THE EFFECT OF POLYPHENOL EXTRACTION ON ENZYME DEGRADATION OF BORDERED PIT TORI[1]

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ABSTRACT

Extraction of polyphenols from the heartwood of loblolly pine, Fraser fir, and red spruce, followed by pectinase and cellulase treatments, resulted in the removal of chemical constituents and the creation of openings in the torus of bordered-pit membranes. The results suggest that during heartwood formation polyphenols rather than lignin are produced and deposited within the torus.

Additional keywords: Bordered pit membrane, torus, pectinase, cellulase, lignin, polyphenols, polyphenol extraction, chemical composition, ultrastructure.

INTRODUCTION

One of the first indications that lignin might not be present in the torus of longitudinal tracheid bordered pit-pairs was the staining experiments of Bamber (1961). Additional support for the absence of lignin was the complete removal of the amorphous substance present in sapwood tori of southern pine as a result of pectinase treatments (Nicholas and Thomas 1968). After pectinase treatment only microfibrils remained in the torus, implying that the removed substance was pectin. Failure of pectinase to remove pectin and thus create openings in tori from the heartwood zone was assumed to be due to the deposition of polyphenols during heartwood formation as their presence would effectively decrease enzyme accessibility.

Treatment of sapwood pit membranes with cellulase and hemicellulase (Nicholas and Thomas 1968) indicated the presence of both cellulose and hemicellulose.

Additional work by Bauch et al. (1968) which involved enzymes, UV microspectrophotometrical analysis, and stains led these authors to the conclusion that lignin was absent from sapwood tori and present in heartwood tori. They further indicated that since this pattern was detected in many species, it was the "normal pattern." Later, Bauch and Berndt (1973) using the same techniques noted that polyphenols are present in sapwood tori of several species. During heartwood formation, in addition to the aromatic substances already present in sapwood tori, lignin is synthesized and added to the pit membrane. Species that, according to their results, follow this pattern are *Abies alba*, *Pseudotsuga menziesii*, *Tsuga canadensis*, and *Chamaecyparis lawsonia*. Both of the above-cited studies indicated the formation of lignin in tori during heartwood formation.

Lignin is defined as a polymeric natural product arising from an enzyme-initiated dehydrogenative polymerization of three primary precursors: trans-coniferyl, *trans*-sinapyl and *trans-p*-coumaryl alcohols. In the case of conifers *trans*-coniferyl alcohol is the main precursor (Sarkanen and Ludwig 1971). Agreement that lignin as defined is not present in sapwood tori exists among the various authors cited above. With the absence of lignin in sapwood tori, it is difficult to conceive the synthesis of lignin in heartwood tori many years after the death of the cell, particularly in view of the fact that substantial evidence exists to indicate that lignification is controlled by the cytoplasm (Wardrop 1971). An equally likely

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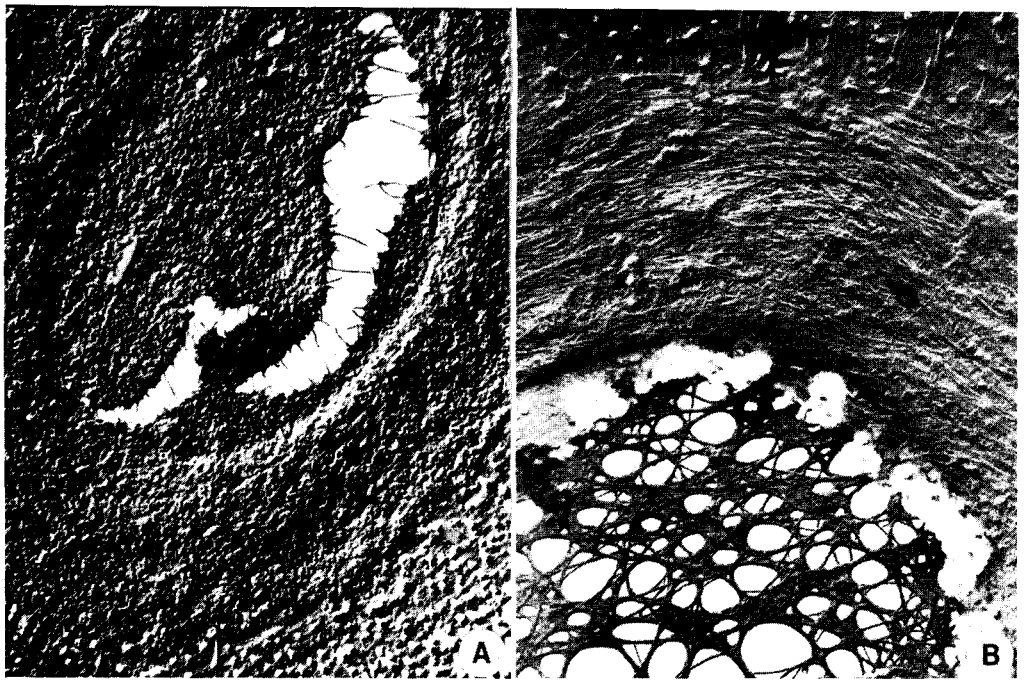

FIG. 1. Tori from sapwood of Fraser fir after treatment with pectinase. A. Nonextracted, split-type opening. (13,000×). B. Extracted, lacelike openings. (20,800×).

explanation for the results obtained by Bauch et al. (1968) and Bauch and Berndt (1973) is the presence of polyphenols in heartwood tori. Normally, the identification of lignin in plant tissue is simple, except when the tissues also contain certain insoluble polyphenols. These constituents have particular characteristics in common with lignins and therefore are often confused with the latter. During heartwood formation, polyphenols are produced by dying parenchyma cells and diffuse into adjacent cell walls.

Since both lignin and phenolic compounds absorb strongly in ultraviolet light, this technique must be used with caution. The series of spectra showing absorption from 700–240 nm for sapwood tori presented by Bauch and Berndt (1973) revealed the presence of aromatic compounds. Considerable variation in absorption can be noted among the individual pit membranes from both sapwood and heartwood. Since the UV spectrum is a composite of the absorption of the compounds present, the variability indicates many different types of compounds present in all sorts of combinations. The UV absorption curves noted after ethanol-benzene extraction could be due to high molecular weight polyphenols not removed by extraction, rather than lignin.

The enzyme peroxidase, which was shown to be present in sapwood tori (Bauch et al. 1968; Bauch and Berndt 1973) has been implicated in several reactions related to phenolic biosynthesis (Conn 1964) as well as the conversion of polyphenols to a higher molecular weight (Thimann 1964). Therefore the presence of peroxidase in sapwood tori does not imply that lignification will follow although peroxidase is believed to be essential for lignification.

The complete removal of sapwood tori by pectinase and cellulase enzymes supports the contention that lignin is not present in sapwood tori. Effective removal of elements of heartwood tori by pectinase and/or

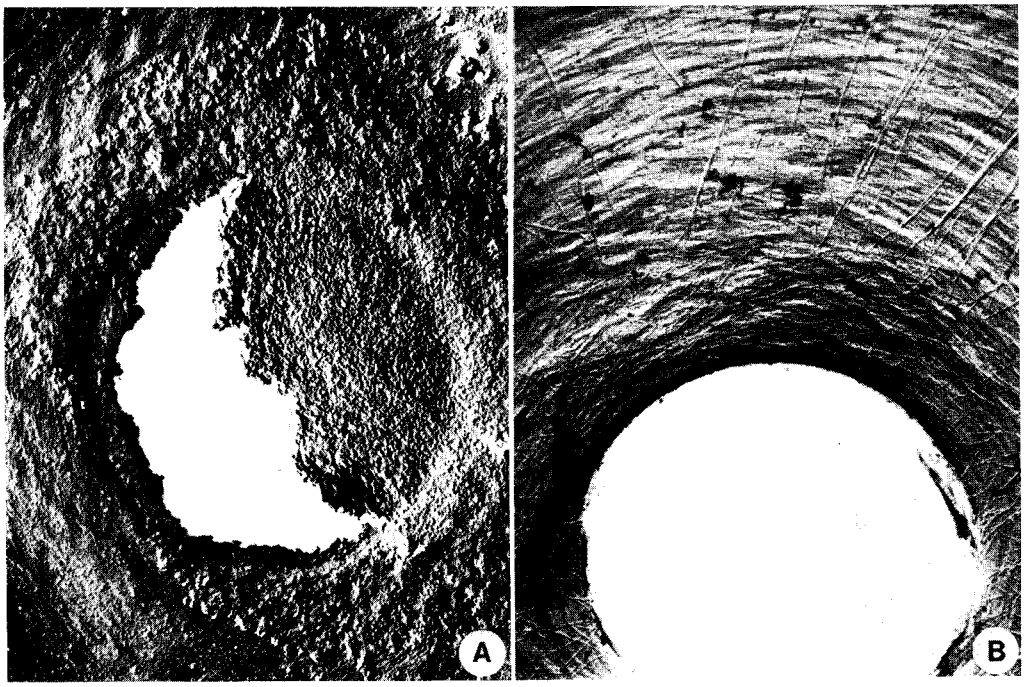

FIG. 2. Tori from sapwood of red spruce. A. Nonextracted, treated with pectinase and cellulase, split-type opening. (13,000×). B. Extracted, treated with pectinase. Note complete removal of torus over the aperture. (13,000×).

cellulase would indicate the absence of lignin. However, since polyphenols effectively block enzyme activity (Firenzuoli et al. 1969), extraction of all or a substantial portion of the polyphenols must be accomplished. The treatment must be sufficiently mild in order not to remove the known constituents of cellulose, hemicellulose, and pectin. Also, the treatment must be such that lignin, if present in the torus, would not be removed. Thus the purpose of this study was the extraction of polyphenols and subsequent treatment with enzymes to determine the presence or absence of lignin in heartwood tori.

METHODS AND MATERIALS

Species

Abies fraseri (Fraser fir) 57-years-old from western North Carolina

Picea rubens (red spruce) 50-years-old from western North Carolina

Pinus taeda (loblolly pine) 60-years-old from eastern North Carolina

From each species individual split-radial specimens (5 mm × 8 mm × 2 mm) were selected from the outermost five growth rings and the innermost five rings.

Enzymes[1]

Pectinase, Technical Grade, Nutritional Biochemicals Corporation; Treatment: 40 mg pectinase per 20 ml of citrate buffer pH 4.5 for 7 days at 45 C

Pectinol, Type 59L, Rohm and Haas Corporation; Treatment: 0.25 g pectinol per 20 ml of ammonium oxalate pH 5.5 for 16 h at 30 C

Cellulase, Ona Zuka, Yakult Biochemical Corporation Ltd., Japan; Treatment: 1 g cellulase per 20 ml of citrate buffer pH 4.5 for 3 days at 45 C

[1] The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Experiment Station of the products named, nor criticism of similar ones not mentioned.

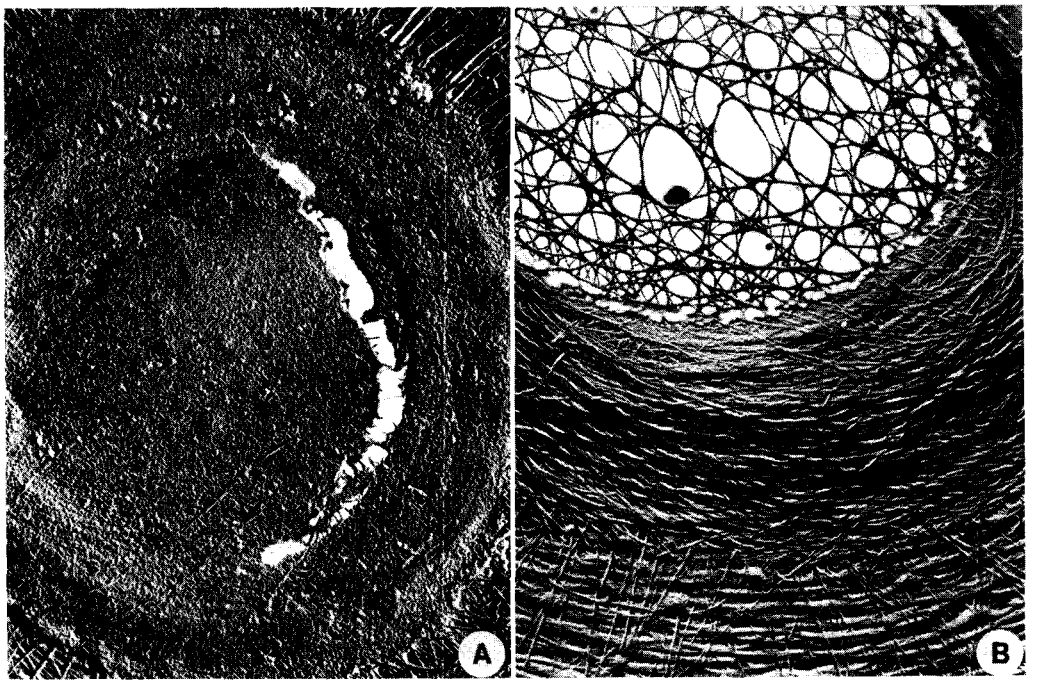

FIG. 3. Tori from sapwood of loblolly pine after treatment with pectinase. A. Nonextracted, split-type opening. (20,800×). B. Extracted, lacelike openings. (13,000×).

Extraction procedure

Sapwood and heartwood specimens were treated for 1 h in 1% NaOH in the absence of oxygen followed by 6 h in a solution of 2% H_2O_2 and 2% Na_2CO_3. Both treatments were performed at 22 C. Specimens were thoroughly washed with distilled water prior to enzyme treatments.

Microscopy

Specimens were replicated by the direct carbon method and examined with light and electron microscopes. Electron micrographs were made with a Siemens 1A electron microscope.

RESULTS AND DISCUSSION

Earlier work with various solvent-extraction systems on heartwood from southern yellow pine species followed by pectinase treatments was not successful in that the torus was not altered except for a mild clean-up. Therefore, for this study two additional species, Fraser fir (*Abies fraseri*) and red spruce (*Picea rubens*), were selected because their heartwood is not discolored and accordingly might contain little or no polyphenols. Consequently, extracted and nonextracted specimens of sapwood and heartwood from loblolly pine (*Pinus taeda*), red spruce, and Fraser fir were treated with pectinase alone or pectinase followed by cellulase.

Although both types of pectinase used, i.e. Pectinol and Pectinase, created openings in the torus, the Pectinol appeared to be slightly more effective. The increased activity was probably due to the higher concentration of the enzyme. Since both were effective, no further distinction is made between the two types.

Pectinase alone or pectinase and cellulase treatments created holes in sapwood tori of extracted and nonextracted specimens from all three species (Figs. 1–3). Both the number of tori with openings and the degree of torus degradation were increased for extracted specimens. The extraction

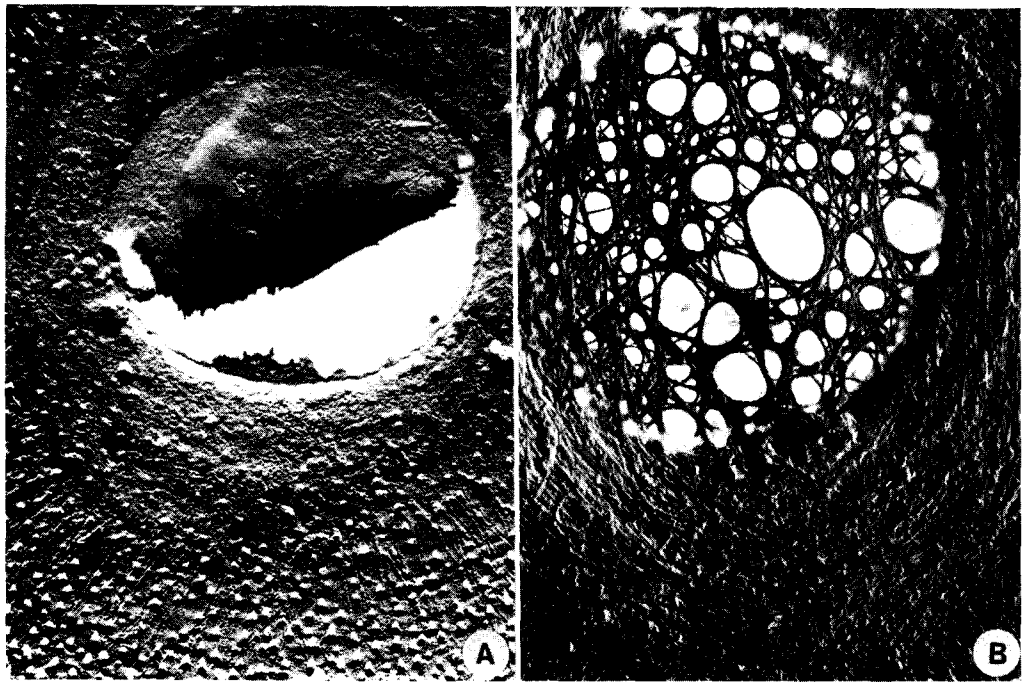

FIG. 4. Tori from heartwood of Fraser fir. A. Nonextracted, treated with pectinase and cellulase, split-type opening. (15,600×). B. Extracted, treated with pectinase, lacelike openings. (20,800×).

process, at least in the case of Fraser fir, apparently removed some low molecular weight polyphenols, which retarded but did not entirely prevent enzyme activity. The presence of polyphenols in sapwood tori of *Abies* was previously noted by Bauch and Berndt (1973). The improved enzyme activity noted as a result of extracting spruce and pine specimens was probably due simply to the removal of surface incrusting materials. Nicholas and Thomas (1968) noted improved pectinase activity after steaming the specimens. In some cases, as shown for red spruce (Fig. 2B), the portion of the torus over the aperture was completely removed in both red spruce and loblolly pine specimens.

Enzyme treatments of nonextracted heartwood produced openings in tori from Fraser fir and red spruce but not in loblolly pine tori (Figs. 4A, 5A, and 6A). The results were quite variable in that for Fraser fir, one specimen might show only 10% of the tori affected and another 80% of the tori with openings. Results for the red spruce were also quite variable; however, doubling the enzyme treatment time gave better results and reduced the variability. The variable enzyme activity noted in the nonextracted heartwood of Fraser fir and red spruce indicates variation in the distribution of polyphenols and/or in their molecular weight. For loblolly pine nonextracted heartwood specimens, the complete lack of enzyme activity apparently indicates a larger concentration and/or of high molecular weight polyphenols. The high concentration is also indicated by the discolored heartwood.

Enzyme treatments produced openings in the heartwood tori of all three species after extraction (Figs. 4B, 5B, and 6B). A higher percentage of the tori contained openings in Fraser fir than in red spruce. For the extracted red spruce heartwood, as with the nonextracted, doubled treatment times were considerably more effective. The enzyme treatments were not as effective on the loblolly pine tori as the openings were smaller and fewer tori were degraded. Presumably

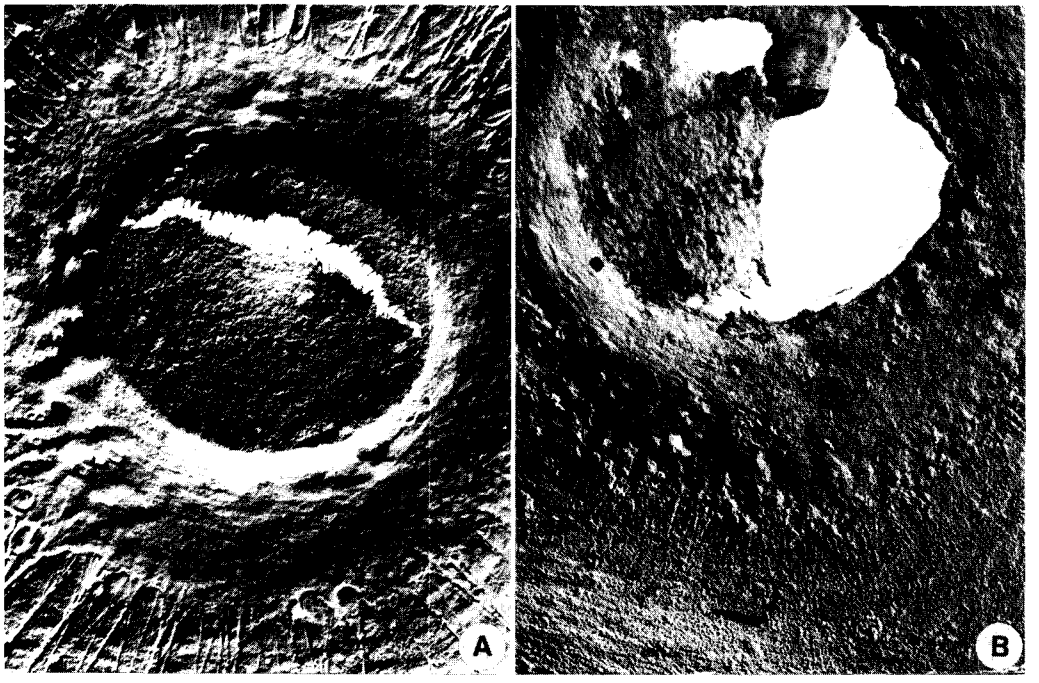

FIG. 5. Tori from heartwood of red spruce treated with pectinase. A. Nonextracted, split-type opening. (13,000×). B. Extracted, split-type opening. (15,600×).

the difficulty was due to the failure of the extraction procedure to remove sufficient polyphenols. It should also be noted that longer enzyme treatment times which improved enzyme performance on red spruce were not utilized with loblolly pine.

Although the specificity of the NaOH and H_2O_2-Na_2CO_3 treatments for extraction of polyphenols without lignin removal was not established in this work, evidence exists as to the validity of this assumption. For example, bark contains both lignin and polyphenols, which precipitate together in the Klason lignin determination procedure. Consequently the polyphenols are removed by extraction with 1% NaOH at 100 C for 1 h. The 72% acid-insoluble residue remaining is considered to represent the true lignin content of the bark (Sarkanen and Hergert 1971). Since the 1% NaOH extraction procedure utilized in this study was performed at only 22 C, a considerably milder treatment that should not remove lignin resulted. The second part of the extraction procedure utilized 2% H_2O_2, a well-known nonlignin removing bleaching agent. Again the conditions utilized in this study were milder than those used in the commercial bleaching process (Rydholm 1964). Also the H_2O_2-Na_2CO_3 extraction removes polyphenols from cottonseed hulls without removing lignin (Chang[2]).

Therefore, the extremely mild extraction conditions with the chemicals selected should result in a lignin-preserving treatment. In fact the extraction procedure was so mild that polyphenols were probably left behind. The residual polyphenols may explain the poor results obtained with enzyme treatments of loblolly pine heartwood which is high in polyphenols.

The fact that pectinase created openings in heartwood tori of three species that received a mild extraction treatment designed to remove only polyphenols offers evidence that lignin is not present in the tori of bordered pit membranes. Therefore, bordered pit membranes consist mainly of cellulose,

[2] Chang, H-M. 1976. Personal communication.

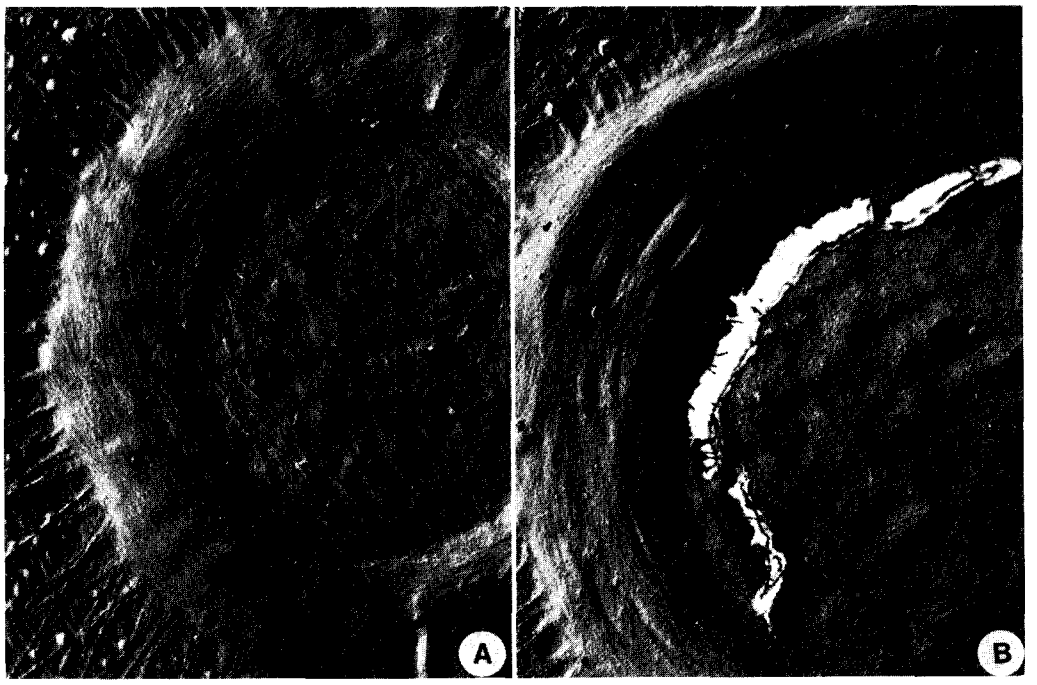

FIG. 6. Tori from heartwood of loblolly pine treated with pectinase. A. Nonextracted. Note lack of opening. (10,400×). B. Extracted, split-type opening. (15,600×).

hemicellulose, and pectin. In addition they will often also contain polyphenols. The presence or absence of polyphenols as well as their concentration is species-dependent.

With regard to the two different types of openings encountered in tori as a result of enzyme treatments, no satisfactory explanation was originated. Figure 1A illustrates the split-type opening and Fig. 1B the lacelike openings encountered. Although some holes in the lacelike pattern might have been produced as a result of sintering (Frey-Wyssling et al. 1956), they were detected only in some enzyme-treated tori. Sintering artifacts add indirect evidence of tori degradation by enzymes as they are most commonly noted with cellulose microfibrils exposed as a result of removal of incrusting materials such as pectin, polyphenols, or lignin.

With the exception of extracted heartwood from loblolly pine, the tori that revealed split-type openings also show an erosion of the tori (compare Fig. 6B to Figs. 1A, 2A, 3A, 4A, 5A, and 5B). The absence of this corroboration of enzyme activity on loblolly pine extracted heartwood specimens raises the question as to whether the openings are the result of enzyme activity or a drying artifact. The fact that these openings were not detected in extracted or nonextracted heartwood controls indicates that either the torus was sufficiently weakened by the enzyme treatment so that drying stresses created the openings or they are the result of enzyme activity only.

Lacelike openings were found in the extracted sapwood of all three species after treatment with pectinase. Apparently the extraction procedure removes some incrusting materials that permit a different type of torus dissolution by the enzyme. The only exception noted to this was the presence of lacelike openings in nonextracted loblolly pine from the outermost ring. Evidently in the recently formed wood of loblolly pine, these incrusting materials have not accumulated.

Although only the Fraser fir heartwood specimens revealed lacelike openings in

both extracted and nonextracted specimens, very few were detected in the nonextracted specimens. The lack of lacelike openings in tori from the heartwood of red spruce and loblolly pine may be the result of an incomplete removal of the polyphenols. Also noted was the fact that tori, regardless of extraction, treated with both pectinase and cellulase revealed only the split-type opening.

Further obscuring the issue was the presence of lacelike openings in the torus of a pit membrane from nonextracted sapwood of loblolly pine and a split-type opening in the torus of an adjacent pit membrane. This observation was made on specimens from the innermost sapwood region in a separate study that was investigating the influence of age of wood on enzyme activity.

Although a satisfactory explanation for the different types of openings encountered was not developed, the fact remains that they were the result of enzyme activity as neither type of opening was detected in untreated specimens.

CONCLUSIONS

The absence of lignin in both sapwood and heartwood tori is suggested by the following:

1. Evidence presented in the literature, with regard to the presence of lignin in heartwood tori, based on UV microspectrophotometry and staining, is equally likely to be indicating the presence of polyphenols.
2. The absence of lignin in sapwood tori is a strong argument for the lack of lignin in heartwood tori as the formation of lignin is controlled by the cytoplasm. Thus, the formation and deposition of lignin years after cell death are highly unlikely.
3. The successful creation of openings in some heartwood tori by pectinase in nonextracted specimens from Fraser fir and red spruce (species with a low polyphenol content) indicates a lack of lignin. The variable results obtained also imply a presence of polyphenols rather than lignin as the deposition of polyphenols is an intercellular diffusion process. Lignin, on the other hand, is a polymerization process controlled by the cytoplasm resulting in an intracellular deposition. The higher variability would be expected from the former process.
4. Polyphenol extraction of heartwood tori followed by pectinase treatment resulted in the removal of tori constituents and the creation of openings. Lignin, if present, would have effectively blocked enzyme activity in the torus. Thus, the success of the treatment suggests the absence of lignin.

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