TISSUE CULTURE OF SECONDARY XYLEM PARENCHYMA OF FOUR SPECIES OF SOUTHERN PINES

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ABSTRACT

Callus was grown from increment core explants of longleaf pine (*Pinus palustris* Mill.), slash pine (*P. elliottii* Engelm.), loblolly pine (*P. taeda* L.), and shortleaf pine (*P. echinata* Mill.) trees that had 30–45 annual rings of sapwood. Callus emanated from vertical and horizontal resin canals and uniserate rays. It originated from epithelial cells and longitudinal and ray parenchyma cells. There was relatively little difference in the amount of callus produced from the outer to inner sapwood of longleaf and slash pines compared to the reduction that occurred in loblolly and shortleaf pines. Production was generally lower in the transition zone, especially in loblolly and shortleaf pines, and virtually nonexistent in the heartwood. Several current theories of heartwood formation are discussed in light of the results.

Keywords: Tissue culture, resin canals, transition zone, sapwood, rays, xylem parenchyma, heartwood formation, *Pinus palustris, P. elliottii, P. taeda, P. echinata.*

INTRODUCTION

Hillis (1987) inferred that because of the experimental difficulties involved and the complexities of the physiological processes occurring at the sapwood/heartwood interface, numerous theories of heartwood formation have been advanced. He reviewed many of these theories and listed a number of questions that must be answered before the processes can be understood and the formation of various types of heartwood explained. Our work in culturing secondary xylem parenchyma from mature southern pine trees, although directed primarily towards attempting to generate plantlets, provides insight into heartwood formation and offers an experimental approach that others may find advantageous in their research.

MATERIALS AND METHODS

Explant materials were collected from four species of southern pines: longleaf (*Pinus palustris* Mill.), slash (*P. elliottii* Engelm.), loblolly (*P. taeda* L.) and shortleaf (*P. echinata* Mill.). All were from 40–60 years in age and had 30–45 rings in sapwood thickness. Longleaf, slash, and loblolly pines were plantation-grown; shortleaf pines were from natural stands. All were growing on the Clemson Experimental Forest in the upper Piedmont of South Carolina.

Explants were increment cores (5 mm in diameter) taken from the trees at about 30 cm above the ground line. Sterile cores were obtained by removing bark with a chisel to ex-

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pose about 5 cm^2 of xylem and rinsing the area well with 95% ethanol. The sample core was extracted with a sterile increment borer, the components of which had been individually wrapped in aluminum foil and autoclaved prior to use. After the bit containing the core had been backed out of the tree, it was covered with sterile foil and the core was transported to the laboratory while still in the bit.

Cores were taken at all seasons of the year. Some trees were sampled in several different seasons. Subsequent sampling in a given tree was in a different sextant and from 3–5 cm above or below the previous sampling. The lower 2 m of the boles of trees sampled in the warmer seasons were sprayed with a benzene hexachloride solution as a necessary protection against bark beetle attack. No other protection was afforded the trees.

In the laboratory the core was removed from the bit while working in a laminar flow hood. Usually the core was sliced into ca. 1-mm disks with a sterile razor blade, although for some purposes whole cores were used. The freehand slicing was governed by disk thickness, disregarding early- and latewood positioning. In a typical experiment 16 disks were taken from the outer sapwood, mid-sapwood, inner sapwood, and heartwood of each core. Average time from field collection through laboratory processing to culture initiation was about three hours.

In the usual culture procedure, disks were placed in 50-ml Erlenmeyer flasks containing 15 ml of liquid DCR basal medium (Gupta and Durzan 1985), which included 10.7 μM naphthaleneacetic acid (NAA) and 4.4 μM benzylaminopurine (BAP). Media were adjusted to pH 6.0 prior to autoclaving. Disks were cultured in the dark at 25 C in a controlled environment shaker at 100 rpm for 4 or 6 weeks with the medium being replaced every 2 weeks.

After 6 weeks in liquid, disks were transferred to a solid (0.6% agar) DCR (+NAA & BAP) medium. Plated disks were kept in the dark at 25 C except for the chloroplast studies. In these, after 6 weeks in the dark, the plated disks were placed under light (white fluorescent and Gro&Sho plant lights combined) for 6 weeks. Transfers or subculturing to fresh media took place at 3- to 4-week intervals. Whole cores were cultured in half-gallon Mason jars with 175 ml of liquid DCR medium in much the same manner as the disks, except that 9.1 μM 2,4-dichlorophenoxyacetic acid was used instead of NAA and BAP. More detailed procedures are given in Hiatt and Allen (1991).

One of the experiments reported later involved sampling two trees each of four southern pine species. Sixteen disks were cut from the outer one-third of the sapwood, inner third, transition+inner sapwood zone, and heartwood of each core. Cutting of transition+inner sapwood disks started at the transition zone–heartwood interface and continued outward until the required 16 disks were obtained. Slicing of the heartwood disks started at the interface and continued inward. Cutting of inner sapwood disks began where cutting of transition+inner sapwood disks stopped. (Transition zone as used here refers to the paler-colored rings surrounding the heartwood.)

RESULTS

Callus could be produced on both faces and the side of a secondary xylem explant (disk) of southern pine (Fig. 1). That on the disk face (tangential surface) could originate from parenchyma cells of horizontal resin canals (Fig. 2), uniserate rays (Fig. 3a, b, c, d), or from a vertical resin canal that happened to be sliced open in cutting the disk (Fig. 4). Callus on the side of the disk (radial surface) came primarily from vertical resin canals (Figs. 4 and 5). However, callus was occasionally observed emanating from horizontal canals or uniserate rays that happened to be cut by the increment borer in taking the sample core. No data were taken on the frequency of such occurrences. Figure 2 shows epithelial cells of a horizontal resin canal that have divided and enlarged to fill the void. Calluses formed by epithelial and by longitudinal parenchyma cells of a vertical resin canal and by ray cells are shown in Fig. 4.

Disks from four species of mature southern

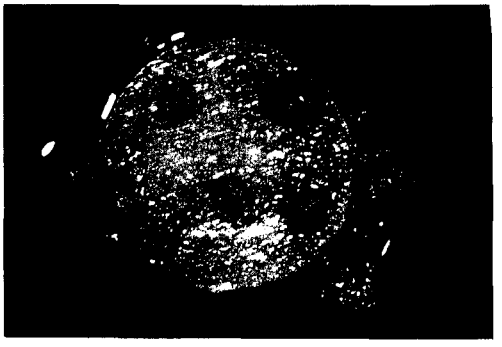

FIG. 1. Callus growth on a disk (from an increment core 5 mm in diameter) taken from near the middle of the sapwood of a 56-year-old longleaf pine tree. The callus growths on the face of the disk originated from horizontal resin canals and those on the side of the disk from vertical canals. The disk had been in liquid culture for 6 weeks and on solid medium for 1 week prior to photographing.

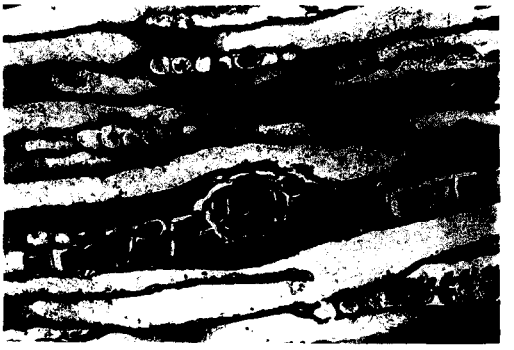

FIG. 2. Horizontal resin canal of longleaf pine from which callus was growing. Epithelial cells have multiplied and enlarged to fill the resin canal, and surrounding parenchyma cells are in an active growing state. (Section taken from just below the upper surface of disk.) (6×.)

pines produced callus in culture (Table 1). More longleaf or slash pine disks produced callus than did those from loblolly or shortleaf pines. Loblolly or shortleaf pine callus production was largely from disks taken from the outer sapwood. Longleaf pine had no significant difference in the number of callus producing disks between the outer and inner sapwood, but the transition+inner sapwood zone had fewer callused disks. Slash pine had no significant difference between the outer sapwood and transition+inner sapwood zone, but the inner sapwood produced fewer callused disks than did the outer sapwood. All of the inner sapwood disks from one slash pine formed callus, but few did from the other tree for some unknown reason. None of the heartwood disks produced callus in this experiment.

Because some inner sapwood disks were included with those of the transition zone in the above experiment, a supplemental test was conducted with six disks from within the transition zone from each of two other longleaf pine trees and eight disks from another slash pine. Thirty-three percent of transition zone disks from one longleaf pine and 50% of the other produced callus, as did 88% of those from the slash pine.

With longleaf or slash pines, there was no difference between vertical resin canals and horizontal resin canals and rays as the source of callus (Table 2). With loblolly or shortleaf pines, virtually all of the callus emanated from vertical resin canals in this experiment.

Vertical resin canals, especially those that had been sliced open during disk cutting, were the major source of callus formed by disks from four longleaf pine trees after four weeks in liquid culture (Table 3). For three trees the number of disks with callus originating from horizontal resin canals and rays increased as time on the solid medium progressed. During this time the percentages of disks with callus from sliced vertical canals decreased simply because a minority of the disks happened to have a sliced vertical canal and most of these

TABLE 1. *Percentages of disks with callus by species and positions in tree bole.*[1]

Positions	Species of pines			
	Longleaf	Slash	Loblolly	Shortleaf
Outer sapwood	100a[2]	100a	70a	21a
Inner sapwood	89a	54b	8bc	2b
Transition + inner	63b	72ab	18b	5b
Heartwood	0c	0c	0c	0b

[1] Data were taken after disks were cultured on solid medium for 26 days following 6 weeks in liquid medium. Data were transformed by $\sqrt{x + 0.5}$ before analyzing.

[2] Percentages in a column followed by same letter are not significantly different at the 95% probability level by the Fisher PLSD test.

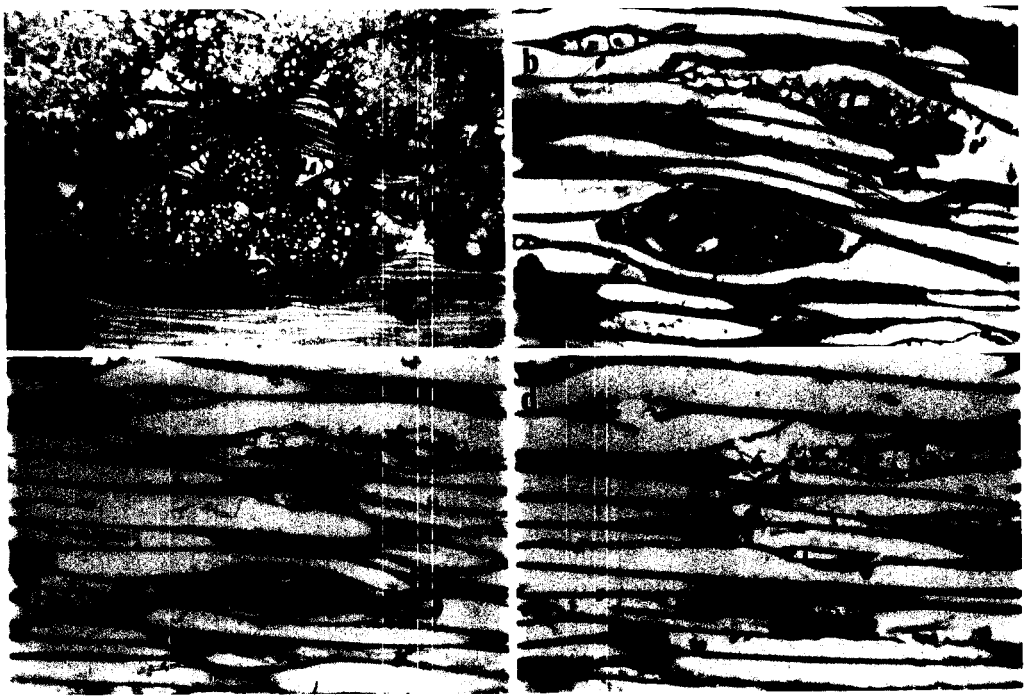

FIG. 3. a. Callus growing from uniserate ray origins on the upper face of a disk of a loblolly pine tree. (No other tree examined had so many active uniserate rays.) (2.4×.) b. One of the uniserate rays with parenchyma cells dividing and enlarging. Section taken very close to the upper surface of disk. (6×.) c. Same ray deeper in disk. (6×.) d. Same ray in the uniserate condition still deeper in the disk. (6×.)

FIG. 4. Longitudinal section through a vertical resin canal of longleaf pine showing callus arising from longitudinal (left pointer) and epithelial (middle pointer) parenchyma cells and from uniserate ray cells (right pointer).

canals formed callus during the 4 weeks in liquid culture.

Individual tree variation was considerable. Thirty-eight percent of Tree G's disks produced callus compared to 97% of those of Tree F. Tree G had no callus originating from horizontal canals and rays, but 93% of Tree E's disks had callus arising from horizontal elements.

Twenty-eight percent of calluses from cultures in two longleaf pine experiments turned green within 3 weeks after being placed in the light. As many of these cultures came from the inner third of the sapwood as from the outer third. Presence of chloroplasts in such green callus has been confirmed (Hiatt and Allen 1991). Some cultures of slash and loblolly pines turned green as well, although no records were kept as to the frequencies. No attempts were made to develop chloroplasts in shortleaf pine callus.

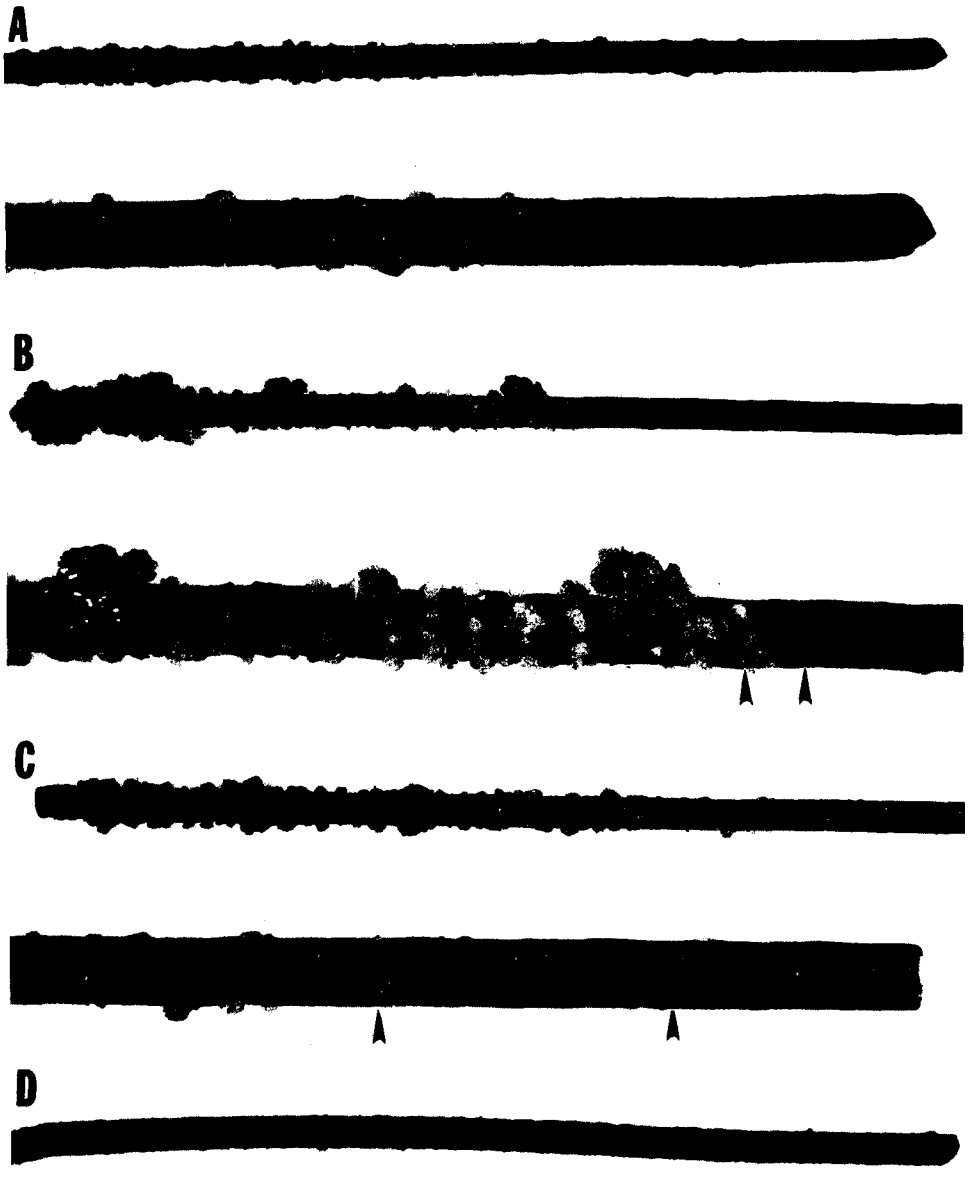

FIG. 5. Callus growth emanating from vertical resin canals in increment cores (5 mm in diameter) taken from four, 56-year-old longleaf pine trees. Cores were cultured in liquid medium for 6 weeks and on solid medium for 1 week prior to photographing. Tree A had 37 rings in sapwood, no distinct transition zone, and callus growth at the heartwood boundary. Tree B had 32 rings in sapwood and one ring in the transition zone (delineated by pointers) where the callus growth is slightly less than that in the adjacent sapwood. Note the one spot of callus in what, by virtue of its coloration, would normally be considered heartwood. Tree C had 32 rings in sapwood and four rings in the transition zone (delineated by pointers) where the callus growth is much reduced. Again there is one spot of callus growth in what would normally be considered heartwood. Tree D had 34 rings of sapwood and no distinct transition zone. Callus growth slow and sparse over the entire sapwood.

TABLE 2. *Origins of callus by species.*[1]

Origins	Species of pines			
	Longleaf	Slash	Loblolly	Shortleaf
Vertical resin canals	22a[2]	41a	92a	100a
Horizontal canals & rays	26a	32a	0b	0b
Both[3]	52a	27a	8b	0b
Total	100	100	100	100

[1] Data were taken 12 days after disks were transferred to solid medium following 6 weeks in liquid medium. Counts were converted to percentages and transformed to angles (angle = arc sin $\sqrt{\text{percent}}$) before analyzing and converted back to percentages for table.
[2] Percentages in a column followed by same letter are not significantly different at the 95% probability level by the Fisher PLSD test.
[3] Callus was emanating from both vertical and horizontal elements of a disk.

OBSERVATIONS AND DISCUSSION

In examining radial fissures in several conifers, Amos (1953) found callus in fissures of *Pinus radiata,* while those of *Pseudotsuga menziesii* and *Picea glauca* had none. He explained that ray parenchyma cells of *P. radiata* have primary walls only and were able to proliferate and form callus, but those of *P. menziesii* and *P. glauca* have secondary thickening that prevented proliferation. White (1967), however, was able to obtain callus cultures from xylem explants of *P. glauca.*

Early investigators working with conifers (Ball 1950; Reinert and White 1956; Bogdanovic and Jelenic 1968; among others) were able to obtain callus from cambium, but White (1967) was the first to grow callus from xylem explants. He determined that it arose from the epithelial cells of resin canals, and he stated: "Cells of the lining of the resin ducts, even those deeply buried in the wood, also de-differentiate and divide." Since then Kondrasheva (1973) working with *Pinus sylvestris* and Pardos (1976) with *Pinus pinaster* have dem-

TABLE 3. *Origins of callus by trees and time in culture.*

Origins	Longleaf pine trees[a]			
	E	F	G	H
Four weeks liquid[b]				
Sliced vertical resin canals on disk face	88[c]	62	100	91
Vertical resin canals on disk side	12	36	0	0
Horizontal resin canals & rays on disk face	0	15	0	9
(% disks callused)	(12)[d]	(61)	(12)	(25)
Four weeks liquid + one week solid[b]				
Sliced vertical resin canals on disk face	36	59	88	73
Vertical resin canals on disk side	15	27	12	23
Horizontal resin canals & rays on disk face	77	43	0	44
(% disks callused)	(61)	(80)	(38)	(49)
Four weeks liquid + 2.4 weeks solid[b]				
Sliced vertical resin canals on disk face	25	[e]	88	57
Vertical resin canals on disk side	21	[e]	12	20
Horizontal resin canals & rays on disk face	93	[e]	0	70
(% disks callused)	(88)	(97)	(38)	(63)

[a] Total sample size in number of disks per tree: Tree E, 64; F, 64; G, 42; H, 89.
[b] Data were taken immediately after disks were transferred to solid medium following 4 weeks in liquid medium, then after 1 week, and again after 2.4 weeks on solid medium.
[c] An entry is the percentage of callused disks that had callus emanating from a given source and time in culture. For example, in the "Four weeks liquid + one week solid" category, 61% of Tree E's disks had callus; of those 36% had callus arising from sliced vertical resin canals, but some of these same disks could have had callus coming from horizontal canals and rays or vertical resin canals and would appear in those categories as well.
[d] An entry in parentheses is the percentage of the total disks with callus for a given tree on a given date and is not a function of the entries above.
[e] Callus had overgrown the disks to where the origins could not be determined without destructive sampling.

onstrated the ability of the resin canals of these species to produce callus. However, Zimmerman and Brown (1971) reported no success in obtaining proliferation of the older ray cells in the xylem of one- and two-year-old twigs of *Pinus elliottii* and *P. palustris*.

As White (1967) and Pardos (1976) found, we observed callus emerging from vertical resin canals, but we also observed it arising from cut ends of horizontal canals and uniserate rays. White (1967), Pardos (1976), and Kondrasheva and Yatsenko-Khmelevsky (1974) all have identified epithelial cells as the source of the callus; however we observed it arising from ray and longitudinal parenchyma cells as well (Figs. 3a, b, c, d, and 4).

The difference in results may have been due to the early investigators' use of strong chemical sterilizers that killed all parenchyma cells near the surface of their explants. Consequently, the viable cells would have been in the interior of the wood sample where those of the vertical resin canals were probably best situated to produce callus. Vertical canals, being larger than horizontal ones (Brown and Panshin 1940), probably facilitated solute movement and provided the most room for cells in the interior of wood samples to divide and grow.

Because our sterile technique did not require sample sterilization, our disks had viable cells on or near their sufaces, especially on the disk faces since they were cut just prior to being placed in the liquid culture medium. Parenchyma cells of horizontal resin canals and rays exposed in taking the increment core (those that would be on the surface of the sides of the disks) may have dried out to a degree while the core was still in the increment borer and during the slicing of the disks prior to their being placed in culture. Because of this, most of the callus on sides of disks originated from vertical resin canals, although a little was observed coming from horizontal canals and rays, particularly where they were in close proximity to callus growing from vertical canals (Fig. 4). Callus growth from vertical canals was readily visible when disks were viewed on solid medium in petri dishes, but determination of callus origins from horizontal canals and rays exposed on sides of disks would have required destructive sampling. Therefore, calluses emerging from sides of disks were grouped under the major source, vertical canals.

Requirements for xylem parenchyma to divide and grow are apparently a sufficient supply of the proper nutrients and growth substances and the room to grow. Movement of solutes is evidently insufficient in fresh wood because only 5% of disks placed directly on solid medium grew callus, whereas averages of 50- to 70% by species were obtained for those maintained in liquid medium for six weeks prior to transferring to solid medium (Hiatt and Allen 1991). After being transferred, callus grew well from the tops and sides of disks even when the callus per se was not in direct contact with the medium. This indicates that movement of solutes through the disks must have improved after liquid culturing.

Although callus did grow on the lower face of a disk, i.e., the face in contact with solid medium, it grew much slower than that on the upper face and side. Fifty-four percent of disks of the four trees of Table 3 had callus on the lower face, while 73% had it growing from the upper face and side. None had callus only on the lower face. Callus growth from the lower face was observed to be only a fraction of that from the upper face or side. This is thought to be due to the contact with the solid medium reducing aeration to the callus and its room to grow. Amos (1953) pointed out that "when a living protoplast is given free access to moist air, a powerful growth stimulus is applied to the cell."

Callus usually grew first from vertical resin canals that were sliced open along the face of a disk, next from cut ends of vertical resin canals, and then from horizontal canals and rays (Table 3). A sliced resin canal favored callus development and growth because it provided a large mass of living cells that was immediately and directly in contact with the liquid culture medium, and that had room to grow.

Relation to heartwood formation

The responses of the explant callus obtained with pine provide a direct measure of the vitality and activity of xylem parenchyma. These parenchyma cells must play major roles in the physiological processes of sapwood, including those involved in formation of heartwood.

Longleaf and slash pines demonstrated no large and consistent differences across the sapwood in the cells' ability to proliferate and grow. That callus from inner sapwood developed chloroplasts as readily as callus from outer sapwood is evidence that proplastids remain functional for the 30–45 years represented here. These two findings do not support the theory of Frey-Wyssling and Bosshard (1959) that heartwood formation is the result of a gradual degradation of the cells' functions with increasing distance from the cambium. However, they do support Bamber's (1976) theory that heartwood formation is a regulatory development process of the tree. In Bamber's theory, death of parenchyma cells is a result of heartwood formation rather than a cause. This concept is further supported by the observation that the transition zones in longleaf and slash pines are capable of producing callus at frequencies approaching those of the inner sapwood.

Nobuchi and Harada (1983) found that the starting point of a ray parenchyma cell's death was at the boundary between the sapwood and the "white" or transition zone in *Cryptomeria japonica* and that the number of living cells showed a gradual decrease from there to the boundary of the heartwood. They concluded that the white zone was a region having irreversible physiological changes toward aging, as contrasted to the sapwood where reversible changes occur. If that is the case, the production of callus from the transition zone indicates that such irreversible changes must not take place simultaneously in all transition zone parenchyma cells.

Frey-Wyssling and Bosshard (1959) reported that active mitochondria were present only in cells of the outer rings of the sapwood, and Bamber (1976) stated that organelles are poorly represented in the inner sapwood of a number of coniferous species. The fact that we found no important differences between the outer and inner sapwood in callus production or chloroplast development seems to contradict these authors' observations. However, the differences in results were more apt to be due to differences among the species studied.

In contrast to longleaf and slash pines, loblolly and shortleaf pine callus was produced primarily by the outer sapwood. Pardos (1976) found that the cell proliferations from resin canals of *P. pinaster* were limited largely to the last five or so rings. The results with the latter three species support the gradual deterioration theory. Such species differences were illustrated by Yamamoto (1982), who showed that the ray parenchyma of *Pinus strobus* and *Pinus densiflora* remained alive across the sapwood and died in the transition zone, while in *Pinus banksiana* the ray parenchyma started dying in the central sapwood and the mortality increased towards the heartwood. These differences demonstrate that the results with one species should not be applied to another without first verifying their fit, and in view of the large tree to tree variation shown in Table 3 and Fig. 5, on a sufficiently broad sample base.

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