A TISSUE CULTURE SYSTEM FOR MATURE TREES USING SECONDARY WOOD GROWTH FOR EXPLANT MATERIAL

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Abstract.--Regeneration of southern pines through tissue culture has proven difficult when using explant material from adult trees. One of the major stumbling blocks in successfully culturing long-lived woody species is rejuvenation. Ontogenetically young secondary wood growth taken near the base of the tree will potentially display juvenile-like culture responses. A sterilized increment borer was used to collect samples of secondary wood growth from trees at least 40 years in age. Samples were taken from four southern pine species: longleaf pine (Pinus palustris Mill.), slash pine (P. elliottii Englem.), loblolly pine (P. taeda L.), and shortleaf pine (P. echinata Mill.). Longleaf and slash pines were more amenable to culture than loblolly and shortleaf pines. Increment cores were sectioned into disks which were placed in liquid medium. After 6 weeks, disks were transferred to solid medium. A high percentage of disks developed callus. In slash and longleaf pines, cultures were produced which grew well and could be maintained for many subcultures. Some of these cultures exhibited chloroplast development, when moved to light. In some cases, the green callus developed an embryogenic appearance.

Keywords: Pinus palustris, P. elliottii, P. taeda, P. echinata, xylem parenchyma, resin ducts.

INTRODUCTION

Strong economic and scientific reasons exist for cloning individual mature forest trees that have demonstrated their superiority over time. However, vegetative propagation with mature conifers has not been economically feasible. A callus system of regeneration should be able to produce a large number of plantlets with little explant material, but regeneration from conifer callus is difficult. Although, there are many cases of success when starting with immature or juvenile tissue, there has been much less success in producing shoots or somatic embryos from callus of mature conifers. Gladfelter and Phillips (1987) report a low frequency of shoot bud formation in mature-tree callus of Pinus eldarica.

Rejuvenation presents a difficult obstacle to regenerating mature woody plants. Tissues that have undergone phase change are not easily converted back to juvenile characteristics (Wareing, 1987). A plausible approach is to use explant material that has not gone through phase change. Hackett (1985) states, "juvenile characteristics such as rooting potential may be preserved at the base

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of plants in ontogenetically young tissues (meristems), while maturation occurs in the periphery of the plant in ontogenetically older but chronologically young tissues." We have based our regeneration studies on explants from the base section of mature southern pine trees, endeavoring to take advantage of ontogenetically less mature tissues. We reasoned that it may be advantageous to start the callus cultures from xylem parenchyma of the inner sapwood which would be ontogenetically much younger than cambium cells at the same tree height.

The ability of ray parenchyma to generate callus tissue in response to wounding has long been known. If bark is removed but the tangentially exposed xylem is kept moist by promptly covering it with foil or plastic film the wound will soon become covered with a wound callus that originates primarily from the ray cells (Zimmerman and Brown, 1971). In studying stress induced cleavages in early wood, Amos (1953) found that the resulting cavities filled with callus in Pinus radiata, whose ray parenchyma are bounded by primary wall, but no callus was formed in the cavities of Psuedotsuga menziesii or Picea glauca whose ray parenchyma become thickened with secondary wall soon after formation. Harris and Barnett (1975) found examples of differentiated callus nodules in cavities of felled Pinus ponderosa. They attributed wood ray cells as the initiation source for this callus.

Xylem parenchyma cells can be cultured. Barker (1953) cultured 50-year-old cells from the medullary sheath region of Tilia americana. Successful conifer callus cultures arising from the resin ducts were obtained from Pinus radiata (Pardos, 1976) and from Pinus sylvestris (Kondrasheva, 1973; Kondrasheva and Yatsenko-Khmelevsky, 1974). White (1967) produced callus cultures from resin ducts "deep in the wood" of 20- to 60-year-old trees of Picea glauca. However, Zimmerman and Brown (1971) were unsuccessful in getting proliferation of the older ray cells in the xylem of 1- and 2-year-old twigs of Pinus elliotti and Pinus palustris.

This work reports the culturing of xylem parenchyma from the sapwood of mature trees of longleaf pine (Pinus palustris Mill.), slash pine (P. elliottii Englem.), loblolly pine (P. taeda L.), and shortleaf pine (P. echinata Mill.).

MATERIALS AND METHODS

Explant Selection

Samples were taken from 4 loblolly pines, 10 slash pines, 21 longleaf pines, and 3 shortleaf pines. Trees were located on the Clemson Experimental Forest, Clemson, South Carolina and were 40-60 years of age, with sapwood ranging from 30-40 rings in thickness. Only trees judged to be of superior vigor and form were used for explant material. After initial studies, longleaf and slash pines were found to be more amenable to culture than loblolly or shortleaf pines, so further studies concentrated on longleaf pine and slash pine.

Explant Collection

Samples of secondary wood growth were taken from older trees with increment borers. The increment borers were separated into their individual parts, wrapped in aluminum foil, and autoclaved prior to use in the field. To remove resin, increment borers were cleaned with 95% ethanol after each use. The tree bark was chopped away from the desired spot, exposing approximately 5 cm^2 of xylem, and the spot was well rinsed with 95% ethanol. A 5 mm diameter core was taken at stump height (approximately 30 cm from ground). Samples were immediately returned to the lab within the borer, where they were removed under a laminar flow hood. Despite the rather crude method of explant collection, no surface sterilization was necessary. Pardos (1976) and White (1967) used either disinfectants or flaming to surface sterilize their explant material. A number of the cores, however, were flamed in initial tests to ensure that callus growth was not originating from cambium cells which might have contaminated the core during sample collection. No callus growth could be attributed to cambium contamination, so flaming was discontinued. The increment cores, approximately 20 cm long, were sliced into 1-2 mm cross sections (disks). The cambium and the heartwood, generally, were excluded and depending upon the age of the parent tree, wood from 3-40 years in age was used.

Cultures were initiated at all times of the year, with some individual trees being sampled at several different times. When multiple samples were taken from the same tree, the cores were taken to one side and slightly above or below previous sample sites. The lower portion of trees sampled in warm weather was sprayed with benzene hexachloride solution as protection against bark beetles.

Supplemental tests were done using wood growth taken from roots of longleaf pine. The soil was removed from around the taproot and a lateral root. The outer covering was cut away and the surface rinsed with 95% ethanol. Sterile increment borers were used to take samples from within the roots. Roots were cultured in the same manner as secondary wood growth taken from the trunk.

Culturing Method

Six to eight wood disks were placed in 50 ml Erlenmeyer flasks containing 15 ml liquid medium. Foam stoppers were used to allow for gas exchange. Flasks were kept in a Lab-line Environ-Shaker at 25°C, in the dark, shaking at 100rpm. Liquid DCR medium (Gupta and Durzan, 1985) was used at pH 6.0 (pH adjusted using either 2N KOH or .15N HC1). Various amounts and combinations of the following growth hormones were used: 6-benzylaminopurine (BAP); a-naphthaleneacetic acid (NAA); and 2,4-dichlorophenoxyacetic acid (2,4-D). DCR medium was prepared at 3% sucrose. Every two weeks the liquid medium was removed, using a sterile pasteur pipet attached to a vacuum line, and replaced with fresh medium. After six weeks the disks were moved to agar (0.6%) solidified plates, and remained in the dark for varying periods of time before being moved to shelves with fluorescent or a combination of fluorescent and GE Gro&Sho plant lights. Cultures were exposed to 16 hours of light and 8 hours of dark. Once on solid medium, cultures were transferred approximately every four weeks. Small (60 X 15 mm) petri dishes were used. Plates were not wrapped with Parafilm^R, but they were kept in clear polycarbonate boxes (Petawawa Germination Boxes,

Spencer-Lemaire Industries Limited, Alberta, Canada) to reduce medium dehydration and to reduce contamination.

RESULTS

Liquid Culture

Studies were conducted using longleaf pine and slash pine to find the optimum period of time required in liquid medium before transfer to solid medium. The medium used for this study was DCR 1 mg/L BAP 2 mg/L NAA (DCR1BAP2NAA). Cultures were transferred to solid medium, at two week intervals, from four weeks to ten weeks after initiation. Disks were also placed immediately upon solid DCR1BAP2NAA plates. Six weeks was chosen as optimum for liquid culture, because cultures exhibited no further visible callus development after six weeks. Less than 5% of the disks without a liquid medium treatment developed callus. In other studies, more than 70% of the disks in cultures of loblolly, slash, and longleaf pines, initially maintained in liquid medium, exhibited some degree of callus development. Approximately 50% of the cultures of shortleaf pine displayed callus development.

Callus Growth

In studies with loblolly pine and shortleaf pine, cultures grew little before callus darkened and growth ceased. Our work has concentrated on longleaf and slash pines, and all the results that follow are from studies performed with these two species. Studies with longleaf pine or slash pine produced callus that could be maintained through many subcultures. Callus cultures on solid medium had better growth if they remained in the dark for several weeks after placement on solid medium. Callus cultures in the dark were mostly opaque with some browning. After cultures were removed to lighted shelves, some of the cultures exhibited a green color. Microscopic examination of the callus confirmed that a portion of the cells had developed chloroplasts. In most cases the green callus existed in conglomeration with dark or opaque callus and increased slowly. Some green callus took on an embryogenic appearance, but no nodules, shoots, etc. were observed.

Callus was obtained from disks taken along the entire width of the sapwood. Disks taken from the heartwood did not show callus development. No difference was found in the percentage of cultures from the inner sapwood versus the outer sapwood that developed chloroplasts.

The source of the callus appeared to be the epithelial cells lining the resin ducts. Callus grew first and most from vertical resin ducts which were sliced along the face of the disks. Callus also grew from the ends of horizontal resin ducts and the ends of nonsliced vertical ducts (Figure 1). There was no evidence of uniserate rays developing callus. Pardos (1976) working with Pinus pinaster and White (1967) working with Picea glauca both denoted resin ducts as the source of callus growth in the xylem. Our research provided no grounds to refute this premise.

Figure 1. Longleaf pine disk with callus growth. The disk was cultured for 6 weeks in liquid DCR 1 mg/L BAP 2 mg/L NAA and one week on solid DCR .5 mg/L BAP .05 mg/L NAA, in the dark at 25°C. The tree was 56 years old and the disk was taken from the inner sapwood.

Longleaf Pine Study

Increment cores were taken from seven longleaf pine trees, in a 55-year-old plantation. One increment core was taken from each tree and 36 disks were sectioned from each core. Twelve disks were placed in each of the following media: DCR 1 mg/L BAP 2 mg/L NAA; DCR 1 mg/L BAP 2 mg/L 2,4-D; or DCR 2 mg/L 2,4-D. Cores were transferred to the same medium solidified with 0.6% agar after six weeks in liquid medium. Seventy-six percent of the all disks developed callus in the **six** weeks while in liquid medium. The differences in the percentage of disks that produced callus were attributed to genetic variation as well as varying responses to media type (Table 1). After seven weeks on solid medium, cultures were moved to lighted shelves (fluorescent lights only). Six weeks after being moved to light, 23% of all cultures had some degree of chloroplast development.

Root Study

Two increment cores were taken above ground (from stump height and ground level) and 2 were taken below ground (from the taproot and a lateral root). Two 56-year-old longleaf pines were utilized for this study. The increment cores were sectioned into 48 disks. Disks were cultured in DCR medium supplemented either with 2 mg/L 2,4-D alone; 2 mg/L 2,4-D plus 1 mg/L BAP; or 3 mg/L BAP alone. Cultures in DCR 3mg/L BAP showed no growth in liquid medium and were discarded from the test. All further results ignore the disks placed in DCR 3 mg/L BAP. After 6 weeks in liquid, disks were transferred to the same medium type plus 0.6% agar. Longleaf pine, 07, had 91% of disks develop callus and

tree 15 had 90% of disks develop callus. The percentage of disks that produced callus from each explant source can be seen in Table 2. Cultures on solid medium were kept in the dark for 11 weeks. Four weeks after cultures were moved to lighted shelves (fluorescent and Gro&Sho plant lights combined) the following percentage of cultures had developed chloroplasts. By explant source: stump height, 57%; ground level, 55%; taproot, 33%; and lateral root, 57%.

TABLE 1. Callus development on longleaf pine disks after 6 weeks in liquid medium.

Tree	DCR Basal Medium plus		
	1 mg/L BAP 2 mg/L NAA	1 mg/L BAP 2 mg/L 2,4-D	2 mg/L 2,4-D
	Disks with callus (percents)		
07	83	92	100
08	83	33	8
09	100	58	83
10	50	42	42
11	100	92	100
12	100	100	100
13	83	75	83

Table 2. Callus development on disks of longleaf pines (07 & 15) taken from the following tree positions; stump height, ground level, taproot and lateral root. Disks maintained for 6 weeks in liquid culture followed by 3 weeks on solid medium (9 weeks).

Tree Position	DCR Basal Medium plus			
	2 mg/L 2,4-D		2 mg/L 2,4-D 1 mg/L BAP	
	6 weeks	9 weeks	6 weeks	9 weeks
	Disks with callus (percents)			
Stump height	83	96	76	100
Ground level	67	79	75	83
Taproot	100	100	100	100
Lateral root	92	100	56	72

DISCUSSION

Callus cultures which continued to increase through many subcultures were derived from xylem parenchyma located in secondary wood growth. Although cells seemed to lose their ability to divide and dedifferentiate once in heartwood, cells the entire width of sapwood can produce viable callus. Cells which are decades old remain vital. These viable cells may be in the minority of all cells present in wood, but enough cells are living to produce callus. Callus derived from these approximately 30-year-old, nonphotosynthetic cells had the ability to produce chloroplasts, indicating that the cells of origin maintain proplastids.

Some cultures produced green callus with an embryogenic appearance. We feel that the potential for obtaining plantlets from these calluses is present, but that the right set of culture conditions has yet to be found. Mature trees are recalcitrant in culture, making regeneration difficult. The parenchyma cells lining the resin duct may be in a position in the tree which is chronologically old, and at the same time ontogenetically young. These cells may retain a high enough degree of juvenility to make them more amenable to in vitro regeneration.

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