MICROPROPAGATION OF *Eucalyptus viminalis*

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Abstract.--Cooperators of the North Carolina State University Hardwood Research Program have selected over 50 Eucalyptus viminalis trees that demonstrated superior growth rates and tolerance to frost in test plantings in southern Georgia and northern Florida. Twenty-eight of these trees have been vegetatively propagated by grafting or rooting at North Carolina State University. These stock plants will be micropropagated as in vitro methods are developed, to establish a seed orchard on lands of Carton de Colombia.

Results are presented for in vitro multiple shoot production, using single node explants. Genotype, node selection, and stock plant vigor influenced axillary shoot production of the original explants. The induction of multiple shoots on excised axillary shoots was not affected by the cytokinin concentrations or durations of treatments tested. When excised axillary shoots were cut into single nodes, there was a slight enhancement of multiple shoot production.

Additional Keywords: tissue culture, vegetative propagation

The need for a hardwood fiber source that would occupy upland sites and therefore be more readily available during wet seasons led members of the North Carolina State University Hardwood Research Program to begin screening seed sources of several species of Eucalyptus in 1971. The objective of this effort was to select species, seed sources, and trees within seed sources that would be fast-growing, of good form, and tolerant to both drought and frost. Over the years more than 100 species and 577 seed sources were tested. Initially, plantings ranged from the coastal plain of North Carolina to northern Alabama. As the program progressed, the plantings were restricted to southern Georgia and northern Florida because of harsh winter temperatures north of that area. The best suited species to this area were determined to be E. viminalis, E. macarthurii, E. nova-anglica and E. camphora.

The failure of any one seed source to consistently produce fast-growing trees that were also frost-tolerant emphasized the need to develop a land race of eucalyptus suitable for planting in the southeastern United States. The seedling seed orchard approach proposed by Purnell and Kellison (1983) for the genetic improvement of other hardwood species could not be used with the eucalypts because the species flowers during the winter months when freezing temperatures destroy the seed-bearing potential of the trees. The decision was thus made to establish a clonal seed orchard farther south, where there would be no danger of freezing temperatures. Container Corporation of America, Fernandina Beach, Florida, in conjunction with one of its South American subsidiaries, Carton de Colombia, provided the funding for the orchard which **is** to be established near Popoyan, Colombia.

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Eucalyptus viminalis, the species showing the most promise of the four mentioned previously, has proven difficult to propagate vegetatively. Attempts to root cuttings from induced epicormic shoots from mature trees, as is done with many other species of eucalypts, have met with minimal success. Grafting, while more successful than rooting cuttings, is also an unreliable method of vegetatively propagating the species. Micropropagation under controlled in vitro conditions may offer a viable alternative as a means to vegetatively propagate selected trees of E. viminalis.

Vegetative propagules have been produced by micropropagation for a number of species of eucalyptus (Hartney, 1983). Franclet and Boulay (1982), Boulay (1983), and Depommier (1981) described methods used by AFOCEL **in** France to successfully regenerate plantlets of E. gunnii and E. dalrympleana, using nodal explants derived from grafted or rooted cutting stock plants. Ages of the original ortets from which stock plants were derived ranged from 2 to 20 years. The general procedure followed in each of these papers included (1) induction of axillary shoot formation on single node explants, (2) multiple shoot formation from excised axillary shoots, (3) shoot elongation, (4) root initiation of excised shoots, and (5) root elongation and transfer to soil. Franclet and Boulay (1982) anticipated producing 25,000 plants per month, using this system.

The objectives of the Hardwood Research Program project are to employ and modify where necessary the techniques outlined by AFOCEL workers to vegetatively propagate 28 clones of E. viminalis. These clones will be shipped to Popoyan, Colombia, S. A., where they will be used to establish a seed orchard. The objectives of this paper are to report on the successes in inducing nodes to produce axillary shoots and the methods tested for multiple shoot formation from excised shoots.

ESTABLISHMENT AND MAINTENANCE OF CLONES

In 1983, cooperators of the Hardwood Research Program selected a total of 41 E. viminalis, E. macarthurii and E. nova-anglica trees that were phenotypically superior in growth rate and frost tolerance. Selections were made from seed source trials and operational test plantings three or more years old. In addition, 14 surviving E. viminalis clones from a previously established seed orchard near Ft. Green Springs, Florida were included, making a total of 55 selections. Twenty-eight of these selections have been vegetatively propagated by rooted cuttings or grafting and are being used as stock plants for the micropropagation work.

Cuttings were rooted following slightly modified techniques of Campinhos and Ikemori (1980). Basal epicormic shoots were induced on mature trees by girdling at a height of one meter. Shoots were cut into four-leaved, two-node cuttings, treated with a rooting powder consisting of 0.8% indolebutyric acid, and placed under intermittent mist. Rooting success was low for these basal sprouts, averaging less than 15% for all clones. Twelve of the 28 clones were propagated in this way. The remaining 16 clones were grafted, using scion material from the upper branches.

The stock plants were maintained in 4.5-1 pots in the greenhouse with an extended photoperiod of 18 hours. They were fertilized weekly with a 0.5 g/1 solution of 20-19-18 (N-P-K) fertilizer and sprayed twice weekly with Benlate, a systemic fungicide. The plants were maintained at a height of 30 - 60 cm by cutting them back at eight-week intervals.

INITIATION OF CULTURES.

Initial explants were derived by cutting shoots into single-node sections and immediately sealing both ends in paraffin. The leaves were then trimmed to approximately four -mm squares. The explants were surface-sterilized for 10 minutes in a 10% solution of commercial bleach with two drops of surfactant added. The wax ends were then cut off, leaving an explant of 8 to 14 mm in length, with 3 to 4 mm above the node. The explants were then placed vertically into the medium, which was contained in sterilized petri plates. They were placed in the dark for one week and then moved to a continuous light environment supplied by two 40-watt, cool white fluorescent bulbs, approximately 15 cm above the top of the plates. The temperature in the culture room was 22° + 2° C.

The basic medium used for all experiments consisted of half-strength salts and vitamins (Murashige and Skoog, 1962),3.0% sucrose, and 0.7% agar. The pH of the media was adjusted to 5.6. For the initial nodal explant studies, 0.5 mg/1 of benzylaminopurine (BAP) and 0.01 mg/1 of napthaleneacetic acid. (NAA) were added.

By the end of the first week in culture, axillary shoots began to emerge from many of the explants. In most cases callusing occurred at the base of the explant and on the leaves where they were touching the media. Callusing occasionally occurred in the nodal region but this did not appear to influence the emergence of axillary shoots. By the third week on the medium, many of the axillary shoots had begun to elongate, some over one cm in length, and were ready to be excised.

As with most vegetative propagation methods, the genotype substantially influenced the response achieved. The responses of three clones that were used in a number of experiments are summarized in Table 1. Growth regulator concentrations were varied slightly from trial to trial but all clones were treated the same within a particular trial, and within a trial the explants for all clones came from stock plants at the same stage after pruning. Clones B16 and B6 consistently had response rates of greater than 50%, while explants from Clnne 7594 never recnnnded at ratec ahnue

Table 1.--Percentage of single-node explants producing axillary shoots in 3 - 4 weeks in four different trials

Clone	Trial 1	2	3	4
	Percent			
2594	33.3	8.6	8.3	-
B15	50.0	68.6	-	75.0
B6	84.6	60.0	50.0	66.7

For a given clone, the percentage of explants producing axillary shoots seemed to be influenced by stock plant vigor and node selection. In an experiment to determine which nodes were most useful, explants were grouped according to their distance from the growing point. The majority of stock plant shoots were no longer than 6 nodes in length, so explants were divided into three groups consisting of two nodes each (Nodes 1-2, 3-4, and 5-6, with Nodes 1-2 being closest to the growing point). After three weeks on the initiation medium, data were recorded for the number of axillary buds and shoots (shoots were distinguished from buds by the visible presence of at least a 1-mm internode) and the length of the longest shoot. The percentage of explants with buds or shoots was calculated. Percent and count data were transformed, using the arc-sine square root and the square root methods, respectively, for the analysis of variance.

The mean response of three clones after three weeks in culture showed that explants derived from Nodes 3 and 4 gave the highest response rate and the greatest number of shoots per plant (Table 2). While fewer explants from Nodes 1 and 2 responded than those from Nodes 5 and 6, those explants that did respond had more and longer shoots. This implies that explants from Nodes 5-6 are slower-growing and probably have many explants with buds that ;lave yet to elongate. There were significant clone effects for all variables but the "clone by node" interaction was not significant, indicating that the influence of node number was consistent for all three clones tested.

Table 2.--Effect of node number on the response of single-node explants after four weeks in culture

Node Number	Percent with Buds or Shoots	Average Shoots per Explant	Average Length Longest Shoot(mm)
1-2	36.1 a[1/]	1.2 ab	3.3 a
3-4	53.3 b	1.5 a	3.4 a
5-6	47.2 ab	0.6 b	2.1 a

[1/] Values within a column followed by the same letter were not significantly different ($P \leq 0.05$) using Duncan's Multiple Range Test.

Stock plant vigor was also found to influence the response of nodal explants. In two separate trials, explants were collected from the same set of nodes and placed on the same medium. In one trial, explants were derived from stock plants that had been repeatedly pruned and fertilized weekly; while in the other experiment, stock plants were slower-growing, had not been fertilized on a weekly schedule, and had not been pruned for several months. For the two clones used in the trials, contamination and mortality rates were considerably higher and the number of explants producing axillary buds or shoots was much lower for the less vigorous stock plants (Table 3). These results emphasize the importance of maintaining pruned and vigorous stock plants as a source of explant material.

Table 3.--Effects of stock plant vigor on the response of nodal explants after 3 weeks in culture

Clone	Stock Vigor	Percent Contaminated	Percent Dead	Percent with Buds or Shoots
2604	Poor	50.0	25.0	25.0
	Good	4.2	4.2	66.7
B15	Poor	58.3	25.0	16.7
	Good	8.3	0	66.7

MULTIPLE SHOOT PRODUCTION

Buds and shoots continued to grow when left attached to the original node, about 3 mm by 3 weeks (Table 2) but reaching 7 to 20 mm by 6 weeks. However, the explants did not continue to make additional buds. Published methods require that shoots be excised both for additional bud production and for growth of shoots in preparation for the rooting stage.

At 3 to 4 weeks, some axillary shoots were sufficiently elongated to be excised and transferred to a new medium. In preliminary trials, excised shoots were placed on the same medium as the original explant (0.5 M.S. + 0.5 mg/1 BAP +0.01 mg/1 NAA) under the same environmental conditions. After 2 to 3 weeks, explants began to turn red, older leaves senesced, and the explant died basipetally. Multiple buds were sometimes formed at the lowest node near the base of the explant. To determine if cytokinin concentrations influence this response, excised shoots from two clones were tested on the original medium, but with either 0.2, 0.5 or 1.0 mg/1 BAP.

Neither clone nor BAP concentrations significantly (PtS 0.05) influenced the number of buds, number of shoots, or shoot length (Table 4). Explants from all treatments responded the same as those from preliminary studies. Some of the explants produced clumps of buds at their base, but none of the buds elongated to more than 5 mm in length during the four weeks. Even at seven weeks, the average shoot length was 2.7 mm.

Table 4.--Effects of BAP concentration on the response of excised axillary shoots

BAP[1/] (mg/1)	Average Buds per Explant	Average Shoots per Explant	Average Length Longest Shoot (mm)
0.2	2.2	1.2	2.7
0.5	2.5	1.2	1.9
1.0	2.9	0.3	2.7

/ Analyses of variance showed no significant treatment effects (P 0.05) for any of the variables measured.

In a second experiment, cytokinin pulses were tested to see if the length of time the explants were treated with BAP would influence their response. Shoots were excised from nodal explants of three clones and placed on the original medium with either 1.0 or 2.0 mg/1 BAP for one, two or three weeks. The explants were then transferred to a medium (Franclet and Boulay, 1982) used for shoot elongation. This medium was the same as the original medium except for a reduced concentration of BAP (0.1 mg/1) and the addition of 1.5% activated charcoal.

Neither the duration nor the concentration of the BAP pulse significantly influenced the explant responses (Tables 5 and 6). The average number of buds and shoots was very similar to the results obtained from the BAP concentration study. Again, explants would respond with red foliage, callusing and senescing from the apex down and the formation of multiple bud clusters at the base of some of the explants. The use of charcoal appeared to make a slight improvement in the response. The average shoot lengths of explants in the pulse experiment (Table 5) were a little longer than those from the BAP concentration study (Table 4). It was noted in previous experiments that when multiple bud explants were transferred to a medium containing charcoal, the buds would begin to expand and elongate for two to three weeks. The explants would then begin to turn pale yellow and callus, probably as a result of the charcoal tying up needed growth regulators. Shoots seldom reached a length suitable for rooting.

Table 5.--Effects of length of BAP pulse on the response of excised axillary shoots

Pulse[1/] (wks)	Average Buds per Explant	Average Shoots per Explant	Average Length Longest Shoot (mm)
1	3.4	1.5	4.3
2	2.9	1.5	4.9
3	3.9	1.2	3.5

[1/] Analyses of variance showed no significant treatment effects ($P \leq 0.05$) for any of the variables measured.

Table 6.--Effects of BAP pulse concentration on the response of excised axillary shoots

BAP[1/] (mg/1)	Average Buds per Explant	Average Shoots per Explant	Average Length Longest Shoot (mm)
1.0	3.2	1.5	4.3
2.0	3.3	1.3	4.3

[1/] Analyses of variance showed no significant treatment effects ($P \leq 0.05$) for any of the variables measured.

The tendency of excised shoots to die back and produce multiple buds at the basal node led to a test of using excised shoots that had been further cut into single-node explants for multiple bud production. The explants were on a medium with 0.2 mg/1 BAP and 0.01 mg/1 NAA for three weeks. The group of single-node explants from one shoot tended to produce more buds and shoots than did comparable entire excised shoots (Table 7). This increase was the result of more explants producing bud clusters rather than an increase in the number of buds per cluster. The increased bud number and increased growth of the buds produced was probably a function of removing the apex from the explant, thus relieving the apical dominance; however, this hypothesis has yet to be tested.

Table 7.--Comparison of the response of single-node versus entire axillary shoots as an explant source for multiple shoot production

Explant Type	Average Buds per Explant	Average Shoots per Explant	Average Length Longest Shoot (mm)
Single Node	4.1	2.9	4.6
Entire Shoot	2.2	1.2	2.5

CONCLUSIONS

Nodal explants of E. viminalis could be induced to produce axillary shoots in vitro. The response varied from clone to clone and was improved by maintaining vigorously growing stock plants and by selecting the proper node. Proper greenhouse care to ensure rapidly growing, disease- and insect-free stock plants seems to be the most critical step for acceptable contamination rates and for the induction of buds and shoots. Axillary shoots, when left intact, continued to elongate through six weeks in culture. However, no additional shoots were initiated on the origianl explant after week four. Therefore, in order to multiply the number of plants for rooting, it is necessary to excise axillary shoots and induce multiple shoot formation.

Excised axillary shoots grew poorly, producing few buds, which failed to elongate. The response of the excised shoots was not influenced by cytokinin concentrations or the duration of these treatments. The addition of charcoal to the media was also ineffective in enhancing shoot elongation.

Boulay (1983) reported similar problems with several frost-tolerant eucalyptus species. He stated that for many clones, frequent subculturing was necessary before the explants would begin to grow vigorously and produce multiple shoots. We believe that this enhanced response after many subcultures probably resulted from better control of shoot vigor and/or node development stage of the in vitro stock plants used for serial subcultures. The influence of in vivo stock vigor and node number on the response of original explants found in our studies supports this hypothesis. From our preliminary data it appears that we can enhance multiple shoot production by selecting single nodes from in vitro-produced axillary shoots rather than using the entire shoot as an explant. However, the buds produced still did not elongate sufficiently for rooting purposes. We will be further investigating the effects of node selection of in vitro-produced axillary shoots to enhance multiple shoot production.

Multiple shoot production and shoot elongation cannot occur under the same in vitro conditions for E. viminalis. Future efforts will also be directed toward development of a separate medium *for shoot elongation of multiple shoot explants* and eventually the rooting of elongated shoots.

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