THE PRESENCE OF CALLOSE IN CORK CELLS[1]

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ABSTRACT

Observations of fluorescence with ultraviolet microscopy and staining with transmission electron microscopy indicate that a substance reacting like callose is present as a plugging material in pitlike areas in the walls of mature cork (phellem) cells.

Keywords: *Pseudotsuga menziesii*, *Abies* spp., *Tsuga heterophylla*, *Acer macrophyllum*, *Quercus suber*, cork, callose, phellem, ultraviolet microscopy, transmission electron microscopy.

INTRODUCTION

Callose is a carbohydrate, which is composed of glucose units linked β-1,3 (Eschrich 1956; Fu et al. 1972), and is present in the walls of sieve and epidermal cells of higher plants (Fahn 1974). Although its function in these cells is unknown, it is thought to act primarily as a plugging material (Currier 1957). Currier and Strugger (1956) suggest that callose might play an even greater role than this in cells and could be distributed more widely in plants than is suspected now.

In phloem tissue of bark, callose can be seen as a plugging material in the sieve areas of the sieve cells. Sitte (1955) studied the cork cell wall of cork oak (*Quercus suber* L.) and observed in the pits a plugging material that he was unable to identify, but referred to as being "alien to suberin." We have observed similar plugging of pitlike areas in the walls of mature phellem (cork) cells in Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] bark (Fig. 1), although to our knowledge the presence of callose in cork cells has not been reported previously.

The presence of callose *in situ* usually is determined by the fluorescence observed because of ultraviolet (UV) light excitation when the callose is stained with resorcin blue or aniline blue (Currier 1957; Eschrich and Currier 1964). Because phenolics in lignin and extractives fluoresce, they can mask any fluorescence produced by callose if they are present in the same cells. Therefore, use of the fluorescence phenomenon for determining the presence of callose is applicable only where these phenolics are not present or can be removed from the cell without removing the callose. Also, the ability to see callose with the transmission electron microscope (TEM) has been hindered by the fact that electron microscopy stains do not differentiate cellulose from callose (Eschrich 1956). Because of these limitations, callose may have been overlooked in many cells. On the basis of results of procedures described below to overcome these limitations for observing callose in these cells, we believe there is substantial evidence for the presence of callose in the phellem cells of Douglas-fir, as well as in true firs (*Abies* spp.), western hemlock [*Tsuga heterophylla* (Raf.) Sarg.], bigleaf maple (*Acer macrophyllum* Pursh), and cork oak.

MATERIALS AND METHODS

Tissue collection

Douglas-fir bark was collected from freshly felled logs and immediately crosscut into ¼-inch slabs from which cork layers were excised with a razor blade.

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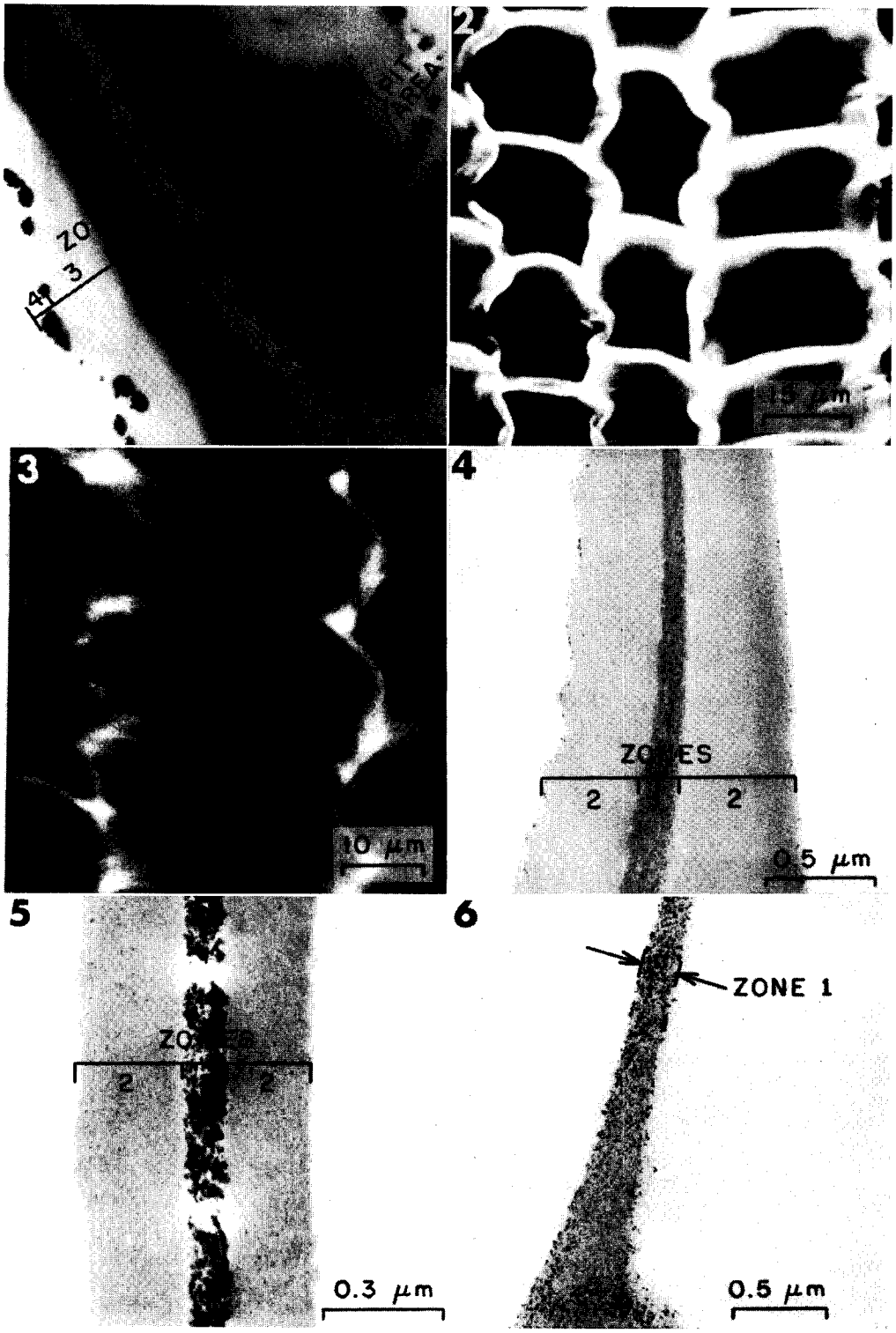
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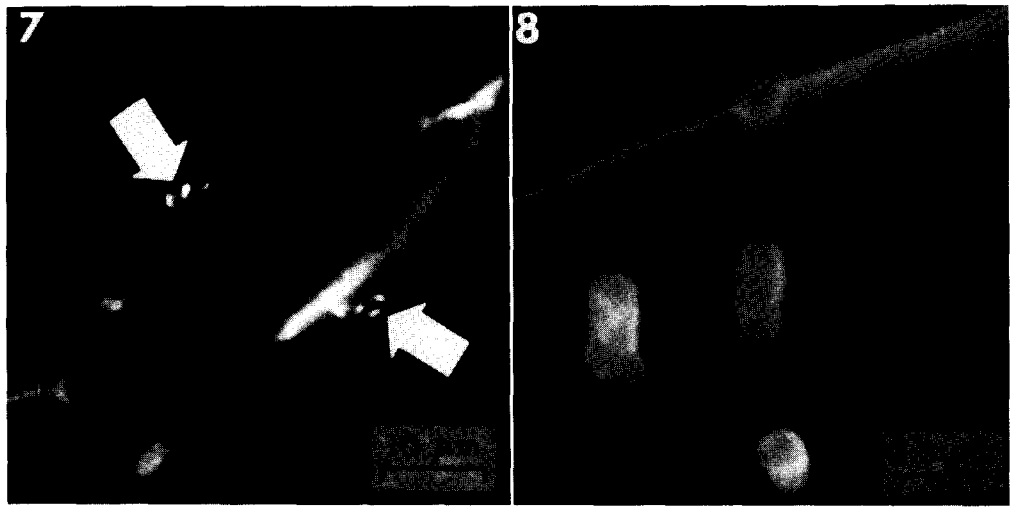

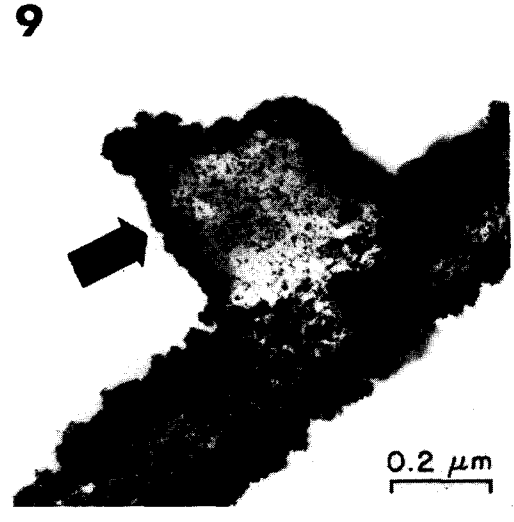

FIG. 7. Fluorescence in cork cells after saponification for 30 min with 3% aqueous NaOH and then stained with aniline blue. The fluorescing pegs (arrows) result from staining with aniline blue and indicate the presence of callose, but the fluorescence in the cell wall is the same as that seen in Fig. 3.

FIG. 8. Scanning electron micrograph of 4 pegs protruding upward from the compound middle lamella of cork, saponified for 30 min with warm 3% aqueous NaOH.

FIG. 9. Longitudinal section through a callose peg (arrow), attached to the compound middle lamella. The black material lining the peg and wall is the metal coating used on these cork cells for examination with SEM before sectioning for TEM (section stained with 1% osmium).

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FIG. 1. Section through adjacent cork cell walls from Douglas-fir bark, which shows layering in the walls. A plugged pitlike area extends through zone 1 (compound middle lamella) and zone 2 of the cell on the right. The section was pretreated with periodate (P) and thiosemicarbazide (TSC), and stained with 1% osmium.

FIG. 2. Fluorescence in transverse section of unextracted and unstained cork cells. The fluorescence is caused by phenolics present in the cell walls.

FIG. 3. Reduction of fluorescences in cork cells after saponification with warm 3% aqueous NaOH for 30 min (unstained transverse section).

FIG. 4. Section of adjacent cork cell walls that have been extracted with benzene-ethanol (section pretreated with P and TSC; stained with 1% osmium).

FIG. 5. Cork cell walls extracted with benzene-ethanol and ammonium oxalate. The two large white areas in the compound middle lamella are unstained substances plugging these areas (section pretreated with P and TSC; stained with 1% osmium).

FIG. 6. Cork cell walls saponified with warm 3% aqueous NaOH for 30 min. Only the compound middle lamella remains (pretreated with P and TSC; stained with 1% osmium).

Chemical treatments and staining

Solvent extractions and selected staining of cork were done on 24-μm-thick sections. The treated sections then were prepared for optical and electron microscope observations. Although Douglas-fir bark was the principal species studied, barks from true firs, western hemlock, bigleaf maple, and cork oak were treated and stained similarly to that developed for Douglas-fir.

Solvent extraction. The extraction was as follows:

1. Benzene-ethanol extraction (1:1 v/v, 24 h at reflux);
2. Absolute ethanol extraction (24 h at reflux);
3. Hot water extraction (24 h at reflux);
4. Ammonium oxalate extraction (0.5% solution, 24 h at reflux) and;
5. Saponification with warm 3% aqueous NaOH (30 min).

Staining and microscopy. Unstained sections were mounted in glycerine for observations of fluorescence with ultraviolet light. To cause callose to fluoresce, sections were stained with a 1% aqueous solution of aniline blue (pH not adjusted) for 30 min (Currier and Strugger 1956). A Zeiss Universal microscope with Zeiss UV attachments was used (Exciter filter BG ¾; barrier filter 50).

Cork prepared for scanning electron microscopy (SEM) was removed from the extraction sequence, solvent exchanged into trifluoromethane, and critical point dried. Surfaces were coated with gold/paladium (60:40) and observed with an I.S.I. mini-scanning electron microscope.

To differentiate cellulose from callose in transmission electron microscopy, the cellulose was stained with 1% aqueous osmium, using a pretreatment procedure described by Seligman et al. (1965). The pretreatment was: soaking 20-μm-thick sections in a freshly made solution of 1% sodium periodate at room temperature for 3 h, rinsing the sections in distilled water, and then soaking them in a fresh aqueous solution of acetic acid (25%), which contained 1% thiosemicarbazide for 1½ h at room temperature. After this treatment, the sections were washed with distilled water and stained with 1% aqueous solution of osmium tetroxide at room temperature for 3 h. Stained samples were embedded in Spurr's media (Spurr 1969), sectioned with a diamond knife, and examined in a Phillips EM-300 transmission electron microscope.

RESULTS AND DISCUSSION

UV microscopy and fluorescence

Untreated and unstained sections of cork exhibited green-yellow fluorescence in the cell wall when observed with the UV microscope (Fig. 2). This fluorescence still was present in the cork cells after cork sections had been extracted chemically through the ammonium oxalate step of the extraction sequence. After saponification, however, the amount of material in the cork sections that fluoresced was reduced (Fig. 3), and the color emitted was pale yellow. Chemical analysis of the solubles from the saponification indicated that they were phenolic, and examination of these phenolics under UV light showed that they exhibited a greenish-yellow fluorescence.

Use of TEM showed that zones 3 and 4 (Fig. 1) had been removed from phellem cells that were extracted with benzene-ethanol (Fig. 4). No further wall layers were removed with hot water and ammonium oxalate extractions (Fig. 5). After saponification, however, only the compound middle lamella remained (Fig. 6). The reduction of fluorescence after saponification (Fig. 3) could be attributed to the removal of zone 2 in the cork cell wall, which is a suberin layer with considerable phenolics present (Litvay and Krahmer 1977).

After the suberin layer was removed, callose could be detected when the callose was stained to fluoresce. Normally, alkaline conditions are used for the staining, which makes callose fluoresce a bright yellow. When we did not adjust the pH of the aqueous solution of aniline blue stain, however, the fluorescence given off by the callose

was yellow-green, which made it more discernible from the compound middle lamella, which fluoresces yellow. In certain locations, the appearance of yellow-green fluorescing "pegs" could be seen easily, which indicated the presence of a calloselike material. Figure 7 is of cork, saponified for 30 min and stained with aniline blue. The diameters of the fluorescing pegs range from 0.2 to 0.3 μm. The distribution of fluorescing pegs is variable, but they were seen in all sections and growth increments of mature Douglas-fir cork studied.

To observe these pegs with electron microscopy, the same cork sections that were stained and used for UV observations were critical point dried, metal coated, and observed in an SEM. Figure 8 is a scanning electron micrograph from the same cork section used in Fig. 7 and shows four of these pegs. After the cork sections had been observed with SEM, they were removed from the specimen stubs and stained with a 1% aqueous solution of osmium tetroxide for 3 h at room temperature. They then were embedded in Spurr's media (Spurr 1969), sectioned, and observed in a TEM. Figure 9 is a longitudinal section of a peg attached to the compound middle lamella (zone 1) of the cell.

Transmission electron microscopy and staining

Pretreating cork sections with sodium periodate and thiosemicarbazide before staining them with osmium tetroxide differentiated callose from cellulose in ultrathin sections observed with TEM. In the pretreatment reaction, periodate cleaves adjacent hydroxyls and simultaneously oxidizes the cleaved hydroxyls to aldehydes. The thiosemicarbazide then condenses with the aldehydes, which leaves an aldehyde-thiosemicarbazone that will readily stain with osmium tetroxide (Seligman et al. 1965). For this reaction to occur, the carbohydrate being oxidized must have adjacent hydroxyls. Cellulose is linked β-1,4 and has adjacent hydroxyls at Carbons 2 and 3, but callose is linked β-1,3 and does not possess adjacent hydroxyls. Thus, cellulose will react and stain with osmium, but callose will not. In areas of a cell wall that contain callose surrounded by cellulose, the callose appears white (unstained), surrounded by dark stained cellulose.

Untreated and pretreated cork, stained with osmium tetroxide, were examined with TEM. Figures 1, 4, 5, and 6, which have already been used to show the removal of wall layers with various solvents, illustrate results of the pretreatment and staining procedure for observation of callose.

The effect of the pretreatment is seen in the compound middle lamella (zone 1). The zone stains darker because of the pretreatment, which indicates that oxidizable carbohydrates are present in this zone. Without the pretreatment, the compound middle lamella is about as light as zone 3 labeled on Fig. 1. In neither Fig. 1 nor Fig. 4 are there white regions, surrounded by dark stained material, to indicate the presence of callose. After sections extracted with benzene-ethanol, however, have been extracted further with hot water or ammonium oxalate, white areas appear in the compound middle lamella (Fig. 5). The diameters of these areas are comparable to the diameters of the fluorescing pegs shown in the UV study. Although these white areas appear in the micrograph as empty holes in the compound middle lamella, no such holes or gaps are seen in sections extracted with the same solvents, but not pretreated before staining. SEM examinations did not show holes in any of this extracted or saponified material.

Because white areas do not appear in the compound middle lamella until after extraction with hot water or ammonium oxalate, other material, such as pectins or hemicelluloses, which are soluble in these solvents, appear to be associated with the callose plugs and must be removed to prevent them from masking the callose. This is partially substantiated by Fu et al. (1972). To isolate callose, they had to treat the carbohydrate with α-amylase and extract it with hot water to remove associated starch and hemicelluloses.

The UV study showed that the callose

pegs extended into the suberin layer (zone 2), although Fig. 5 does not show white areas present in that layer. Again, the callose in the suberin layer could be associated with water insoluble materials similar in composition to those found in the suberin layer, such as phenolics, which stain with osmium and would mask the presence of callose. In the compound middle lamella, however, the materials reacted to this pretreatment and staining in a manner that would suggest the presence of callose in the pitlike areas.

CONCLUSIONS

1) A material that gives a callose reaction to aniline blue under UV light is present in mature saponified cork (phellem) cells of Douglas-fir bark. Similarly, a material is present in the compound middle lamella of cork cells extracted with organic solvents and hot water or ammonium oxalate, which is not stained with osmium tetroxide after treatment with periodate and thiosemicarbazide. Both of these results provide substantial evidence for the presence of a calloselike substance. Observations on mature cork from the true firs, western hemlock, bigleaf maple, and cork oak yielded similar results.

2) Callose in plant cells functions as a plugging material, and in cork cells it is plugging pitlike areas in the cell wall.

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