Wood Growth/Morphology

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Chemistry and functionality of stone cells in loblolly pine outer bark periderms

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Abstract: For the southern pines, interlocking stone cells (sclereids) are a fascinating periderm feature comprising only a small fraction of the outer bark (rhytidome), yet contributing greatly to its density/hardness. This study addresses the long-overdue need to carry out wet chemical analyses on periderm and obliterated phloem layers, the former herein limited to the stone cells. Extractives distributions by polarity were similar between loblolly pine (*Pinus taeda* L.) periderm and wood samples; the total extractives content of the obliterated phloem was threefold higher. The claim that stone cells are highly lignified held true, with lignin contents that were 20 % higher than those for the wood.

Keywords: cork; lignin; phloem; periderm; rhytidome; sclereids

1 Introduction

Cell division at the vascular cambium in a growing tree continues the formation of annual rings of wood (xylem) inwards, and new phloem tissue outwards, the latter adding to inner bark (Haas et al. 2022). The wood that is formed each year remains for the life of the tree, initially transporting water and nutrients pulled from the soil, and continuing to provide structural support for the crown to meet the tree's light requirements (Agusti and Blázquez 2020); however, the functionality of the wood changes over time with its transport role ceasing as the living sapwood changes into essentially dead heartwood (Taylor et al. 2002). Similarly, inner bark serves an essential transport function, carrying photosynthates from the leaves to the support structures (e.g., branches, bole, and roots). As inner bark declines in this functionality, another meristematic tissue is formed, that being the cork cambium (phellogen).

Like the vascular cambium, the cork cambium forms layers of cells inwards (phelloderm) and outwards (phellem), the latter also known as "cork" (Campilho et al. 2020). Together these three layers constitute a periderm. For the southern pines, a group of hard pines (e.g., *Pinus taeda* L., *Pinus palustris* Mill., *Pinus elliottii* Engelm., etc.) in the southeastern United States, periderms comprise about 10 % of the outer bark, also known as rhytidome (Howard 1971). The innermost periderm serves the function of sealing off inner phloem tissue that will continue living from outer phloem which becomes nonconducting phloem (Evert 2006), and ultimately obliterated phloem, through further sieve cell collapse and parenchyma cell expansion (Howard 1971). The alternating layers of rigid periderm and spongy obliterated phloem, outside the innermost periderm, constitute the outer bark, and thus the inner bark includes the remaining phloem between the innermost periderm and the vascular cambium. While the above synopsis of bark development is generally applicable, for a few species (e.g. *Quercus suber* L.) the cork cambium can live as long as the tree, continuously producing cork (Leite and Pereira 2017).

The most thorough descriptions of southern pine bark anatomy are those in a series of reports dating back 50 years (Howard 1971; Martin 1969; Martin and Crist 1970). In these reports one will find fascinating illustrations and pictures of the cog-like interlocking of the thick-walled stone cells (sclereids) in the highest density layer (phellem) within the periderm; Evert (2006) refers to these cells as brachysclereids and illustrates the variety of shapes in which they occur. The function of the phellem cells (mainly stone cells) in the southern pines is simply suggested as being "protective" (Howard 1971). For Sitka spruce (*Picea sitchensis* (Bong.) Carr.), the protective roles of the stone cells are more explicitly (and recently) described, providing resistance against insect feeding via the wearing down of larval mouth parts, and/or interfering with larval digestive processes (King et al. 2011; Krokene 2016; Whitehill et al. 2016b, 2019).

In concert with the processes of phloem development, and its subsequent partitioning and transformation, is the

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biosynthesis and deposition of secondary metabolites commonly called extractives. The inherent toxicity and/or hydrophobicity of the extractives is commonly accepted as being essential to the resistance of the outer bark to attack by insects and fungal pathogens (Franceschi et al. 2005). Regarding outer bark cell wall chemistry, the stone cells of the southern pines are suggested to be highly lignified (Howard 1971; Martin and Crist 1970), yet no quantitative data have been reported to date to substantiate this claim. A compositional analysis of Sitka spruce stone cells conducted by Whitehill et al. (2016a) showed a very high Klason lignin content, approaching 48 % after an alkali treatment to remove cell wall bound phenolics long known to interfere with this lignin determination (Labosky 1979; McGinnis and Parikh 1975).

Chemical characterizations of southern pine bark have largely used whole bark (Huang et al. 2011; Labosky 1979; McGinnis and Parikh 1975; Pan et al. 2013); however, in some cases an effort was made to analyze separately the inner bark and outer bark components (Eberhardt 2012; Pearl and Buchanan 1976). Relying on the dense nature of stone cell clusters or layers, grinding and sieving operations have been used for the valorization of conifer barks by partitioning by differences in cellular structure/rigidity/density (Eberhardt and Reed 2006; Miranda et al. 2017). In a study by Whitehill et al. (2016a), the rigidity/density of stone cell clusters in Sitka spruce bark was used for the isolation and analysis of stone cell rich samples; alternatively, laser microdissection was used by Li et al. (2007) to isolate bark stone cell clusters from Norway spruce (*Picea abies* (L.) H.Karst.) for the characterization of phenolic glycosides. Herein, long-overdue wet chemical analysis results for pine bark stone cells are reported, and thus available to other researchers to refute or substantiate long-standing assumptions about how the chemistry and structure of bark contribute to its essential role as a chemical and physical barrier. Since the objective of the study was to carry out chemical characterizations of outer bark layers, microscopy of cross sections was not carried out to estimate, by image areas, the proportions of periderm and obliterated phloem tissue.

2 Materials and methods

2.1 Bark collection and partitioning

Freshly peeled southern pine bark was collected at a plywood plant ring-debarking station continuously processing loblolly pine (*P. taeda* L.) logs. A subsample comprised of the largest pieces (mostly 2–8 cm wide [tangential direction] and 12–15 cm long [longitudinal direction]) was rinsed with a mild detergent solution (0.05 % Triton X-100) and then water to remove dirt and bark dust (all water used was deionized, i.e., lab-grade). After drying under ambient conditions in the laboratory, small square sections (1 cm × 1 cm, tangential plane) were cut with a woodworking bandsaw. A razor blade was used to remove conducting (and nonconducting) phloem tissues by slicing between the two innermost periderms; note that periderm and obliterated phloem layers were readily visible with the naked eye. Flakes of highly weathered outer bark were removed from the opposite surface. The remaining blocks (roughly 2 mm–5 mm thick, radial direction) were sliced with a razor blade to obtain periderm and obliterated phloem samples. Periderm samples were gently scraped to ensure the exclusion of any adhering obliterated phloem tissue. Loblolly pine wood from the same source was used as a control for the chemical characterizations.

2.2 Microscopy of macerated tissues

Periderm and obliterated phloem tissues were macerated by treating a few thin tissue sections in a 30 % hydrogen peroxide:water:glacial acetic acid (1:4:5) solution (Ruzin 1999) at 56 °C for a 4-day period. After carefully removing the spent maceration solution with a Pasteur pipet, the mostly bleached periderm and obliterated phloem tissues were further macerated (3 additional days) with new maceration solution. Once the maceration treatment was complete, the samples were gently rinsed with an excess of water before subjecting to vigorous agitation in aqueous ethanol (50 % v/v). Observations were carried out by polarized light microscopy (Olympus BH-2).

2.3 Chemical characterization

All samples were ground in a Wiley mill (20-mesh); aliquots (5 g) were sequentially extracted in a Soxhlet apparatus using hexane, ethyl ether, ethanol (95 %), and then water, in this order, to provide an extraction that was exhaustive, and to quantify the extractives by weight along a gradient of increasing solvent polarity. Organic extracts were dried by rotary evaporation to afford oils that were collected in vials, dried further under a stream of nitrogen and gentle heating (50 °C), and then dried to completeness *in vacuo* before weighing. The aqueous extracts and wet extractive-free bark tissues were recovered by freeze-drying.

One-gram samples of dry extractive-free bark tissue were subjected to alkaline extraction with 1 % sodium hydroxide in water (50 ml) to remove suberin and phenolic

acids (Labosky 1979; McGinnis and Parikh 1975). After heating the suspensions in a hot water bath (~100 °C) for 1 h, the resultant slurries were vacuum filtered (Whatman GF/A), rinsed with hot water (~100 °C, 2 × 50 ml), 10 % acetic acid in water (RT, 2 × 25 ml), and finally hot water until the filtrates were free of acid. Retentates were freeze-dried before weighing to determine recoveries (periderm, 0.86 g; obliterated phloem, 0.57 g).

Samples of extractive-free and alkaline-treated tissue were subjected to Klason lignin and polysaccharide sugar analyses using an established method (Davis 1998). The resulting acid-insoluble residue, or Klason lignin, was dried (100 °C) and weighed. Sugars in the hydrolysates were determined by anion-exchange high-performance liquid chromatography on a system equipped with a Carbo-Pac PA1 column and detection using a pulsed amperometric detector (Davis 1998). Nitrogen contents (Galbraith Laboratories, Knoxville, TN) were determined to be <0.1 %, indicating that any interference from proteins for the Klason lignin content determinations, especially with the obliterated phloem tissue, would be negligible. Lignin contents were also determined by the acetyl bromide method (Morrison 1972) using wood as a control. An absorptivity value of 23.30 l g^{-1} cm^{-1}, determined for a softwood lignin (Johnson et al. 1961), was used to calculate the lignin contents. Wet chemical analysis results from a loblolly pine whole bark sample (Eberhardt 2012) are provided for comparison. All determinations are averages of duplicate analyses.

3 Results and discussion

Observations by polarized light microscopy verified that the loblolly pine outer bark (rhytidome) samples were exclusively comprised of either periderm or obliterated phloem tissue. Figure 1 shows photographs taken under polarized light, with the periderm showing only stone cells (sclereids); as to be expected, the obliterated phloem sample shows mostly parenchyma and sieve cells. Note that loblolly pine periderms are not exclusively comprised of sclereids, but technique used to prepare the periderm samples (slicing/scraping) afforded samples with few cork cells. Compared to macerated Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) bark (Cardoso et al. 2019), the loblolly pine bark samples do not show the presence of any fiber-sclereids.

Table 1 provides the extractive yields for the separated periderm (almost exclusively stone cells) and obliterated phloem tissues. Using a series of neutral solvents of increasing polarity, there is a simple degree of extractives partitioning, and thereby, an indication of the nature of the extractives present (Eberhardt 2012; Hafizoğlu et al. 1997; Krogell et al. 2012; McGinnis and Parikh 1975; Vangeel et al. 2023). Whereas the hexane-soluble extractives constituted about 25 % of the total extractives removed from the periderm, the corresponding value from the obliterated phloem was less than 6 %. It can be reasoned that the periderm, the tissue responsible for sealing off the outer bark from the phloem, should be rich in non-polar extractives to contribute to the moisture barrier properties. In contrast, for the obliterated phloem, the polar extractives (e.g. proanthocyandins) are favored as shown by more than 90 % of all extractives being removed with ethanol and then water as the solvent; residual photosynthates may also be present. These results appear to be the first to demonstrate different extractive polarities and presumed barrier properties between these two tissues in a pine outer bark.

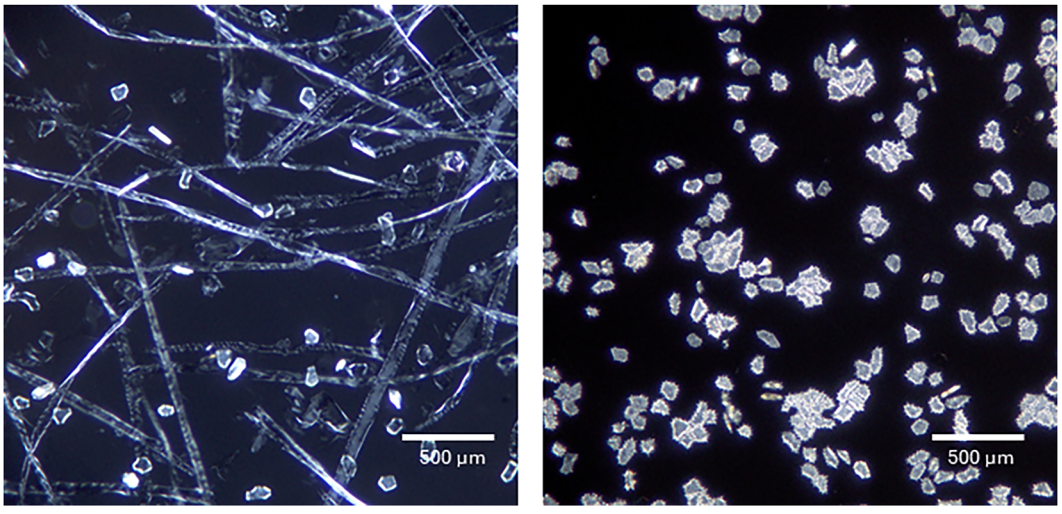

Figure 1: Photographs of macerated loblolly pine outer bark layers showing mostly parenchyma and sieve cells from obliterated phloem samples (left image) and stone cells from the periderm samples (right image).

Table 1: Extractive yields (% w/w of initial dry mass of bark or wood) from neutral solvent and alkaline extractions.

Extract	Periderm	Obliterated phloem	Wood (xylem)	Whole outer bark (rhytidome)
Hexane	1.4	0.9	1.4	1.7
Ethyl ether	0.5	0.5	0.3	0.5
Ethanol	2.4	9.4	1.2	6.5
Water	1.3	4.8	1.6	4.9
Total (neutral solvents)	5.6	15.6	4.5	13.6
Alkaline extract	15.8	37.9	nd	20.8

nd, not determined.

Prior results from the extraction of a whole outer bark sample from the same industrial source (Eberhardt 2012) are provided for comparison, and as expected, were largely intermediate those of the separated tissues. The hexane extract is somewhat the exception; this is attributed to the use of the entire thickness of outer bark, not sectioning blocks like used herein, from which the outermost (and most weathered) layers were discarded. The sample of loblolly pine wood that was processed as a control gave results that paralleled those for the periderm. This result can be attributed to the periderm and wood both being comprised mostly of cells with thick walls, albeit distinctly different cell types (stone cells vs. tracheids). The values for total extractives content of the periderm (5.6 %) and wood (4.5 %) were similar each other, and both were substantially lower than that for the obliterated phloem (15.6 %). At this juncture it is necessary to note that the proanthocyanidins in bark are not completely removed using neutral solvents (Matthews et al. 1997). These compounds, and other interfering substances (phenolic acids, suberin), are factors in the high lignin contents reported for solvent extracted barks by the Klason method. The suberin contents of *Pinus pinaster* Ait. and *Pinus pinea* L. bark have been determined to be relatively low (1.5 and 2.5 % respectively) for whole bark samples (Nunes et al. 1996, 1999). Given these findings, and the fact that the periderm samples prepared here were essentially free of cork cells, the contribution of suberin to the lignin content is likely negligible.

Alkaline (1 % NaOH) extractions have been employed before the acid hydrolysis steps to obtain more realistic values for lignin content (Labosky 1979; McGinnis and Parikh 1975). The amount of material removed by alkaline extraction is substantial, with the value for the obliterated phloem (37.9 %) being more than twice that for the periderm (15.8 %), the latter value being closer to the general value (12 %) reported for loblolly pine wood (Pettersen 1984).

Consistent with Klason lignin determinations for solvent-extracted pine bark, the lignin contents of the periderm and obliterated phloem samples were high relative to that for the corresponding wood (Table 2). The value for the periderm was about 25 % lower following the alkaline extraction process; the corresponding value for the obliterated phloem was about 50 % lower. The caveat with these values, although being more in line with those for solvent extracted wood, is that the alkaline extraction can solubilize some of the hemicelluloses.

Whitehill et al. (2016b) state that the toughness and recalcitrance of the stone cells in Sitka spruce are attributed to their thick cell walls and "high lignin content." Indeed, for the alkaline extracted samples (see Table 2), the Klason lignin content of the loblolly pine periderm stone cells (32.3 %) is higher than that for the thin-walled cells of the obliterated phloem (20.9 %), and even the wood (26.4 %). These values are confirmed by those obtained by the acetyl bromide method with the tissues typically subjected to neutral solvent extraction alone. On average, the lignin content for the periderm appears to be 20 % higher than that for the wood that was used as the control. Since alkaline extraction is not normally carried out before the acetyl bromide method, those values are provided in Table 2 only to show the consequence of combining the two laboratory methods.

The acetyl bromide method is an alternative for lignin quantification that is generally accepted as being more amenable to non-wood samples (Hatfield et al. 1999; Morrison 1972). Results for the solvent extracted samples are comparable to those for the Klason lignin values from alkaline extracted samples. It is acknowledged that correcting for the potentially large losses of material by alkaline extraction can be a source of error when back calculating the lignin contents on a dry weight basis prior to any extractions (Eberhardt 2012). Herein, the acetyl bromide lignin contents of the whole outer bark samples before and after alkaline extraction were similar, and the acetyl bromide lignin content of the periderm after alkaline extraction was still higher than the lignin content values reported for the wood. In a study by Whitehill et al. (2016a), Sitka spruce stone cells were isolated and extracted to remove soluble and cell-wall bound phenolics, albeit using an alkaline extraction with a lower base concentration, lower temperature, but longer extraction time. The lignin contents from weevil susceptible and resistant genotypes were both over 40 %. Those values seem to be unusually high given the lignin contents presented herein and in other studies of pine bark (Fradinho et al. 2002; Hafizoğlu et al. 1997; Labosky 1979; McGinnis and Parikh 1975).

Table 2: Lignin contents (% w/w of initial dry mass of bark or wood) determined for samples following neutral solvent and alkaline extractions.

Extraction method	Lignin method	Periderm	Obliterated phloem	Wood (xylem)	Whole outer bark (rhytidome)
Neutral solvents	Klason	42.8	44.4	26.4	46.2
	Acetyl bromide	31.2	19.4	26.6	21.0
Neutral solvents and 1 % NaOH	Klason	32.3	20.9	nd	30.2
	Acetyl bromide	27.7	13.3	nd	19.9

nd, not determined.

Finally, Table 3 shows the polysaccharide sugars quantified for the outer bark and wood samples. As expected, the results for the whole outer bark are intermediate between those for the periderm and obliterated phloem tissues. Comparing the periderm to the obliterated phloem, the values for the backbone sugars, glucose, xylose, and mannose, are all higher for the periderm. The two main hemicelluloses in loblolly pine wood are the galactoglucomannans and the arabinoglucuronoxylans (Jones and Painter 1957, 1959; Rowell et al. 2013). The glucose is primarily derived from cellulose. It can be surmised that the thicker cell walls are higher in lignin content but also in polysaccharides. The more interesting comparison is between the periderm and the wood, with the former being richer in the xylans and the latter being richer in the glucomannans. These results are consistent with sugar contents determined by a comparison of Sitka spruce stone cells and xylem (Whitehill et al. 2016a). Finally, sugars were analyzed from the alkaline-treated periderm, but are not reported in Table 3, because the purpose of the method is to achieve more representative Klason lignin (not sugar) values; however, it is worth noting in passing that the values for the main sugars, glucose (26.9 %) xylose (7.7 %), and mannose (4.8 %) were similar to the values reported for the periderm treated with neutral solvents alone. Thus, it appears that hemicellulose losses from the alkaline treatment of the periderm were minimal.

Table 3: Polysaccharide sugar contents (% w/w of initial dry mass of bark or wood) determined for samples following neutral solvent extraction.

Polysaccharide sugar	Periderm	Obliterated phloem	Wood (xylem)	Whole outer bark (rhytidome)
Arabinose	1.0	2.6	1.0	1.1
Galactose	1.5	3.6	1.9	2.4
Rhamnose	0.1	0.5	0.1	0.2
Glucose	25.6	18.3	41.6	19.1
Xylose	8.2	2.6	5.9	5.6
Mannose	6.6	2.2	11.4	3.6
Total	43.0	29.8	61.9	32.0

4 Conclusions

The current study addresses the long-overdue need to carry out wet chemical analyses on the periderm and obliterated phloem layers comprising a pine outer bark. Microscopy of the periderm sample prepared for chemical analysis confirmed that it was comprised almost exclusively of stone cells. The distribution of extractives appeared consistent with periderm and obliterated phloem functions. Quantitative data now demonstrate that stone cells are highly lignified, even more than the corresponding wood. Finally, since the sugar contents of the periderm before and after alkaline treatment were quite similar, the relatively high lignin content detected by the Klason method on the alkaline extracted samples is not likely an artifact from high hemicellulose removal by this treatment. Overall, outer bark chemical composition data were consistent with expectations of bark barrier properties of moisture retention to the inside, and the exclusion of feeding insects and fungal pathogens to the outside.

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