GROWTH AND DIFFERENTIATION OF AMERICAN CHESTNUT CALLUS CULTURES

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ABSTRACT.--The effects of various auxins and auxin and cytokinin concentrations on growth and differentiation of American chestnut callus cultures were studied. Growth was greatest on 2.0 mg/1 NAA or 0.5 mg/1 2,4,5,-T with 0.5 mg/1 kinetin. Differentiation of procambia and primary xylem occurred on 0.5 mg/1 2,4-D or 2.0 mg/1 IAA with 0.5 mg/1 kinetin. Growth increased as auxin concentration was increased and cytokinin concentration was decreased. Shoot-like tissues formed on epicotyl callus cultures grown on BAP, 2-iP, kinetin, or adenine sulfate.

The idea of plantlet formation from tissue cultures of tree species has excited many future-minded foresters. Plantlet formation has resulted from cultures of Coffea arabica L. (Sondahl and Sharp, 1977), Populus spp. L. (Chalupa, 1974; Venverloo, 1973, Winton, 1970, 1971), Ulmus americana L. (Durzan and Lopushanski, 1975), and U. Campestris L.(Chalupa, 1975), showing that some angiosperms are capable of being propagated in vitro. Tissue culture is a possible technique for the vegetative propagation of species, such as American chestnut [Castanea dentata (March) Borkh.], which respond poorly to the conventional methods of grafting or rooting cuttings (Keys, 1978). A reliable system for propagating American or hybrid chestnuts will be of value, since researchers are still searching for such trees which are resistant to the chestnut blight caused by Endothia parasitica (Murr) A. & A. (Keys, Cech and MacDonald; 1975; Samman and Thor; 1976). For this reason, plantlet formation from American chestnut tissue cultures is being attempted.

The purpose of this research is to determine the cultural conditions necessary for in vitro plantlet formation of American chestnut. This paper reports the effect of various growth regulators on callus tissue from mature stems and etiolated epicotyls.

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MATERIALS AND METHODS

Mature stem callus. Stems approximately one inch in diameter were collected from tree of five-inch D.B.H. and surface-sterilized by flaming in alcohol. Cambial sections approximately 8 mm X 15 mm were aseptically excised and placed on Murashige and Skoog (MS) medium to initiate callus. This tissue was subcultured on the same medium at six-to-eight week intervals until enough tissue was available for experimentation.

Three trials were made, using MS basal medium, to test the effect of:

1) various growth regulators[1]
   a) 2.0 mg/l indoleacetic acid (IAA)
   b) 2.0 mg/l naphthaleneacetic acid (NAA)
   c) 0.5 mg/l 2,4-dichlorophenoxy acid (2,4-D)
   d) 0.5 mg/l 2,4,5-trichlorophenoxy acetic acid (2,4,5-T)
   e) 9.3 mg/l 2-chloroethanol (EC)
2) hormone ratios
   a) 0.2 mg/l NAA and 1.0 mg/l kinetin
   b) 0.5 mg/l NAA and 0.5 mg/l kinetin
   c) 1.0 mg/l NAA and 0.2 mg/l kinetin
3) kinetin concentrations of 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M. in combination with 0.5 mg/l NAA.

In trials 1 and 2, four flasks per treatment in each of four completely-randomized replications were placed in continuous darkness at 27+2 degrees C. for eight weeks. The same number of flasks was also placed in a 12-hour photoperiod of 250 f.c. Grolux and flourescent light. In the third trial three flasks per treatment in each of three replications were placed in continuous darkness.

Fresh and dry weights were anlayzed using analysis of variance and modified LSD. Linear regression was run on trials 2 and 3. For histological studies, some cultures in trial 1 were fixed in formalin: acetic acid and stained with safranin o-fast green.

Epicotyl callus. Preliminary results showed that callus from etiolated epicotyls of American chestnut was capable of differentiation. So stratified seeds were germinated in a dark room to promote etiolation. Prior to secondary-leaf formation, the epicotyls were cut off and sterilized in 50% Clorox and 0.1% Alconox for ten minutes. They were washed four times in sterile, distilled water. Sections approximately one centimeter long were asceptically inoculated on the medium shown in Table 1 supplemented with:

'Treatments a-d were in combination with a 0.5 mg/1 kinetin.

1) 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}M. 6-benzylaminopurine (BAP), (6- x, X-dimethylally (amino) purine) (2-iP), kinetin (K), or adenine sulfate (AS) with 2.0 mg/1 NAA.
2) 2.0 mg/1 NAA without a cytokinin

Three flasks per treatment in each of three replications were placed in continuous light (250 f.c. Grolux and flourescent) of 27+2 degrees C.

Fresh and dry weights were analyzed using analysis of variance and linear regression. Some cultures were fixed in formalin: acetic acid or Randolph's Navaschin solution and stained in safranin O-fast green.

Table 1. Medium used for epicotyl cultures.

Constituent	Concentration (mg/l)	Constituent	Concentration (mg/l)
KNO_3	1900.0	myo-Inositol	100.0
NH_4NO_3	1650.0	Glycine	2.0
$MgSO_4 \cdot 7H_2O$	370.0	Nicotinic acid	0.5
$CaCl_2.2H_2O$	440.0	Pyridoxine-HCl	0.5
KH_2PO_4	595.0	Thiamine-HCl	0.1
$MnSO_4 \cdot 4H_2O$	22.3	NAA	2.0
$ZnSO_4 \cdot 7H_2O$	8.6	Sucrose	20,000
H_3BO_3	6.2	Difco Bactoagar	8,000
Na_2MoO_2	0.05	pH	5.6
KI	1.66		
$CuSO_4 \cdot 5H_2O$	0.05		
$CoCl_2$	0.05		
$FeSO_4 \cdot 7H_2O$	58.6		
NaEDTA	79.3		

Mature stem callus. The greatest callus formation occurred in the 2,4,5-T and NAA treatments (Table 2). Fresh weight increase was significantly greater at the 1% level for cultures grown on 2,4,5 -T or NAA than on IAA or 2,4-D. Dry weight of cultures grown on IAA did not differ statistically from those grown on 2,4,5-T or NAA. EC stimulated little or no callus formation.

Light significantly inhibited fresh weight increase at the 1% level of cultures grown on all growth regulators but EC. Light significantly inhibited dry weight of cultures grown on NAA or IAA, at the 1% level.

Histological examination indicates that growth regulators that stimulate intense callus proliferation do not favor differentiation. Cultures grown on 2,4,5-T or NAA were parenchymatous with random cell-division. Cultures grown on 2,4-D or IAA, however, developed procambial strands and primary xylem elements. Though vascular systems did not develop completely, these cultures were partially organized. The highest degree of differentiation occured on 2,4-D. No definite shoot or root primordia were evident (Fig. 1).

Table 2. Effect of hormone and light on stem callus tissue.

	Fresh Weight Increase (mg)			Dry Weight (mg)		
Hormone	Overall Average	Continuous darkness	12-hour Photoperiod	Overall Average	Continuous darkness	12-hour Photoperiod
NAA	9,102	11,642	6,562	661	722	599
IAA	6,528	9,066	3,990	635	764	507
2,4,5-T	9,133	10,922	7,344	650	633	668
2,4,-D	3,038	3,278	2,797	408	393	424
EC	587	332	843	168	128	208

Callus proliferation was greatest in cultures grown on the high auxin:cytokinin ratio. Growth was positively correlated with NAA concentration, and negatively correlated with kinetin concentration. This correlation was linear and significant at the 1% level, with r-values for fresh weight increase and dry weight of 0.79 and Q,79 respectively (Table 3). Again, light inhibited callus proliferation. Two cultures grown on 1.0 mg/1 NAA and 0.2 mg/1 kinetin in continuous darkness developed small protuberances from the upper surface, but these failed to develop into shoots.

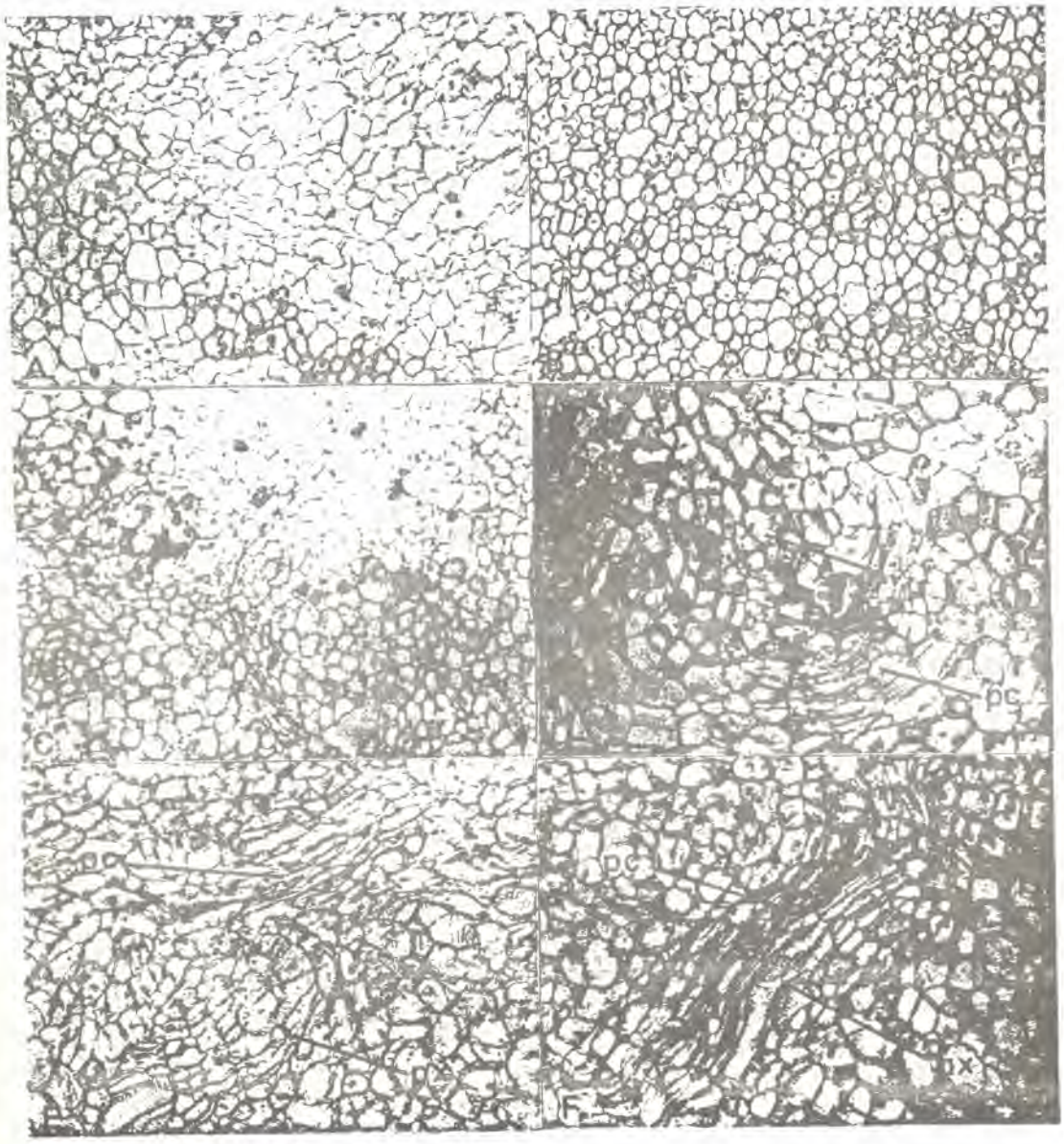

Fig. 1. The effect of various growth regulators on differentiation. (A-B) Parenchymatous callus growth on NAA and 2,4,5-T, respectively. (C) A possible shoot primordium in a culture grown on IAA. (D) Primary xylem (px) and procambium (pc) found in a culture grown on IAA. (E-F) Primary xylem and procambium found in culture grown on 2,4-D.

Table 3. Effect of light and auxin: cytokinin ratio on stem callus tissue.

Auxin Concentration (mg/l)	Cytokinin Concentration (mg/l)	Fresh weight increase (mg)		Dry weight (mg)	
		Continuous darkness	12-hour Photoperiod	Continuous darkness	12-hour Photoperiod
0.2	1.0	88	38	47	45
0.5	0.5	5,987	1,868	495	280
1.0	0.2	6,815	5,527	461	513

In Trial 3 the negative correlation of callus growth to cytokinin concentration was substantiated. Again, this trend was linear and significant at the 1% level with r-values of -0.488 for fresh weight increase and -0.566 for dry weight. Kinetin concentrations greater than 1 X 10^{-6} M inhibited growth in comparison to the control (Table 4).

Table 4. Effect of kinetin concentration on stem callus tissue.

Kinetin Concentration (M)	Fresh Weight Increase (mg)	Dry Weight (mg)
1 X 10^{-8}	2471	240
1 X 10^{-7}	2050	216
1 X 10^{-6}	1094	151
1 X 10^{-5}	34	38
1 X 10^{-4}	-56	35
Control	1343	167

Enicotyl callus. Although differences among means for fresh weight increase and dry weight did not differ statistically, growth showed a curvilinear response. The lack of significant differences could be attributed to the small sample size (3 to 8 cultures per treatment). Peak fresh weight increases occurred in cultures grown on 10^{9} M AS or BAP, and on 10^{-7} M 2-iP or K (Fig. 2). Dry weight was similar except for cultures grown on 2-iP, which peaked at a concentration of 10-8M. Note the high fresh weight increase in comparison to dry weight in cultures grown on 10-7M 2-iP. This result indicates a highly vacuolated callus. The calli in this treatment externally resembled the calli from mature stems in that they were nodular and white. The other calli were compact and eventually turned green. Since callus formed on the control cultures, an exogenous cytokinin is not necessary for callus initiation. However, the presence of an exogenous cytokinin generally stimulated callus proliferation (Table 5).

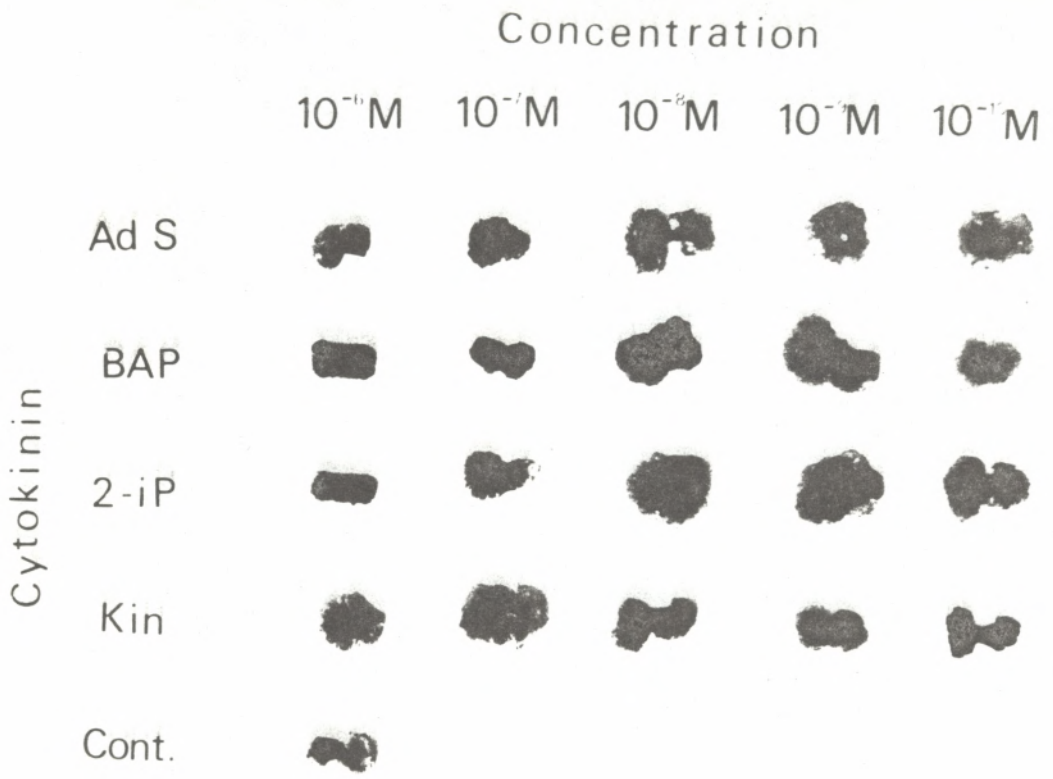

Fig. 2. Growth of epicotyl callus.

Table 5. Effect of hormone and hormone concentration on growth of epicotyl callus.*

Concentration (M.)	Adenine sulfate		BAP		2-iP		Kinetin	
	Fresh Weight (mg.)	Dry Weight (mg.)	Fresh Weight (mg.)	Dry Weight (mg.)	Fresh Weight (mg.)	Dry Weight (mg.)	Fresh Weight (mg.)	Dry Weight (mg.)
1×10^{-10}	519	54	510	44	457	40	320	37
1×10^{-9}	634	59	697	55	456	49	386	46
1×10^{-8}	535	58	614	61	798	63	431	42
1×10^{-7}	273	31	275	36	1125	47	683	58
1×10^{-6}	324	36	281	33	244	32	581	51

* The control averaged 317 mg. fresh weight and 32 mg dry weight.

Differentiation occurred within four weeks. This response occurred on all the cytokinins, but was not consistent on any of them. The differentiated tissue looked like young shoots. Longitudinal sections showed xylem initial formation. But well-developed apical meristems, vascular systems, or procambial strands did not occur (Fig. 3). This differentiation occurred most frequently in cultures grown on 10^{-8}M BAP, 10^{-9}M 2-iP, or 10^{-6}M K. Cultures grown on 10^{-8}M BAP showed the greatest frequency of shoot-like initials per culture. The most developed tissues occurred in cultures grown on 10^{-} M 2-iP (Table 6). None of these tissues developed into shoots and, upon subculture, formed callus.

Table 6. Effect of hormone and hormone concentration on differentiation.

Concentration (M.)	Number of cultures with shoot-like initials			
	Adenine sulfate	BAP	2-iP	Kinetin
1×10^{-10}	1	1	1	0
1×10^{-9}	0	1	3	0
1×10^{-8}	1	4	0	0
1×10^{-7}	0	1	0	1
1×10^{-6}	1	0	0	3

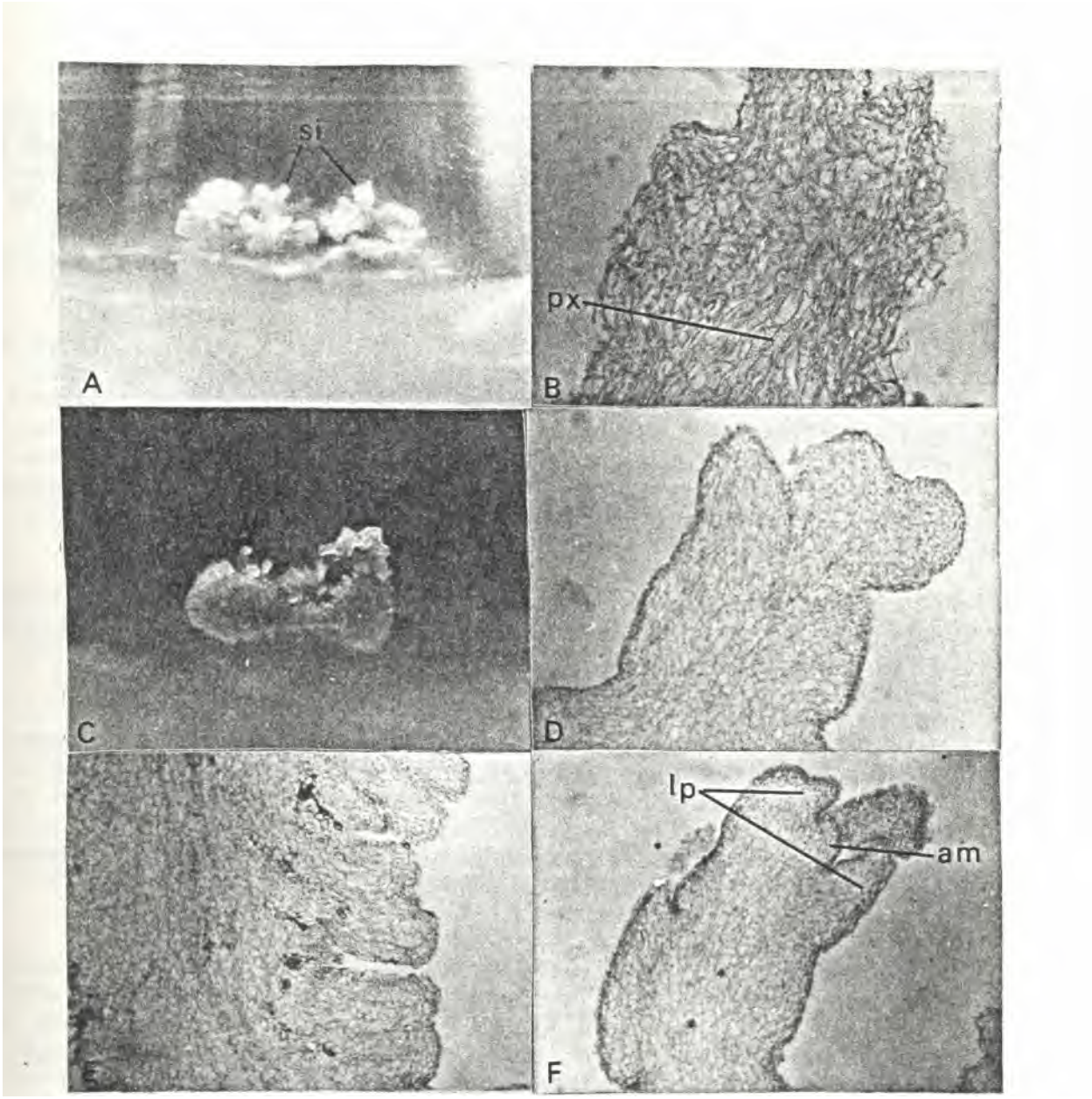

Fig. 3. Differentiation of epicotyl callus. (A) Culture grown on 10^{-9}M 2-iP showing "shoot initials" (si) (B) Longitudinal section of a "shoot initial" started on 2-iP showing primary xylem (px). (C) Culture grown on 10^{-8}M BAP showing "shoot initials". (D-E) Sections of a "shoot initial" and callus showing organized growth. (F) Section of a "shoot initial" started on 10^{-8}M adenine sulfate showing possible apical meristem (am) and leaf primordia (1p).

DISCUSSION AND CONCLUSIONS

The callus tissue of C. dentata is sensitive to exogenous growth regulators. Callus proliferation and internal differentiation can be controlled by the particular auxin which is applied and its concentration. For the purpose of in vitro plantlet formation, 2,4-D or IAA are more desirable than 2,4,5-T or NAA. Higher concentrations of auxin (1.0 mg/1 of NAA) increase callus growth and seem to stimulate differentiation.

The cytokinin used and its concentration will also affect callus growth and differentiaton. Concentrations of AS, BAP, 2-iP or K exceeding 10^{-6}M inhibit callus growth and differentiation. Optimal concentrations are 10^{-7} to 10^{-8}M. The highest frequency of differentiated tissue occurs in cultures grown on BAP or K; however, the most developed tissue occurs in cultures grown on 2-iP. Maintaining cultures for longer periods might show which of the cytokinins are most useful.

Callus from the physiologically younger epicotyls differentiated more readily than that from mature stems. This tissue will, therefore, be more useful in developing a reliable technique for plantlet production. Once the key cultural requirements are determined, the system could be tested using mature tissue to determine its usefulness for clonal propagation of blight-resistant parents. Seckinger, McCown, and Struckmeyer (1973) reported the formation of "organoids" containing procambial strands and vascular systems from cultures of red oak (Quercus rubra L.) seedlings. These "organoids" also failed to form plantlets. It is possible that many members of the Fagaceae will require these key cultural factors for in vitro plantlet formation.

These preliminary results are certainly not sufficient to categorically state that in <u>vitro</u> propagation of C. dentata can be obtained, They show that this tissue possesses the ability to differentiate in culture. Therefore, the possibility of in vitro propagation exists. Many factors such as light intensity and quality, medium pH and consistency, and the various tissue sources are yet to be tested. Research on these factors will be conducted.

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