Chapter 16

Phenolics and Compartmentalization in the Sapwood of Broad-Leaved Trees*

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Contents

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16.1 Importance of Phenolics in the Growth and Defense of Trees

Tree survival depends on the chemistry of phenolic compounds, a broad class of chemicals characterized by a hydroxylated benzene ring. In trees, phenolics occur frequently as polymers, acids, or glycosylated esters[1] and perform diverse functions. For example, lignin, a phenylpropane heteropolymer, provides structural strength to wood.[2] The induced production of phenols is a common response to injury and infection for both woody and nonwoody plants.[3,4] Phenolics also deter herbivores from feeding on leaves,[5] slow the spread of pathogens in roots and bark,[6] confer some measure of durability to heartwood,[7] and with other secondary metabolites, tend to limit the spread of infection in the sapwood of conifers[8,9] and broad-leaved trees.[10,11] This report focuses on methods to detect, measure, and identify phenolics as markers of the response of sapwood to wounding and infection in broad-leaved trees.

16.2 Compartmentalization of Discoloration and Decay

16.2.1 Stage I: Tree Response

Living trees respond to injury and infection. The wounding of sapwood initiates a cascade of spatial and temporal processes collectively termed "compartmentalization."[11-13] Compartmentalization is the boundary-setting process that tends to limit the loss of normal sapwood function following wounding. Sapwood decay occurs within compartments. The effectiveness of compartmentalization to limit the spread of infection and the loss of normal functioning is based on both constitutive and inducible features of tree defense and protection.

During most of the year, water in stemwood is under tension. When conducting xylem is wounded, water columns snap and emboli are introduced into the conducting elements. Tyloses and plugs are formed.[14] These events do not require infection, although inoculation with microorganisms may occur during stage I of compartmentalization. This stage is nonspecific and tends to minimize the volume of sapwood killed by desiccation and consequently made available for colonization by microorganisms. Stage I initiates a shift in oxidative metabolism to the shikimic acid and acetate pathways.[11] A waxy layer of suberin may be synthesized at the boundary between healthy and wound-altered sapwood to minimize dessication and reduce the spread of microorganisms.[15-17] The metabolic shift consumes readily assimilable starch, lipids, and simple sugars in advance of potential exploitation by microorganisms. Constitutive and newly synthesized phenolics oxidize and discolor the wood. Oxidative polymerization precipitates phenolics and phenolic–protein complexes, reducing their availability for microbial breakdown and utilization.

In a separate process, shifts in enzymes and growth regulators in the vascular cambium result in the formation of a barrier zone, an anatomically distinct tissue that separates wood formed after wounding from wood present at the time of wounding.[12,18-20] If wounded during the growing season, the barrier zone will form in that same year. If wounded during the dormant season, the barrier zone will form in the following year. Initially, barrier zone parenchyma contain large amounts of starch. With time and perhaps due to the spreading of infection, the stored starch is mobilized and converted to phenolics. Unless the barrier zone is breached, wound-initiated decay is limited to wood present at the time of wounding.

16.2.2 Stage II: Interactions of Microorganisms and Sapwood

As microorganisms interact with constitutive and induced tree defenses, the wood altered by wounding enters stage II of compartmentalization. In this stage, microorganisms may degrade or reduce the toxicity of phenolics in the discolored wood.[21-23] Also in stage II, the tree may form a phenol-enriched boundary layer between healthy sapwood and columns of wound-initiated discoloration.[11,24,25] This boundary may be discontinuous and usually follows ray plates in the radial plane and ring boundaries in the tangential plane. Although the phenolic-enriched column boundary layer (CBL) may be effective in limiting the spread of the decay process, the layer can be breached and reformed at a greater distance from the wound.[25] A CBL can be breached by cracks, boring insects, or particularly aggressive pathogens. Some vascular wilt pathogens spread in advance of column boundary formation, reducing the effectiveness of the boundary.[26] Some canker-rot fungi degrade boundary layer phenolics, reducing their effectiveness.[27]

16.2.3 Stage III: Wood Breakdown

The microorganisms that colonize sapwood after wounding are to some degree tolerant of phenolics present in nonwounded sapwood (e.g., gallic acid and catechin). The ability of some nonhymenomycetous "pioneer" fungi to detoxify phenols may be evidence of a microbial succession in wood through the stages of the decay process.[21,22,28]

16.3 Classification and Synthesis of Sapwood Phenolics

16.3.1 Overview

Practical interest in phenolics predates history because of the ability of some phenolics to tan or preserve leather from animal hides. Such phenolics were called tannins. As time has passed, some esterified or simple phenolics still are referred to as tannins, though their ability to precipitate macromolecules and preserve leather has not been demonstrated.[1,4] Terminology based on practical properties continues to be used, but is being replaced by a more fundamental nomenclature. Tannins occur in two major groups: proanthocyandins (formerly referred to as condensed tannins or leucoanthocyanins) that tend to polymerize upon hydrolytic treatment, and gallic acid derivatives (polymers or sugar esters, formerly referred to as hydrolyzable tannins) that hydrolyze readily to yield phenolic acids and sugars. The larger proanthocyandins (up to 20,000 Da) are regarded as the more primitive group, as they are found in ferns and conifers as well as in angiosperms. Tannins that are structurally based on gallic acid (such as ellagic acid and galloyl esters) are smaller (up to 3000 Da) and are found only in dicotyledons.[4]

16.3.2 Schematic of Biosynthesis

The fine details of the biosynthsesis of plant phenolics are complex and still are being determined. A single phenolic can be formed by different pathways, though a single route frequently is predominant. Phenolic synthesis requires a shift in carbohydrate utilization away from energy-yielding metabolism. Phenolic synthesis is expensive and not merely a metabolic dead end for "waste products". The most immediate shift in utilization is the increased allocation of glucose to the pentose phosphate pathway (PPP).

Erythrose-4-phosphate derived from the PPP and phosphoenolpyruvate diverted from the energy-yielding glycolytic sequence enter the shikimic acid pathway.[25] Through a number of steps, shikimic acid is formed. Immediate precursors of shikimic acid may form gallic acid directly or be converted to the amino acid phenylalanine.[26,27] For phenolic synthesis, phenylalanine can be diverted from protein synthesis and deaminated to yield cinnamic acid. Cinnamic acid can then be converted to gallic acid or used in proanthocyandin synthesis. Proanthocyandins (e.g., catechin) are flavonoids formed from a phenylpropanoid skeleton derived from cinnamic acid. Added to this skeleton are 2- and 3-carbon units obtained from acetyl-CoA diverted from the TCA cycle and lipid biosynthesis, and from malonyl-CoA, which is itself derived from acetyl-CoA through the acetate pathway. Although difficult to quantify reliably on a whole-tree basis, phenolic production represents a true metabolic cost to the tree.

16.4 Research Techniques

16.4.1 Experimental Subjects and Treatments

16.4.1.1 Species Selection

The selection of experimental subjects and design must be tailored to suit the research question. Much of the research on the role of phenolics in compartmentalization has entailed quantitative analysis of total phenolics to answer questions about the spatial position of tree defense mechanisms and changes in wood quality.[11,25] In these studies, phenol concentration was used as a marker of the position and timing of metabolic shifts in the wood of living trees. Such research required the collection and analysis of small, spatially discrete samples, which were then extracted and analyzed by simple techniques. Conversely, some chemotaxonomic studies have included the collection of large, bulked, and frequently heterogenous samples that were then rigorously extracted and analyzed in an effort to maintain structural integrity and avoid the formation of artifacts through polymerization or degradation. Both approaches can be valid, depending on the nature of the research question.

Much of the research on phenolics and wound response has focused on maple trees, especially *Acer rubrum*, *A. saccharum*, and *A. saccharinum*. These species are desirable as experimental subjects as they do not form true heartwood during maturation.[12] Consequently, any column of discoloration is due to the response to wounding rather than the formation of heartwood. Species of *Acer* and *Populus*, among others, are also useful in that freshly exposed CBL are distinctly colored and readily distinguished from surrounding tissues:[11,25,26] Those species that form clonal sprout clumps can be used to test the influence of genetics by comparing wound responses within and between clones.[28]

16.4.1.2 Prescribed Wounding

Research on chemical changes due to the tree wound response frequently includes the use of prescribed wounding which standardizes wound position, severity, frequency, and timing. Wounding techniques include boring,[22,33,34] bark removal,[35] or a combination of the two.[13]

16.4.2 Sampling

Effective compartmentalization depends in part on the position and time of formation of column boundary layers, marginal interfaces, and barrier zones. Chemical analysis of these layers or margins requires the sampling of small, well-defined volumes of wood. Differences in phenol concentration are large over short (<5 mm) physical distances.[11,22,36]

For analysis, stem or branch sections are sawn from living or recently felled trees and cut into discs of a convenient thickness (e.g., 2 to 5 cm). Blocks for chemical or histological analysis can then be sawn or split from the discs and frozen or fixed. Depending on the subsequent chemical analysis, wood samples can be air or oven-dried or frozen and freeze-dried.

16.4.2.1 Shaving

A carpenter's block plane was used to sample thin layers of chemically distinct wood tissues.[11,22,36] A wood wafer (0.5 × 3 × 5 cm) was clamped in a benchtop vise with the radial surface exposed. With each stroke of the plane, the shaving curl was collected. Following trimming, if necessary, individual shavings were pooled to yield homogeneous collections of column margin, CBL, or barrier zone. Although shaving was slow, it allowed the collection of greater quantities of CBL than other methods. The thin shavings were easily milled prior to solvent extraction.

16.4.2.2 Slicing

Sample strips (5 × 10 × 60 to 70 mm) were sliced with a coping saw from bars of wood that contained sapwood, discolored wood, and CBL or marginal tissue.[25] Each strip was then sliced at 1-mm intervals. Slices (5 × 10 × 1 mm) were extracted and analyzed. Slicing allowed the analysis of individual contiguous pieces of wood without pooling within or between trees.

16.4.2.3 Gouging

Woodworker gouges have been used to collect small, well-defined pieces of wood.[33] They are also useful in that contiguous gouged pieces can be used for chemical analysis and the isolation of microorganisms. Unfortunately, gouged pieces frequently must be pooled to obtain a sample adequate for chemical analysis.

16.4.2.4 Drilling

Drill shavings were collected from sample blocks with a 1.6-mm drill bit.[11] Drilling was facilitated with a drill press adjusted to penetrate to a maximum or fixed distance in the wood. Depending on the phenol concentration of the wood tissues, it may be necessary to pool shavings to obtain an adequate sample for extraction.

16.4.3 Extraction

Various extraction regimes have been used to study the role of phenolics in the compartmentalization response in wood (Table 16.1). Chemotaxonomic investigations of broad-leaved foliage have revealed the need to use fresh leaves and cold, nonaqueous methanol to avoid the interconversion of phenolic glycosides.[38] This also could be expected to be true for phenolics in wood. The use of aqueous ethanol or water as solvents is acceptable for determining a qualitative shift in oxidative metabolism that may be associated with compartmentalization.

16.4.4 Chemical Analysis

16.4.4.1 Total Phenolics

Total phenolic concentrations have frequently been measured using the Folin reagent as in the Folin–Dennis[39] or Folin–Ciocalteau[40] techniques. With these methods, an oxidant, the Folin reagent, is mixed with the sample extracts followed by the addition of saturated sodium carbonate. After 1 h incubation, the absorbance of the resulting phosphomolybdic acid complex is measured with

TABLE 16.1
Representative Procedures to Extract Phenolics from Wood

Solvent and extraction conditions	Wood/solvent (w:v)	Ref.
Methanol, cold (temperature not reported), with sonication followed by partitioning in methanol/hexane/water (5:5:1)	1:5	46
Methanol, 4°C, about 5–10 h, repeated once	1:20	24
Methanol (50%), 40°C, 10 min. repeated 3–4 times	Unspecified	50
Methanol, Soxhlet extraction, 8 h	1:50	23,51
Methanol, Soxhlet extraction, 16 h	1:200	33,34
Ethanol, 76% aqueous, 70°C, 1 h	1:200	25,32,36
Boiling water with reflux, 1 h	1:50	11
	1:100	22,32,37
Ethyl acetate which was then extracted with $NaHCO_3$, acidified, and back extracted with the original solvent	1:4	52
Dichloromethane, processed as above	1:8	52

a spectrophotometer (725-nm wavelength). Concentrations are estimated using a standard curve of absorbance of known concentrations of a specific phenolic, such as gallic acid. The Folin reagent can react and colorize with nonphenolic reductants, especially in foliage extracts, and thereby overestimate phenol concentration. Analysis of aliquots of sample extracts before and following the addition of a phenol-adsorbing resin can provide an estimate of the effect of nonphenolic reductants on optical absorbance.[41]

16.4.4.2 Separation Techniques

16.4.4.2.1 Planar Chromatography. Thin-layer (TLC) and paper chromatography[42] can be used to isolate and identify plant phenolics. These two-dimensional techniques also can suggest solvent systems to be attempted in more advanced separation methods; for example, high-performance liquid chromatography (HPLC) and droplet countercurrent chromatography.[43] Several systems have been used for the TLC of sapwood phenolics (Table 16.2). Development systems frequently contain mixtures of solvents of different degrees of polarity. Phenolic hydroxyl groups are polar and hydrophilic. As the degree of hydroxylation increases, the phenolic molecule becomes more polar, interacts more strongly with the solid adsorbent phase, and is partitioned in the more polar solvent to an increasing degree. The carboxyl group of phenolic acids also adds to the polarity and water solubility of the phenolic molecule. An organic acid such as acetic or formic acid can additionally affect the relative mobilities of a mixture of phenolics by suppressing the ionization of the carboxylic and hydroxylic groups.[44]

Spots can be detected on the developed chromatogram using native UV fluorescence or by reaction with chromogenic reagents.[44,45] Alternatively, the antimicrobial properties of developed spots can be bioassayed through the inhibition of mycelial growth[24] or the inhibition of germination of spores[46] sprayed on the developed chromatogram.

16.4.4.2.2 High-Performance Liquid Chromatography. The use of HPLC allows the identification of sapwood phenolics with greatly improved resolution compared to planar techniques. In addition to chemotaxonomic interest, detailed analysis may improve the scientific understanding of the interaction between wounded, living trees and colonizing insects and microorganisms. However, high-resolution analysis requires greater care in sample extraction and processing to avoid the formation of chemical artifacts through degradation or polymerization.[38] Although not yet used widely to assess phenolics in sapwood, several HPLC systems have been useful in identifying phenolics involved with the wound response (Table 16.3).

TABLE 16.2
Representative Procedures for Thin-Layer Chromatography of Phenolics in Wood

Adsorbent	Development systems	Detection method	Ref.
Silica gel	Unidimensional, chloroform/methanol (96:4, v/v) chloroform/methanol (9:1, v/v) acetone/hexane (2:1, v/v)	UV (254, 365 nm) absorbance diazotized *p*-nitroaniline, 2% $FeCl_3$ in methanol, inhibition of growth of mycelial fragments	24
Silica gel	Unidimensional, chloroform/methanol/water (75:22:3)	Inhibition of spore germination 0.1 *M* $FeCl_3$-$K_3Fe(CN)_6$	46
Cellulose	Unidimensional, water-saturated chloroform/acetic acid (6:1:2 v/v) followed with a mixture of aqueous acetic acid (7%) and sodium acetate (0.03%)	UV absorbance (254 nm)	53
Cellulose	Bidimensional, butanol/acetic acid/water (6:1:2) followed with a mixture of aqueous acetic acid (7%) and sodium acetate (0.03%)	1% $FeCl_3$ diazotized *p*-nitroaniline UV of eluted spots (50% ethanol)	23

TABLE 16.3
Representative Procedures for the High-Performance Liquid Chromatography of Phenolics from Wood

Column packing	Eluent	Detection	Ref.
Phenomenex ODS 2	Gradient, 20–85L methanol in 2% tetradhydrofuran in water	UV absorbance at 280 nm (phenolic glycosides) and 254 nm (catechol)	46
Viosfer	Isocratic, methanol in water (15:85, v/v) previously treated with trifluoracetic acid, 1 g L^{-1}	Cochromatography with authentic standards	54
Partisil 5	Isocratic, ethyl acetate/methanol (95:5, v/v)	UV absorbance at 354 nm	24
Nucleosil C_{18}	Two-step linear gradient mixture of 1.5% aqueous H_3PO_4 and water/methanol/acetonitrile	Cochromatography with authentic standards and mass spectroscopy	53

16.4.5 Histology

Histological analysis can show the spatial positioning of phenolic layers that are part of compartmentalization in sapwood. Histology also can illustrate the temporal and spatial relationships of phenolic incorporation with related processes such as the suberization of CBL and altered anatomy in barrier zones.[24]

Small blocks (about 1 cm^3) of wood are excised and fixed for histological analysis. Numerous fixatives have been used and usually are based on acrolein, formaldehyde, or a formaldehyde/acetic acid/ethanol mixture.[20,47] Frequently, fixed blocks are sectioned without embedment. Various stains have been used to localize phenolics (Table 16.4).

16.5 Summary

Phenolic production is an investment made by trees toward their long-term survival. This investment represents a strategic tradeoff in energy allocation toward phenolic biosynthesis and away from

TABLE 16.4
Representative Stains Used for the Histochemistry of Phenolics in Wood

Stain	Preparation	Ref.
Ferric sulfate	0.5–1.0% in 0.1 *N* HCl	55
Ferrous chloride	1% in water	28
Ferric chloride	2% in 95% ethanol	56
Aniline potassium iodate	0.5 mL in 20 mL 0.5 *M* KIO_3 plus 5 mL water	
Diazotized sulfanilic acid and others	5 mL 0.025 *M* in 50 mL acetate buffer, pH 4.8	
Toluidine blue O	0.05% in benzoate buffer, pH 6.8	20,57
Substituted chlorimine (DCQ)	0.5 mL of 0.5% ethanolic solution of DCQ in 10 mL borate buffer, pH 9.4	58
Vanillin	1% in 70% sulfuric acid	
Diazotized *o*-toludine nitrous acid reagent		24

growth and reproduction. This investment is costly both in the use of structural carbon and on the energy required to make and break structural bonds. In part, this tradeoff is the basis for the relationship of slow growth rate, great longevity, and the production of defensive chemicals such as phenolics.[48,49] For tree species that produce heartwood rich in extractives, this tradeoff can be sizeable.[7] A compromise between extremes may be made by tree species that rely heavily on the inducible compartmentalization process, including phenolic biosynthesis.

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