.3

Relative Intensity of staining in tissues by chloronaphthol or syringaldazine in the presence of hydrogen peroxide

Source of Tissue	Tissue	Stained by: Chloronaphthol Cytoplasm [a]	Chloronaphthol Wall	Syringaldazine Cytoplasm	Syringaldazine Wall
Stem:	Cortex	±[b]	-	-	-
	Phloem	+	-	-	+
	Xylem	++	-	-	++
	Pith	±	±	-	±
Callus:					
5 µM 2,4-D	Tissue Surface	+	±	-	+
	Parenchyma	±	-	-	±
10 µM NAA + 1 µM BAP	Tissue Surface	+	±	-	+
	Parenchyma	±	-	-	±
	Tracheids	+	-	-	+
No Hormones Added	Tissue Surface	+	±	-	++
	Parenchyma	±	-	-	±
	Tracheids	+	-	-	+

[a] Cytoplasm = cell contents were stained; wall = cell walls were stained.

[b] Intensity of staining as seen through a light microscope: - Not stained; ± A few cells lightly stained; + stained; ++ Very darkly stained.

cannot be ruled out. The pink color of the syringaldazine chromophore began fading from 2,4-D-grown callus walls within one or two minutes after addition of syringaldazine and H_2O_2, while stem cells and callus cells grown on media without added hormones remained strongly colored for at least 20 minutes. Cells cultured on NAA + BAP started fading after 8 to 10 minutes. The chloronaphthol chromophore remained stable for several hours.

Peroxidase Purification and Kinetic Studies

Mäder and coworkers (1975) isolated 3 peroxidase isozyme groups from cell walls of tobacco. The most anionic isoforms, extracted from cell walls by salt, showed the highest rate of polymerization of cinnamyl alcohols. These isozymes have been shown to have very high activity with syringaldazine as substrate (Goldberg *et al.*, 1986). Therefore, peroxidase activity was measured in both soluble and wall-associated, salt-extracted protein fractions (Table 7.4). Because cell wall peroxidases are secreted from cells, peroxidase activity was also measured in liquid media of cultured tissues.

Peroxidase activity was low in crude protein fractions from all tissues. The syringaldazine chromophore faded especially quickly in the soluble fractions from tissues cultured on media containing 2,4-D, and, to some extent, from NAA plus BAP tissues. Therefore, measurements with crude homogenates may underestimate peroxidase activity. After the enzyme fractions were purified by gel filtration with Sephadex G-100, the chromophore did not fade throughout the period of measurement. Peroxidases may bind, polymerize or degrade auxins and phenolics, and these reactions could interfere with the measurement of peroxidase activity. However, when 5 μM 2,4-D or 10 μM NAA and 1 μM BAP were added

Table 7.4

Peroxidase assays on purified protein fractions from *P. trichocarpa* cells using syringaldazine as substrate

Tissue	Fraction [a]	Crude Fraction [c]		After G-100 Column		After G-100, FPLC		
		ΔOD/min/ µg protein	ΔOD/min/ g fw	ΔOD/min/ µg Protein	ΔOD/min/ g fw	ΔOD/min/ µg Protein	ΔOD/min/ g fw	Yield [d]
Stem	Soluble	ND [b]	ND	0.004	0.18	0.008	0.03	17
		ND	ND	0.003	0.11	0.007	0.01	9
	Ionic	0.02	2.55	0.43	2.78	0.73	0.35	13
		0.03	3.92	0.47	3.04	0.75	0.35	12
Callus								
5 µM 2,4-D	Soluble	0.02	1.08	0.07	4.46	2.40	0.72	16
		0.02	1.09	0.09	3.70	2.33	0.55	15
	Ionic	0.07	3.94	0.14	5.60	0.64	0.71	13
		0.08	4.01	0.20	6.04	0.63	0.61	10
	Medium	0.05	4.46	0.35	8.60	0.75	1.24	14
		0.07	4.73	0.40	9.92	0.83	1.32	13
10 µM NAA + 1 µM BAP	Soluble	0.03	3.16	0.44	11.4	1.51	1.41	12
		0.04	3.13	0.37	11.5	1.62	1.37	12
	Ionic	0.09	5.80	0.98	11.2	2.87	1.36	12
		0.10	5.86	1.10	13.1	2.77	1.65	13
	Medium	0.01	8.69	0.94	14.1	2.20	2.00	14
		0.02	10.45	0.94	13.3	2.17	1.62	12
No Hormones Added	Soluble	0.04	6.33	0.32	7.80	0.99	1.01	13
		0.04	6.31	0.39	8.29	1.11	0.85	10

(Continued)

Table 7.4, Continued

Tissue	Fraction [a]	Crude Fraction [c] ΔOD/min /µg protein	Crude Fraction [c] ΔOD/min /g fw	After G-100 Column ΔOD/min /µg Protein	After G-100 Column ΔOD/min /g fw	After G-100, FPLC ΔOD/min /µg Protein	After G-100, FPLC ΔOD/min /g fw	Yield [d]
	Ionic	0.17	2.49	0.67	14.9	2.79	2.83	19
		0.17	2.35	0.71	14.1	2.77	2.45	17
	Medium	0.09	11.04	0.33	13.2	2.39	2.51	19
		0.08	9.90	0.31	14.7	2.57	2.21	15

[a] See Table 7.1 for explanation.

[b] ND = not detected; data from 2 experiments, n = 2 each.

[c] The chromophore faded quickly in assays of crude fractions in these tissues and therefore measurements may be less accurate.

[d] Difference in yield between amounts of protein recovered after Sephadex G-100 and FPLC column recoveries.

to partially purified protein, there was no change in peroxidase activity when either syringaldazine or guaiacol were used as substrate. Thus, growth hormones did not inhibit peroxidase activity *in vitro*.

In FPLC-purified protein from stem tissue (Table 7.4), peroxidase-specific activity in the ionic fraction was 10 times greater than that from the soluble fraction. Activities in the ionic fraction from cultured tissues were lowest in callus grown on media containing 2,4-D and highest from tissues grown on NAA plus BAP or without added hormones. Liquid media from callus tissues contained peroxidase activity which was similar to that from the ionic fractions. After the ionic fraction had been extracted, cell walls still contained a high level of activity as measured by syringaldazine oxidation, but this activity could not be accurately quantitated. Thus, not all the peroxidase activity was extracted from the cell walls.

Kinetic studies were performed using soluble and ionic fractions from tissues cultured on media containing NAA plus BAP after purification by FPLC. Guaiacol is a widely used peroxidase substrate, and its activity was compared with that of syringaldazine. Initial velocities were linear for at least 1 minute with up to 1 µg protein preparation. Double reciprocal plots were constructed from initial velocity data. The plots for both the soluble and ionic fractions are shown in Figure 7.1. The Michaelis-Menten constant (K_M) for syringaldazine was at least 5700 times lower than for guaiacol. The K_M values for syringaldazine were similar for both fractions (about 6.7 to 6.9 X 10^{-7} M), but the K_M for guaiacol in the soluble fractions was about half of the value for the ionic fraction (Figure 7.1).

Figure 7.2 shows the effects of pH on the oxidation rates of syringaldazine and guaiacol. Oxidation of syringaldazine and guaiacol showed similar pH profiles. The

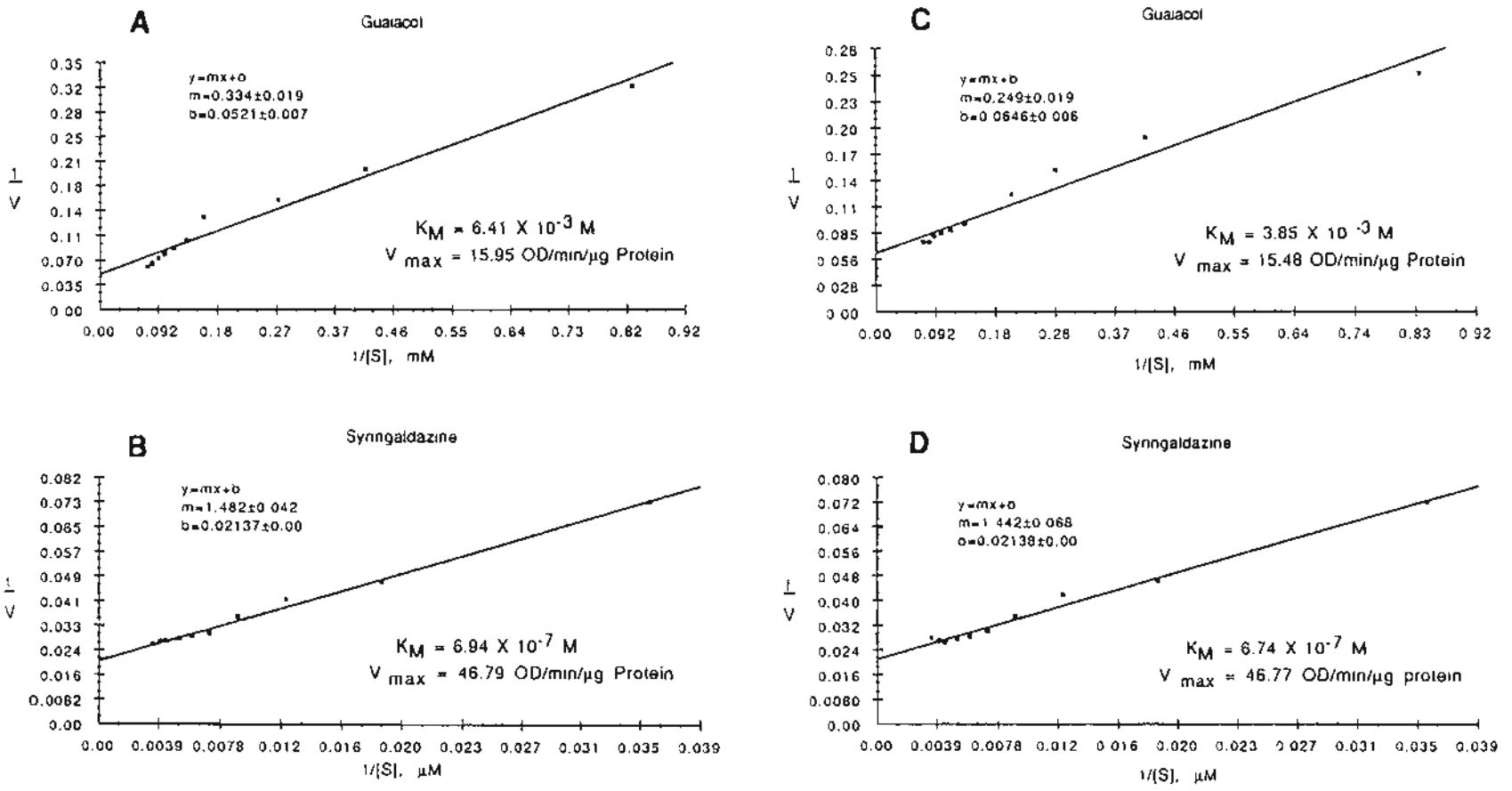

Figure 7.1 Lineweaver Burke plots of peroxidase activity using guaiacol (**A**, **C**) and syringaldazine (**B**,**D**) as substrates. Soluble (**A**, **B**) and ionic (**C**,**D**) peroxidase fractions were obtained from callus cultured on 10 µM NAA and 1 µM BAP and purified by FPLC.

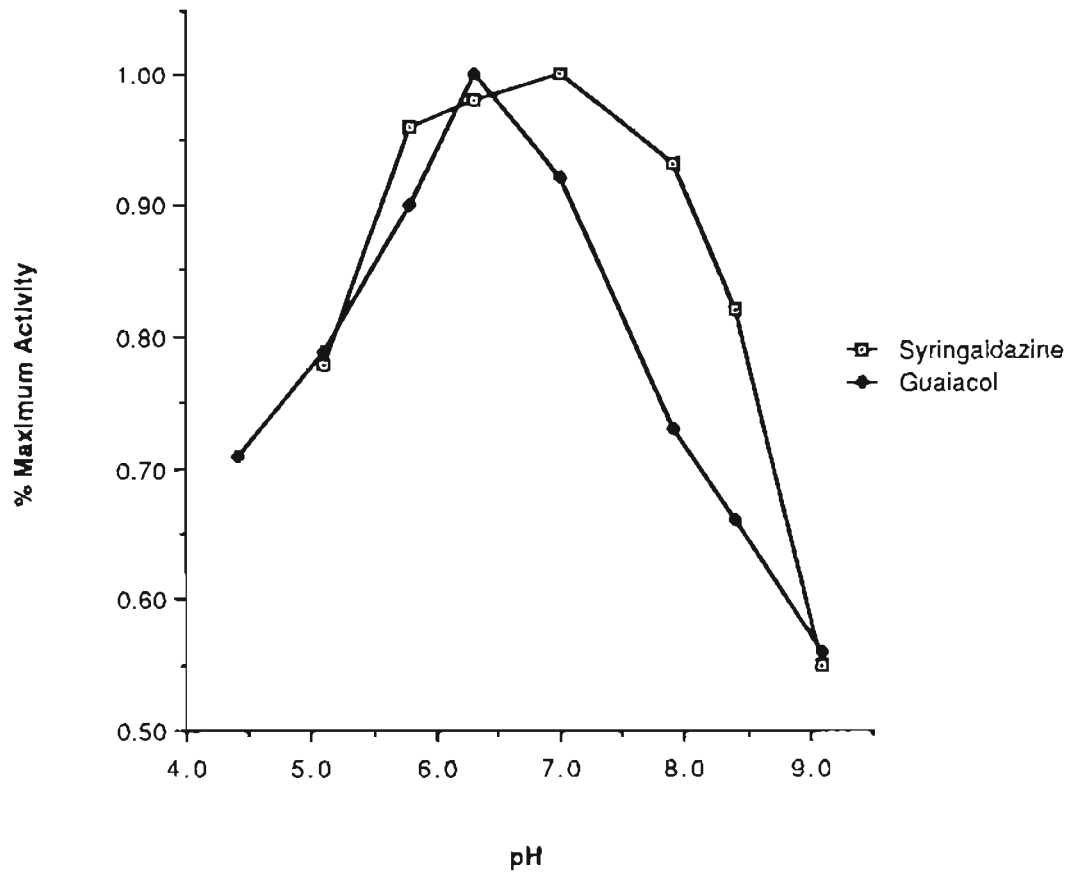

Figure 7.2 Effect of pH on the oxidation rates of syringaldazine and guaiacol using FPLC purified soluble protein fraction from callus cultured on 10 µM NAA and 1 µM BAP and expressed as per cent of the maximal activity.

pH optimum for syringaldazine was 7.0, while optimal pH for guaiacol was slightly lower. Maximal activity occurred at lower H_2O_2 levels with syringaldazine as hydrogen donor as compared with guaiacol (Figure 7.3). Based on elution profiles from G-100 and FPLC columns, molecular weights of syringaldazine oxidase isozymes were estimated to be between 45,000 and 50,000 daltons.

Isoelectric focusing of peroxidases

FPLC Purified peroxidases from cell wall extracts were separated on ultrathin isoelectric focusing gels. Identical protein lanes were added to each half of the gel. After development, the gels were cut, and one half was stained with syringaldazine and the other with chloronaphthol (Figure 7.4). A number of bands were strongly stained with chloronaphthol and H_2O_2, and a few weakly stained bands were also seen. The amount of protein applied for each sample is shown under each group of lanes. Most of the bands from the purified protein fractions contained anionic peroxidases. Major syringaldazine bands were all strongly anionic, with isoelectric points at 3.5 and below. It is quite possible that some isoelectric points were lower than 3.0, since resolution was poor below pH 3.5. All 2,4-D-grown tissue fractions contained a syringaldazine band at PI 3.4, while stem and the tissues cultured on other hormone treatments showed a band at 3.3.

Densitometer scans of the gels showed differences in staining intensity (Figure 7.5). When gels were stained with syringaldazine, the background was fairly high due to deposition of syringaldazine crystals on the gel surface. The most anionic bands showed the greatest staining intensity.

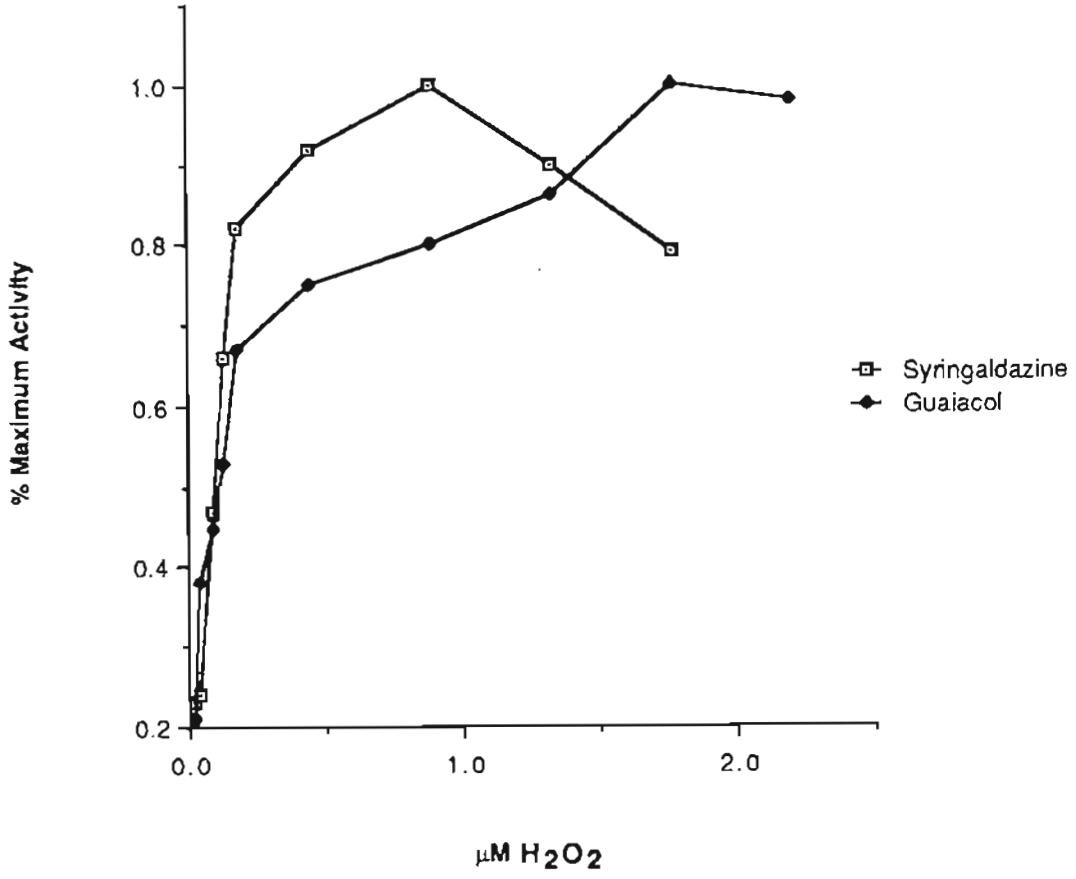

Figure 7.3 Effect of H_2O_2 concentration on the oxidation rates of syringaldazine and guaiacol using FPLC purified soluble protein fraction from callus cultured on 10 μM NAA and 1 μM BAP and expressed as per cent of the maximal activity.

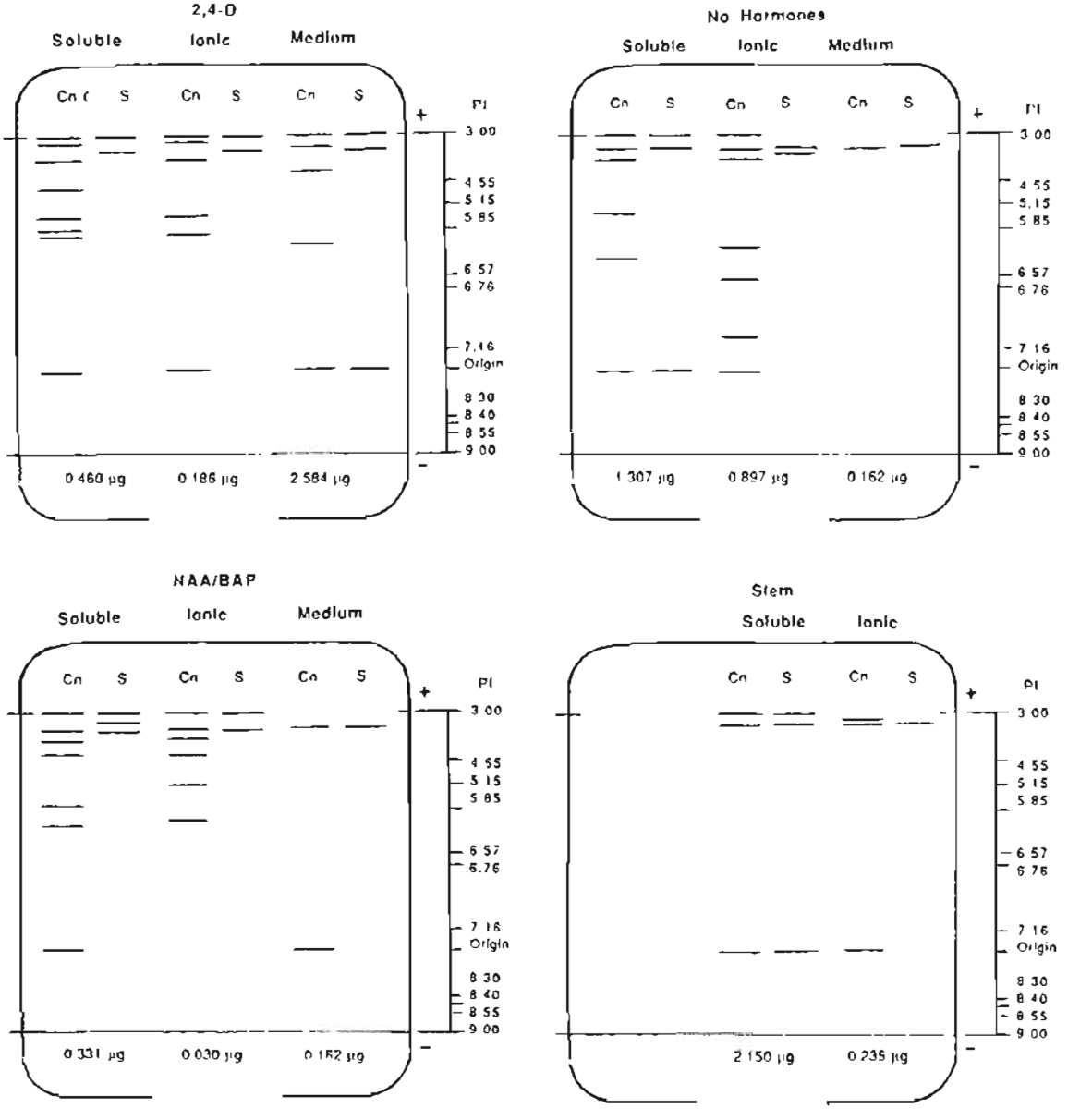

Figure 7.4 Liquid medium or cellular peroxidases from stem or callus tissues cultured on media containing 5 µM 2,4-D, 10 µM NAA + 1 µM BAP or without hormones were run on isoelectric focusing gels and stained for peroxidase with chloronaphthol (Cn) or syringaldazine (S). Lanes were redrawn so syringaldazine and chloronaphthol lanes could be compared more directly.

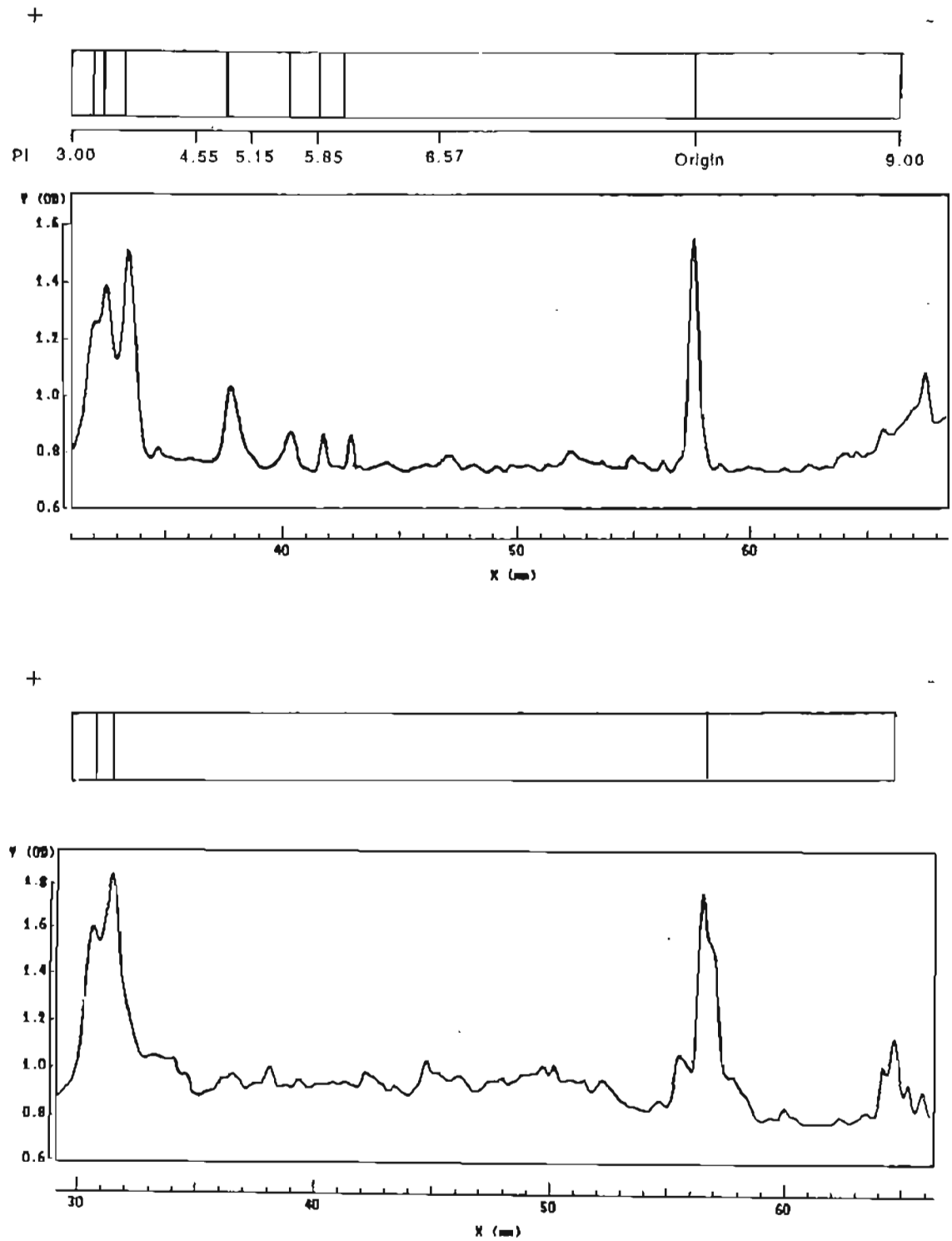

Figure 7.5 Densitometer scans of IEF gels containing peroxidase isozymes from the FPLC purified soluble fraction of callus cultured on 5 µM 2,4-D. Gels were stained with H_2O_2 and **A** Chloronaphthol or **B** Syringaldazine.

Discussion

Both PAL and peroxidases play a role in the biosynthesis of lignin. Increased PAL activity has been associated with xylogenesis in cultured tissues (Fukuda and Komamine, 1982). When calculated on a protein or fresh weight basis, PAL activity in cultured *P. trichocarpa* tissues was positively correlated with increased tracheid differentiation and polyphenol levels.

Histological staining with syringaldazine and chloronaphthol indicated that at least two classes of peroxidases were present in *P. trichocarpa* tissues. Syringaldazine stained the cell walls, while chloronaphthol stained cell cytoplasm. A lower amount of staining activity was observed in 2,4-D-grown tissues, although this may have been due to the instability of the chromophore.

Since cell wall staining indicated that syringaldazine-stained peroxidases were found primarily in the cell wall instead of in the cytosol, it was expected that peroxidases extracted from walls would exhibit higher syringaldazine oxidase activity than the soluble fractions. Results, however, showed very high activity associated with both soluble and wall-associated fractions from some tissues. Furthermore, isozyme patterns for soluble and wall-associated fractions were similar. Peroxidases released from cell walls during grinding may be found in the soluble fractions, and cytoplasmic peroxidases probably adhered to walls during grinding, thus appearing to be wall-associated isozymes. Therefore, isozymes in the ionic fractions were probably not strictly wall-associated proteins, and soluble fractions are likely to contain some wall-associated proteins. These possibilities may be further investigated by studying the proteins more closely.

There are numerous peroxidase isozymes in plants, and these are likely to perform a variety of different functions. Peroxidases were measured in

cytoplasmic and wall-associated protein fractions as well as in the liquid media. Soluble fractions always had lower peroxidase activity with syringaldazine. In cultured tissues, ionic and liquid medium fractions had similar specific activities, suggesting that some of the peroxidases secreted to the walls were also found in the medium. Since these proteins had high activity with syringaldazine, isozymes found in the ionic fractions and culture medium may be involved in the biosynthesis of polyphenols. Lowest activities were seen in fractions from 2,4-D-grown cells that formed no tracheids and possessed lower levels of polyphenol, while cultured tissues that formed tracheids possessed much higher activity. At the time of harvest, cultured cells were in the process of differentiation and polyphenol formation. It is therefore reasonable to assume that certain peroxidase isozymes involved in polymerization would be more abundant.

Peroxidase activity in the soluble fraction from 2,4-D callus was higher than that in any of the other tissues (Table 7.4). This activity was low in the soluble fraction from tissues cultured on media containing NAA and BAP, and it was even lower in fractions from very slowly-growing tissues cultured on media without added hormones. Soluble fraction activity, therefore, was greatest in tissues which were growing the fastest.

Anionic isoperoxidases have been correlated with cell wall lignification (Imberty *et al.*, 1985; Mäder, 1986). Several anionic peroxidase isozymes were identified by staining of isoelectric focusing gels with chloronaphthol (Figure 7.4). In tracheid producing tissues, more chloronaphthol-stained bands were obtained from ionic fractions than from medium. This may be due to wall contamination by cytoplasmic peroxidases. In 2,4-D-grown cells, the pattern of

isozymes from the medium fraction was more complex than in the other tissues, suggesting that 2,4-D may affect the transport of peroxidases out of the cell.

The most anionic bands stained very well with syringaldazine. Although staining intensity could not be accurately measured, it appeared that more intensely stained bands were from ionic fractions taken from tissues grown on media containing NAA and BAP or without added hormones. however, very similar syringaldazine-stained protein bands were found in cells that varied markedly in the type of polymer produced. Peroxidase isozymes that are involved in lignification may also polymerize the polyphenol found in 2,4-D-grown cells.

CHAPTER 8

Discussion

The purpose of this investigation was to study the regulation of polyphenol biosynthesis using *Populus trichocarpa* as a model woody angiosperm. *P. trichocarpa* is a rapidly growing species of poplar that shows a high capacity for regeneration. This species was therefore useful for *in vitro* monitoring of responses to exogenous plant hormones.

Venverloo (1971), Fukuda (1983) and Fukuda *et al.* (1988) suggested that structures of lignin in cultured tissues may be different from those of intact plants. They noted that, although similar oxidation products were recovered, depolymerization of callus MWL gave lower yields as well as a lower syringyl/guaiacyl ratio as compared with MWL from the corresponding woody plants. A major problem to be addressed in this thesis, then, was to determine whether *P. trichocarpa* callus produced true lignin.

Measurement of polyphenol in cell walls of cultured tissues

Two initial concerns to be addressed were cell wall preparation and measurement of polyphenol content. Chang *et al.* (1975) found that the process of preparing MWL may lead to additional methoxylations and acetylations. Since part of the task at hand was to quantitate monomeric subunits in cell walls which may differ in methoxylation patterns, a minimum of wall processing was done. The cells were homogenized and extracted at room temperature extraction with water and neutral organic solvents. Although preservation of natural methylation patterns was an important advantage in cell wall analysis, it precluded direct comparison with yields

obtained by investigators who used different cell wall preparations for their experiments.

Polyphenol content was measured spectrophotometrically at 280 nm following acetyl bromide digestion. It was assumed that all material that absorbed light at 280 nm was of phenolic origin. The presence of substantial amounts of protein containing tryptophan or tyrosine may have led to an overestimation of polyphenol content. A low extinction coefficient from solublized bovine serum albumin and horseradish peroxidase relative to polyphenol suggested that this would not be a significant problem. A further complication was the use of coniferyl alcohol DHP as a polyphenol standard. Since the extinction coefficients at 280 nm of the various phenolics in cell walls differ from that of coniferyl alcohol DHP, spectrophotometric measurements should only be considered as estimates of the actual polyphenol content.

Relationship of hormone treatment to cell wall polyphenol in cultured *P. trichocarpa*

A second major question this thesis addressed was that of hormone modulation of polyphenol in cell walls of cultured tissues. A system was set up in which to study the regulation of cell wall polyphenol by modification of hormone treatments. Although *in vitro* regulation of polyphenol biosynthesis may not parallel that occuring *in vivo*, a tissue culture system can be easily manipulated so that differences in the effects of specific hormone treatments can be monitored on genetically identical or nearly identical tissues grown under identical conditions. The resulting polyphenol can then be characterized by content and composition, and the differences correlated with

hormone treatment. This type of information may be useful for further investigations into the genetic basis of polyphenol biosynthesis.

The addition of cytokinin to cultured tobacco (Koblitz, 1962) and *Zinnia* tissues (Fukuda and Komamine, 1982) led to increased lignification and tracheid development. Increases in lignin formation and tracheid development have also been associated with elevated activities of peroxidases or of phenylpropanoid pathway enzymes such as PAL (Masuda *et al.*, 1983). Using cytokinin dose responses, we found that both polyphenol content and tracheid number were affected by the amount of exogenous cytokinin in the growth medium. Since the effects of both 2,4-D and NAA plus BAP on polyphenol level and tracheid development are reversible, it can be assumed that responses to these hormones were mediated by the growth regulator present in the medium rather than by some permanent change in the cells. It was thus possible to study the effects of different hormones using stock cultures that had been maintained on other media.

Cytokinin concentration affected the polyphenol content and tracheid development in different ways. Polyphenol content in the tissues showed an increase that was proportional to cytokinin dose, while tracheid development showed a biphasic response to cytokinin. Thus, polyphenol content was not directly related to tracheid formation.

The polyphenol content in 2,4-D-grown tissues was only about 70% of that in tissues cultured on media containing NAA plus BAP or without added hormones. Results from copper hydrolysis and permanganate oxidation showed a 3 to 6-fold higher yield in syringic acid recovered from NAA plus BAP-grown tissues as compared with tissues cultured on 2,4-D. Less than a 2-fold difference in the amount of P and V was noted in the cultured tissues. Compared to oxidation without copper

treatment, the yields from copper pretreated wall material showed greater yields of S as polyphenol levels rose and tracheid differentiation increased. Thus, treatments which increased polyphenol levels and tracheid differentiation altered the composition of the polyphenol.

There was also a relationship between polyphenol composition in cultured tissues and the type of hormone present in the culture medium. Tissues that were cultured on medium containing increasing cytokinin doses yielded more syringic and less *p*-hydroxybenzoic acid after oxidation, while addition of 2,4-D led to increased P rather than S. Thus, 2,4-D and cytokinin had very different effects on polyphenol composition. Furthermore, when tissues from highly habituated cell lines (R > 1) were cultured on media containing cytokinin, growth was slower, and the polyphenol contained less P than when cultured on media lacking cytokinin. Polyphenol in less habituated tissues (R < 1) contained more P when grown on cytokinin-containing media. Thus, cytokinin affected polyphenol composition differently in highly habituated or less habituated cell lines.

Approximately 15 to 20 % of recovered permanganate oxidation products was *p*-Hydroxybenzoic acid. It was impossible, however, to determine whether this compound was derived from oxidized phenylpropanoid units or was originally as the the benzoic acid. Smith (1955 a,b) noted that *Populus tremula* contained a high proportion of *p*-hydroxybenzoic acid which was released by alkaline hydrolysis of MWL preparations. Many other species of Salicaceae (including *Populus* species) are known to contain *p*-hydroxybenzoic or *p*-coumaryl residues (Venverloo 1971). Lapierre and Rolando (1988) found no evidence for the presence of *p*-coumaryl alcohol residues in *P. euramericana* wood. It is commonly believed that the majority of P units recovered after oxidation are derived from acids that were esterified to the

walls. We found that approximately 65 to 85% of the acids removed from *P. trichocarpa* tissues by alkaline and enzymatic hydrolysis were *p*-hyroxybenzoic or *p*-coumaric acid. Although the majority of these were *p*-hydroxybenzoic acid, recovered *p*-hydroxybenzoic acid could account for only 0.4 to 0.5% of the estimated polyphenol in the cell wall preparations, or less than 1 or 2% of the total permanganate oxidation products. This is not enough to explain the presence of P units recovered from the oxidized polyphenol.

Depending on the hormone treatment, different levels of C6-C3 residues were recovered from walls of cultured tissues by thioacidolysis. Only trace amounts of phenylpropanoid residues could be removed from the walls of 2,4-D-grown tissues. Tissues cultured on NAA plus BAP contained more than 3 times the amount of thioacidolysis products as in tissues cultured on 2,4-D, while tissues cultured on media without hormones contained about 13 times the amount recovered from tissues cultured on NAA and BAP. The majority of the recovered residues were derived from coniferyl alcohol. Approximately 9 times more thiolated phenylpropanoid derivatives were recovered from stem than from callus cultured on media without added hormones, and the majority of these were from syringyl and coniferyl alcohols. Although none of the treatments led to production of polyphenol where the majority of the phenolics were like those found in stem, the polyphenol in tissues cultured on media lacking hormones was most similar to stem.

Tissues which produced tracheids yielded greater amounts of thioacidolysis products, but all callus tissues gave low yields as compared to stem. There are two possible reasons for these differences. First, polymerized cinnamyl alcohol units may have been present in the polyphenol, but bonds between them could not be broken by thioacidolysis. If carbon-carbon bonds were present on a residue, it would not be

removed as a monomeric unit. Furthermore, a number of non-tracheid cells in tissues grown on NAA plus BAP or media without hormones could be stained with Safranin-O or Mäule reagents, indicating that they contained lignin or at least a lignin-like compound. These results suggested that the linkages in 2,4-D callus were very different from those in stem, but that tracheid-forming tissues were more likely to contain lignin. A second explanation for the thioacidolysis data is that callus tissues, especially when grown on 2,4-D, may contain very few polymerized cinnamyl alcohol units. These units were probably incorporated only into tracheids. Evidence from staining for lignin and peroxidases suggests that this is likely.

Phenylalanine ammonia lyase controls the availability of polyphenol precursors. Variation in polyphenol content may result, in part, from hormonal regulation of the activity of this enzyme. Fukuda and Komamine (1982) found high levels of PAL activity in cultured *Zinnia* mesophyll cells that were active in lignin synthesis and tracheid differentiaion. The PAL activity in *P. trichocarpa* stem was at least twice that found in callus. Activity in tissues cultured without hormones was greater than in tissues cultured on media containing NAA plus BAP. Lowest PAL activity was detected in 2,4-D-grown tissues. The differences in specific activity of PAL most closely paralleled levels of tracheid differentiation in the cultured tissues.

To examine the question of whether polyphenol composition was controlled at the polymerization step, we tested soluble and ionically bound protein fractions and liquid media for syringaldazine peroxidase activity. The ionic and liquid media fractions from tissues cultured on media containing NAA plus BAP or no added hormones had very high peroxidase activity, but fractions from 2,4-D-grown tissues contained only 20 to 30% as much activity. The soluble fraction from 2,4-D-grown tissues, however, showed high peroxidase activity with syringaldazine as a substrate, while

that from tissues grown with NAA plus BAP or without added hormones possessed 66% or 44% of this amount, respectively. Syringaldazine peroxidase activity was high in the liquid media and in ionic protein fractions from tissues that formed tracheids but not in fast-growing tissues cultured on 2,4-D. Thus, the soluble fraction activity may be related to tissue growth. Tissues cultured on media without added hormones grew very slowly, and the soluble protein fraction from this tissue also showed a low level of activity.

Anionic peroxidase isozymes have been implicated in the formation of lignin in intact plants (Mäder *et al.*, 1980). This conclusion has been based on localization studies (Mäder, 1986) response to wounding (Espelie *et al.*, 1986) and organ distribution (Lagrimini and Rothstein, 1987). We have found that all the callus tissues contained anionic peroxidases. The friability of the 2,4-D-grown tissues may have resulted from the reduced cross-linking which could have been mediated by low activity of cell wall peroxidases.

Characteristics of somatic clones of *P. trichocarpa* and their responses to exogenous hormones

Somatic cloning holds promise as a technique for selecting variants with improved yields, disease resistance, and nutrition (Murashige, 1974). Somaclonal variation can provide new options for increased genetic variability at a relatively rapid pace and without the use of sophisticated technology (Larkin and Scowcroft, 1981). This approach was used to select for variants with higher or lower cell wall polyphenol. Despite much clonal variation in polyphenol levels, a number of cell lines were obtained which contained polyphenol levels that were consistently higher or lower than the population mean when cultured on media containing 2,4-D. If stable cell

lines were transferred to media containing NAA or NAA plus BAP, the amount of polyphenol measured in cells increased. Cell lines whose walls contained less polyphenol when cultured on media containing 2,4-D also had lower polyphenol levels on media containing NAA or NAA plus BAP. Thus, the distinction between high and low polyphenol levels was maintained. Differences between high and low polyphenol content were also preserved through the cycle of shoot or root regeneration. Therefore, plants with lower or higher polyphenol levels may be obtained from tissue culture.

Cultured tissues may become habituated for growth hormones. Meins and co-workers carried out several studies with tobacco showing that cytokinin habituation was a heritable epigenetic change which was inducable and reversible (for review, see Meins, 1983). Since we found that higher levels of endogenous cytokinins in culture media led to increased polyphenol levels in *P. trichocarpa* tissues, it was possible that variant cell lines containing higher polyphenol levels were more highly habituated for cytokinin. Levels of cytokinin habituation, however, were not cor-related with polyphenol content. Therefore, different concentrations of polyphenol in clones did not appear to result from habituation.

In summary, the studies described in this thesis provide evidence that plant hormones play an important role in controlling polyphenol biosynthesis in cultured *P. trichocarpa* tissues. With this model angiosperm, it was possible to study the structure and amount of phenolics formed in the cell walls. Results showed that the amount and composition of polyphenol varied with cytokinin concentration. Most of the phenolics associated with purified walls of cultured *P. trichocarpa* tissues were not part of a typical lignin polymer. Either the phenolics were not cinnamyl alcohols, or these alcohols were cross-linked in a manner different from lignin. Cultured

tissues did appear to produce true lignin, although in much lower amounts than wood. The concentration of lignin varied with the amount and type of growth regulator supplied in the culture medium, and was associated with the formation of tracheids. Furthermore, with this system, it was possible to screen cloned cell lines for lower than normal stable polyphenol levels and to regenerate a plant with lower than normal lignin content.

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