

**CLONAL PROPAGATION OF ASPEN FROM
TISSUE CULTURE**

Project 2351

Report Ten

A Progress Report

to

PIONEERING RESEARCH COMMITTEE

October 15, 1970

THE INSTITUTE OF PAPER CHEMISTRY

Appleton, Wisconsin

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Appleton, Wisconsin

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SUMMARY

The growth of aspen stock tissues and the initiation of shoots both showed erratic variations during the year. Seasonal variations in growth rate were measured for 19 months, but definite and reoccurring patterns were not apparent. The initiation of shoots followed the general frequency of callus growth, and on slow-growing callus tissue relatively few shoots were formed which were large enough to use in rooting trials. Shoots over 1-cm. tall were excised and their bases thrust into agar medium or soil, and after several weeks in the light the shoots turned green. Relatively high frequencies of rooting were obtained with Medium 104-IBA, a supplemented defined medium with the auxin indolebutyric acid added. A method was discovered where single excised shoots proliferated on Medium BAP-.05 into 20-50 new shoots, some of which were then separated and rooted. Of the many excised shoots which rooted, four survived and are still growing. In addition, complete plants were produced for the second tree species, a tetraploid quaking aspen from Sweden.

INTRODUCTION

Last year, in Report Nine, we described the first known reproduction of a tree species from 3-year-old triploid quaking-aspen callus tissue. From among several large experiments in the fall of 1968, seven shoots rooted while still attached to the callus and four survived and grew into normal trees (1). The first tree was planted in the lawn of The Institute of Paper Chemistry on July 11, 1969, when 122-cm. tall (about 4 feet) and nine months old from the time the shoot was initiated on the callus tissue. Tree I grew several side branches during May-June, 1970, and added another 45 cm. (1.5 feet) in height (Fig. 1). The tree appears healthy and most of the stem damage from last year (probably from rodents) has callused over. Some fungus infection may have invaded the lower stem, but we hope that future rapid growth will confine the fungus and prevent an early decline in health. Over two dozen swollen buds were produced this year, which may be male flower buds. Flowering is unusual in quaking aspen on trees less than 6-7 years old.

Tree II is still in the greenhouse in a box of soil. No side branches grew this year, but the height increased from 127 to 167 cm., for a total height of 5.5 feet. Several swollen buds were also produced. This tree has been accepted for planting in the National Arboretum in Washington, D. C., by its Director, Mr. Henry T. Skinner. Mr. Arnold Grummer, of the Institute Staff, worked out the agreement and is arranging for the tree to be shipped to Washington.

Unconfirmed plans now call for placing Tree III on the Capitol grounds in Madison, Wis., and Tree IV is scheduled to be planted near a memorial to the late Professor Scott S. Pauley, School of Forestry, University of Minnesota, in Gunn Park, Grand Rapids, Minnesota. Professor Pauley was an international authority



Figure 1. Tree I, the First Tree Reproduced from Tissue Culture, was 20 Months Old and 5.5-Feet Tall in June, 1970

on aspens and was also the thesis advisor of Dr. Winton, the principal investigator of this project.

Since the growth of the original four trees from callus tissue, no other complete plants have survived, but several small trees are growing from shoots excised from callus and rooted in medium. All trees produced so far are from the same callus tissue of triploid quaking aspen Clone T-2-56. The same clone has been propagated extensively from root sprouts, but the importance of the trees from tissue culture is that differentiated tree tissue was used to grow undifferentiated callus tissue, and it was undifferentiated cells which then differentiated into vegetative shoots and eventually into trees. This reversion to the undifferentiated state may be essential for the clonal propagation of plus-trees of many commercial tree species, which cannot be propagated by normal vegetative means of rooting branch cuttings or by grafting. If so, then the problem is probably involved with the two processes of juvenility associated with vegetative growth, and adult tissue concerned with flowering, which many workers now believe are mutually incompatible. Dr. Robert Pharis, at the University of Calgary, has collected evidence that the production of gibberellin hormones cause the cessation of juvenility and shoot initiation, and starts the adult phase of plant growth concerned with reproduction by flowering. We are extremely interested in the several research programs being conducted in this field around the country, because our eventual success in developing a general method of clonal propagation for both hardwood and conifer tree species may be dependent upon the juvenility of tissue when callus is initiated.

During the past year, our studies with aspen have been mainly in the three areas of the growth of stock cultures from both old and new sources, the initiation of shoots from callus tissue, and rooting of shoots excised from callus.

NUTRIENT MEDIUM

The original Medium 1 (2), used as the basic medium throughout this study, was modified from Wolter and Skoog medium (3) by omitting ammonium nitrate, increasing inositol, decreasing the agar and the levels of nicotinic acid, thiamine, and pyridoxine, and exchanging the amounts of calcium and potassium nitrate (Table I). In tests run several years ago, neither WS medium or Medium 1 gave consistently faster growth of stock callus tissue, but the quality of firm white tissue appeared to be better on Medium 1. Stock tissue was grown on Medium 1, 100, and 104. Medium 100 is Medium 1 plus all of the groups of supplements listed in Table II at 10 mg./liter. Medium 104 is Medium 100 minus Group 4 (tryptophan), and was designated as Medium 107 in past reports. Some callus tissue was grown on Medium IAA, made by substituting 2,4-D (2,4-dichlorophenoxyacetic acid) with 10 mg./liter of indole-3-acetic acid. Other tissue was transferred to Murashige and Skoog medium (4), modified as shown in Table I.

During the past year, triploid quaking aspen callus has been cut (subcultured) into inocula, 3-mm. cubed, each month and transferred to fresh Medium 1, 100, or 104. The fresh-weight increments are reported in the next section. Also, each month, some inocula were placed on BAP media made by omitting 2,4-D and substituting 6-benzylaminopurine for kinetin in various amounts. BAP media were used for shoot initiation and will be discussed in a later section.

All nutrient media were made with water which was first deionized in a mixed-resin cartridge then distilled in a Corning glass still Model AG-1B. Media were compounded from stock solutions, the pH adjusted to 5.6-5.8 with 1% NH_4OH , and all components autoclaved for 20 minutes at 15 p.s.i. Hot media were dispensed into glass Petri dishes, at 30 ml. per dish, which were then cooled and stored

TABLE I

COMPONENTS OF MEDIA IN MG./LITER

Component	Medium 1 (1968)	Wolter & Skoog (1966)	Murashige & Skoog (1962)
MgSO ₄ ·7H ₂ O	764	764	370
Na ₂ SO ₄	425	425	--
KNO ₃	425	170	1,900
KH ₂ PO ₄	--	--	170
Ca(NO ₃) ₂ ·4H ₂ O	170	425	--
KCl	140	140	--
NaH ₂ PO ₄ ·H ₂ O	35	35	--
NH ₄ NO ₃	--	50	1,650
Fe (EDTA)	5.5	5.5	27.8
MnSO ₄ ·H ₂ O	9	9	22.3 (16.9) ^a
ZnSO ₄ ·7H ₂ O	3.2	3.2	8.6 (10.6) ^a
H ₃ BO ₃	3.2	3.2	6.2
KI	1.6	1.6	0.83
Na ₂ MoO ₄ ·2H ₂ O	--	--	0.25
CuSO ₄ ·5H ₂ O	--	--	0.025
CoCl ₂ ·6H ₂ O	--	--	0.025
Inositol	100	10	100
Nicotinic acid	0.05	0.5	0.5
Thiamine·HCl	0.01	0.1	0.1
Pyridoxine·HCl	0.01	0.1	0.1
Glycine	--	--	2 (0.0) ^a
Kinetin	1	1	0.64 or 2.56
2,4-D	0.04	0.04	--
IAA	--	--	4
Sucrose	20,000	20,000	30,000
Agar	8,000	10,000	10,000

^aThe actual amounts used are shown in parenthesis.

over the weekend to allow surface water to evaporate and contaminant spores to germinate.

TABLE II

L-AMINO ACID, VITAMIN, AND NUCLEOTIDE SUPPLEMENTS^a

<u>Group 1</u>	<u>Group 2</u>	<u>Group 4</u>
Alanine	Leucine	Tryptophan
Asparagine	Lysine	<u>Group 5</u>
Aspartic acid	Methionine	Folic acid
Citrulline	Phenylalanine	<u>Group 6</u>
Cysteine	Threonine	Adenosine-3'-(2')-phosphoric acid
Glutamic acid	Tyrosine	Cytidine-3'-(2')-phosphoric acid
Glycine	Valine	Guanosine-3'-(2')-phosphoric acid
Histidine	<u>Group 3</u>	Thymine (5-methyl uracil)
Hydroxyproline	Arginine	Uridine-3'-(2')-phosphoric acid
Isoleucine	Ornithine	
Proline		
Serine		

^aEach supplement was supplied at 10 mg./liter.

STOCK CULTURES OF TRIPLOID QUAKING ASPEN

Stock cultures of triploid quaking aspen tissue were derived from callus initiated from root sprouts of Clone T-2-56, on 1/23/67. Sterile segments 5-7 mm. long were plunged halfway into Medium 1, with the basal end up, and were left in a dark incubator at 27°F. After 5-6 weeks, cells from the cambium region of dividing cells, beneath the bark, had emerged onto the basal end of each segment and formed a firm white mass of cells which were undifferentiated individually or collectively. Callus was cut from the parental segment, subcultured monthly, and grown independently on Medium 1 for one year. Some callus was transferred to Medium 100 and 104, and grown for another year on all three media. These tissues were used for shoot initiation and tree growth, reported last year in Report Nine.

The best age of tissue for shoot initiation was established last year as four-week-old callus from the previous subculture. However, the rate of tissue growth varied among passages throughout the year. During this past year, more growth studies were run for stock cultures maintained at 4-week passages, as well as for tissue subcultured every five or six weeks.

SEASONAL GROWTH VARIATIONS

During 1967-68, monthly qualitative assessments were made of the relative rates of growth between stock cultures. In earlier tests, already reported, tissue growing on Medium 104 always had the fastest rate of growth and the best firm white tissue. The slowest growth was on Medium 1, and Medium 100 gave intermediate rates. However, the absolute increment of each culture varied throughout the season. The slowest growth for all tissues was in late winter and late summer, with the fastest growth in the fall, coincident with the best shoot initiation. Beginning in February, 1969, initial and final fresh weights were recorded for 15 inocula (among

three dishes) for each of the three stock cultures growing on Medium 1, 100, or 104. These weights were used to calculate the growth factor by the formula, $GF = \text{final} - \text{initial}/\text{initial}$. The inocula consistently ranged between 30-40 mg. each, so the growth factor is a sufficient parameter to look for general relationships between the growth increments of the three cultures.

The growth factors are plotted for each stock tissue in Fig. 2, for constant four-week passages during the 19 months of record. The two major peaks for Tissue 104 are separated by nine months, whereas lower peaks for Tissue 100 occur every 2-4 months. With a few exceptions, Tissue 1 generally produced less increment than the other two tissues. In a few cases, callus pieces of Tissue 1 were larger after four weeks, but Tissues 100 and 104 usually were significantly larger at the end of 5-6 weeks. For the seven months where data from two years are available, the growth factors are superimposed in Fig. 3 for Tissue 100 and 104. For Tissue 104, correlation appears poor during the late winter months, but better in summer. Correlation may be better for Tissue 100, but both tissues have peaked during summer for the past two years, which is the opposite of 1967-68 growth observations.

OPTIMUM DURATION OF PASSAGES

The total fresh-weight growth increment was measured two ways for passages longer than four weeks in duration. The first way was to return the inocula of the regular, monthly stock cultures to their dishes after the four-week weighing, and weigh them again after five or six weeks. The second method was to start separate cultures of Tissue 104, subculturing every five weeks for one culture and every six weeks for the other.

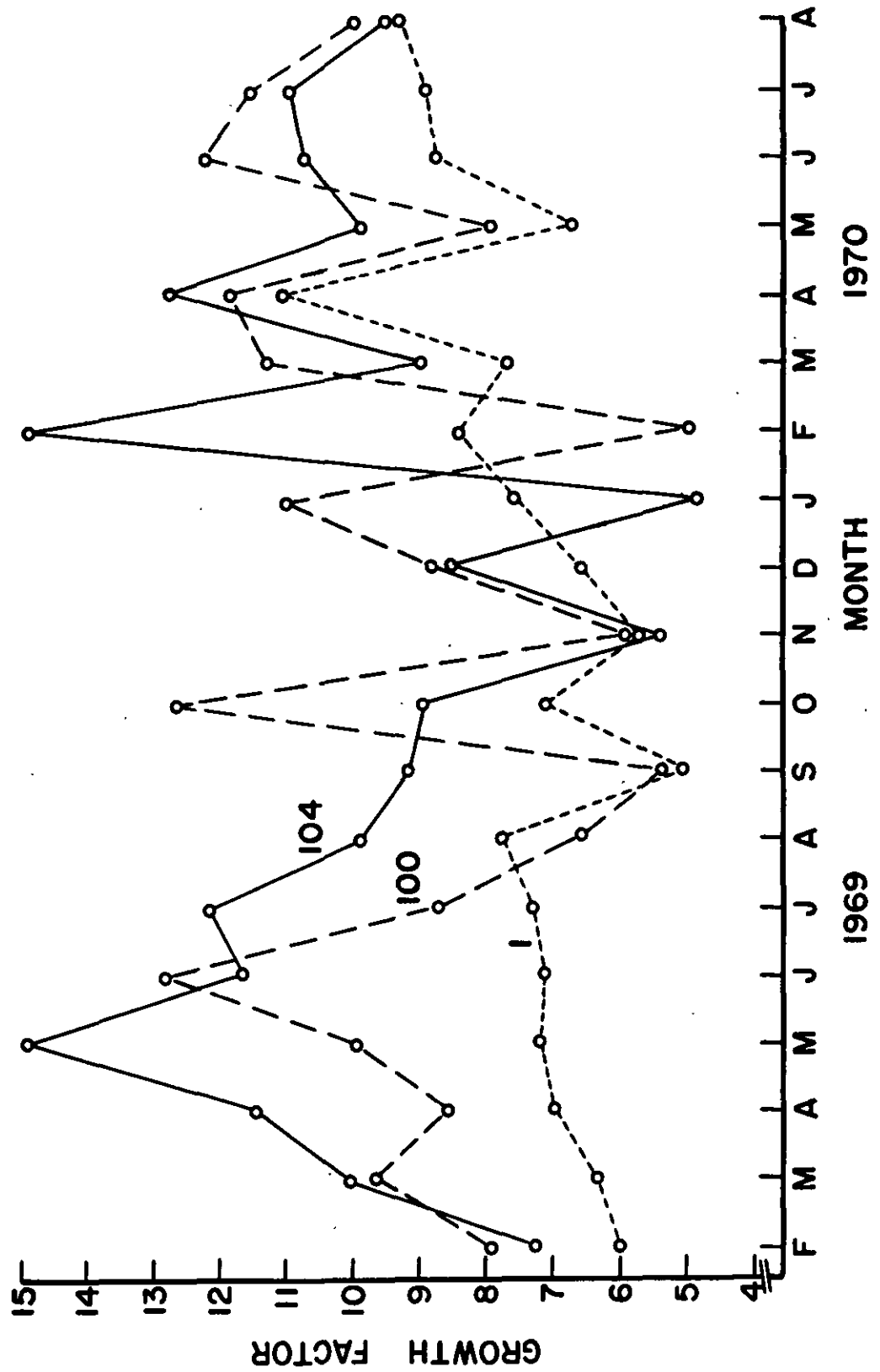


Figure 2. Growth Factors for Stock Tissues on Medium 1, 100, and 104 for Fresh-Weight Increments at the End of Four-Week Passages

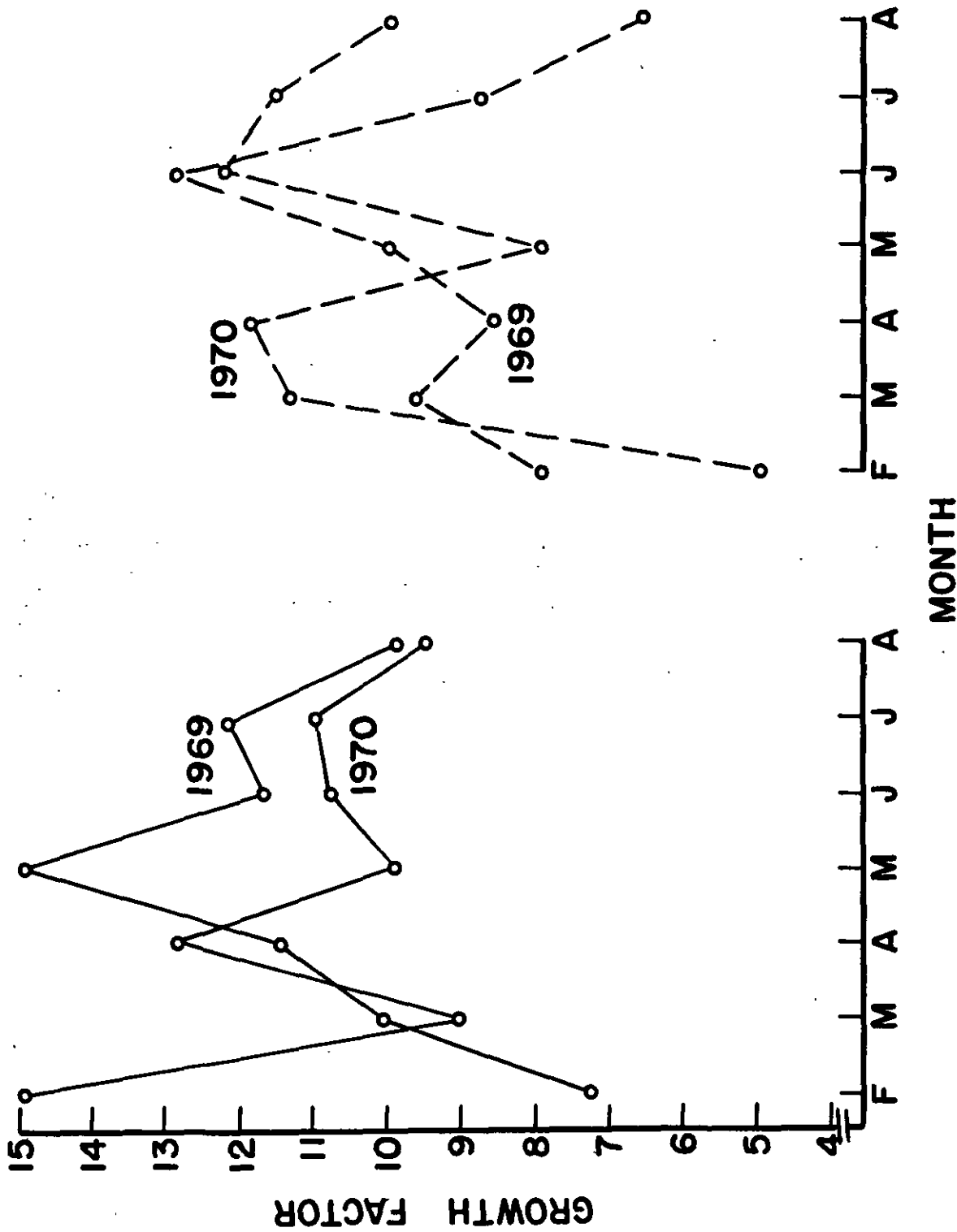


Figure 3. Growth Factors for the Same Months in Different Years are Superimposed for Tissue 104 (Left) and Tissue 100 (Right)

The first type of measurement is summarized in Table III for three studies during the past year. The five- and six-week growth factors show the additional increment of stock tissue grown during the one or two weeks past their normal passage time of four weeks. The four-week growth factors are plotted in the grafts of Fig. 2 and 3. For July, 1969, the four-week GF values were widely separated for the three tissues, and the additional two weeks of growth more than doubled the GF for Tissues 100 and 104, but not for Tissue 1. For November, the GF values were all nearly the same, but were fairly low amounts, and the additional one week of growth did not appreciably increase the GF for any tissue. On the other hand, all tissues had a rather high four-week GF for August, 1970, and the additional one week of growth increased the GF by one-third for Tissues 1 and 104, and almost doubled for Tissue 104.

TABLE III

GROWTH FACTORS FOR TRIPLOID QUAKING ASPEN TISSUE^a ON THREE MEDIA
AFTER PASSAGES OF FOUR, FIVE, AND SIX WEEKS

Medium	4 Weeks	5 Weeks	6 Weeks
July, 1969			
1	7.7		8.2
100	6.6		18.2
104	9.8		19.3
November, 1969			
1	5.7	8.8	
100	5.7	8.4	
104	5.4	10.0	
August, 1970			
1	9.4	12.9	
100	9.9	19.6	
104	9.5	13.4	

^aThis was stock tissue subcultured every four weeks, whose growth factors are reported in Fig. 2 and 3. In 1969, tissue was returned to dishes after the 4-week weights were taken and the same pieces weighed again after an additional one or two weeks. In 1970, 10 pieces from each medium were weighed after four weeks and five pieces were left undisturbed for five weeks.

One dish, with five inocula of each tissue, is shown in Fig. 4-6 after four, six, and eight weeks of growth, respectively, in July, 1969. In this case, growth was unusually slow after four weeks, and the additional two weeks (for a 6-week passage) brought the size of callus pieces up to about normal for each tissue during 4-week passages of rapid growth. After eight weeks of growth, the callus on all media was turning brown, was of poor quality, and was discarded.

In the second type of measurement for passage duration, some Tissue 104 was subcultured every five weeks beginning in April, and every six weeks starting in February, 1970. In August, initial and final wet weights were taken and the fresh-weight increment calculated by difference, for 15 inocula for each passage duration. The results are shown in Table IV.

TABLE IV

MEASUREMENTS OF TISSUE 104 FOR ONE PASSAGE, AFTER SEVERAL
CONSECUTIVE PASSAGES OF FIVE OR SIX WEEKS

Passage, weeks	Initial Wt., mg.	Increment, ^a mg.	Growth Factor
5	39.3 ± 4.9	727.0 ± 122.0	18.5
6	38.4 ± 6.8	729.6 ± 118.7	19.0

^aFresh-weight increment = final wet weight - initial wet weight.
Variations are shown as one standard deviation above and below
the mean.

On the basis of information already given in this report, it is evident that the same tissue does not grow at the same rate from month to month; neither do the three tissues of the same aspen clone always grow in the same relative amounts from month to month. The media are chemically defined and the composition of each has remained constant during this study, but seasonal variations are still

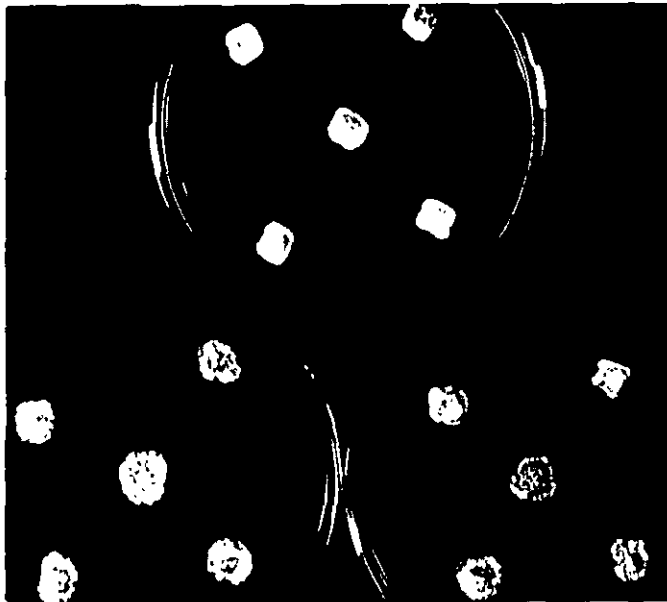


Figure 4. Stock Tissue on Medium 1 (Top), Medium 100 (Left), and Medium 10⁴ (Right) After the Regular Four-Week Passage of July, 1969



Figure 5. The Same Callus Inocula After an Additional Two Weeks in the Dark, Showing Optimal Growth and Quality of Firm White Tissue

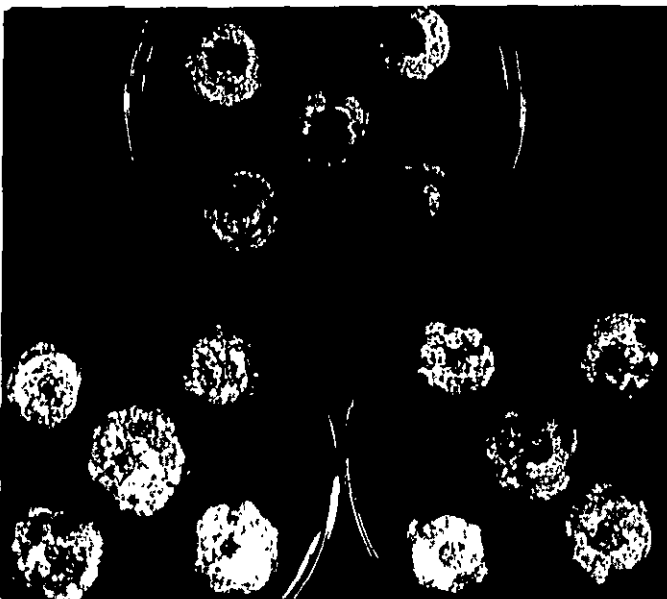


Figure 6. The Same Inocula at the End of Eight Weeks, Showing Rapid Degeneration of Tissue

observed in growth. The explanation could be due either to changes in the environment, or to truly endogenous or internal changes. We have tried to minimize external changes in pH, air moisture, temperature, and exposure to light during handling, but we do not have control over all these factors. On the other hand, the quantitative data for the past 19 months show growth peaks for most tissues in the spring and winter, whereas earlier, subjective observations seemed to show a major growth peak in the fall. The apparent changing pattern of the growth cycle from year to year favors an endogenous cause, but does not eliminate interaction or confounding independent actions of both internal and external causes. Because of the apparent relationship between the high rate of growth of firm white callus tissue of stock cultures and the subsequent ease of shoot initiation from inocula, this changing growth pattern remains an unresolved question in our main objective of efficient clonal propagation of aspen species from tissue cultures. Time considerations, however, have not allowed too high a priority to be placed on this problem, and we hope that continued, but abridged, experiments will shed new light on the answer.

We now feel that we can allow stock cultures to grow as many weeks as are necessary, such as five or six weeks, in order to attain an optimum size and tissue quality before subculturing. New callus cultures established last year will be maintained in this manner. At the same time, we plan to maintain our present stock tissues on a four-week passage, to further study the seasonal variation of growth.

OVEN-DRIED WEIGHT OF STOCK TISSUE 104

Past measurements have shown that firm white callus tissue, on any of the three stock media, generally ranged from 7-8% oven-dried weight. Soft and friable callus generally varies from 1-2% oven-dried weight. Oven-dried weights were again measured for stock Tissue 104, at the end of its normal four-week

passage in August, 1970, for five of the ten pieces which were weighed at the end of four weeks. The value was $8.5 \pm 0.9\%$ oven-dried weight, of the fresh-weight increment of 234.1 ± 57.5 mg. The average inoculum fresh weight was 32.7 ± 3.3 mg.

The fresh-weight growth factor of the five pieces in one dish, used for oven-dried weights, was 7.2 compared with 9.5 calculated for the ten pieces used to measure the growth increment. In order to test for mean differences between callus on the three dishes, the growth factor was calculated separately for the five pieces (one dish) at 11.9, or half again as large as the average GF of the ten pieces (two dishes). The five inocula averaged 31.7 ± 2.7 mg. initially, and the fresh-weight increment was 376.7 ± 34.9 , pointing up the occurrence of growth variations among pieces in dishes of the same culture. Inocula in the past have generally been more uniform than this, but this anomaly is the reason we use 15 inocula among three dishes as a basis of growth measurements.

GROWTH ON IAA MEDIA

Some of each of the three stock tissues were subcultured to Murashige and Skoog media MS-1 and MS-2. Both contained 4 mg./liter IAA, but the first had 0.64 and the second had 2.56 mg./liter kinetin. After four weeks, Tissue 104 had grown better on both of the MS media, Tissue 1 the poorest, and Tissue 100 intermediate. Generally, growth was better on MS-1 than on MS-2, so only tissue on MS-1 was subcultured to fresh medium. In addition, each stock tissue was grown on Medium IAA-10, made by substituting 0.04 mg./liter 2,4-D for 10 mg./liter IAA in Medium 1. Figure 7 shows one of three plates per tissue for each of the Media MS-1 and MS-2, along with one dish of each stock tissue on Media 1, 100, or 104. Figure 8 shows four inocula per treatment of each stock tissue grown for five weeks on MS-1, IAA-10, or stock Media 1, 100, or 104.

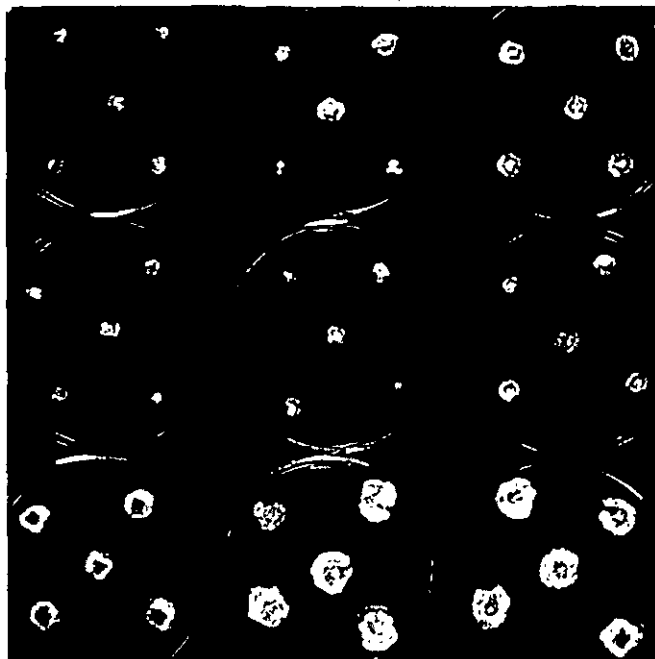


Figure 7. Stock Tissues (Left to Right) 1, 100, and 10^4 Grown on Media (Top to Bottom) MS-1, IAA-10, and Stock Medium for Each Tissue in the Bottom Row. Inocula Were Cut from Four-Week-Old Tissue

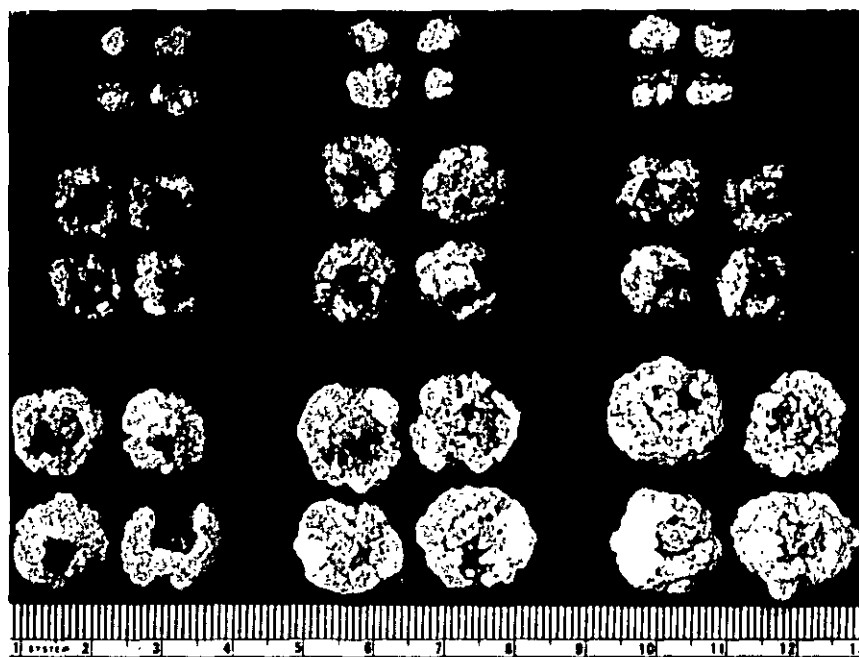


Figure 8. Aspen Stock Tissue Grown for Five Weeks in the Dark on Medium MS-1 (Top), IAA-10 (Middle), and Stock Media (Bottom). Stock Tissue (Left to Right) 1, 100, and 10^4

In the MS media, the lower level of kinetin was more beneficial than the high level, but growth was still extremely slow on MS-1. Stock Tissue 100 appeared to grow better than Tissue 104, and both grew larger with more white tissue than Tissue 1. Inocula on Medium IAA-10 grew intermediate in size between tissue on MS-1 and their stock medium, again with the best response apparently with stock Tissue 100. The stock tissues, themselves, show the usual relative growth rates of firm white callus, with the fastest growth on Medium 104, then 100, and both better than on Medium 1.

Tissues are being maintained on IAA media, even though the growth is slower and the quality less than on 2,4-D stock media. We hope to initiate shoots on IAA-grown stock tissue easier than from tissue grown on Medium 1, 100, or 104 containing 2,4-D. We, and other workers, have some evidence that 2,4-D may inhibit shoot initiation for several months from callus initiation, or for at least several months out of the year. IAA apparently is not inhibitory to early shoot initiation.

NEW CALLUS INITIATION

TRIPLOID ASPEN

About a dozen naturally occurring clones of triploid quaking aspen have been located in the Lake States region, and most have been reproduced vegetatively and are growing near Appleton in the Institute nursery at Greenville. Callus was initiated from four clones in 1968 (Report Eight, page 56), but the only tissue of interest was isolated from Clone T-38-59, located near Trout Creek, Michigan. Callus from this clone grew faster than from our standard Clone T-2-56, also located in the Upper Peninsula near Bruce Crossing.

Last fall, roots from six triploid clones were collected from locally grown ramets and planted in the greenhouse. Root sprouts were produced and, in February, stems 8-12 inches tall were excised two inches above the soil, and callus was initiated by methods described earlier in this report for the stock cultures. After five weeks on Medium 1 in the dark, firm white callus, on horizontal segments of T-38-59, far outgrew callus from any other clone. The next best firm white tissue was on T-2-56, although faster-growing friable callus grew on T-9-59, another clone from Bruce Crossing. The best segments from each clone are seen in Fig. 9.



Figure 9. Callus Tissue Initiated on Stem Segments of Triploid Quaking Aspen Root Sprouts, After Five Weeks in the Dark on Medium 1. Clones from Left to Right:
T-1-65 T-9-59
T-1-62 T-38-59
T-2-56 T-71-59

Figure 10 shows two pieces of tissue from each clone, at the end of the second monthly passage after the callus was isolated from the stem segments. Again, the best firm white tissue was from Clone T-38-59 (Fig. 10-5); and callus from T-2-56 (Fig. 10-3) was one of the poorer tissues.

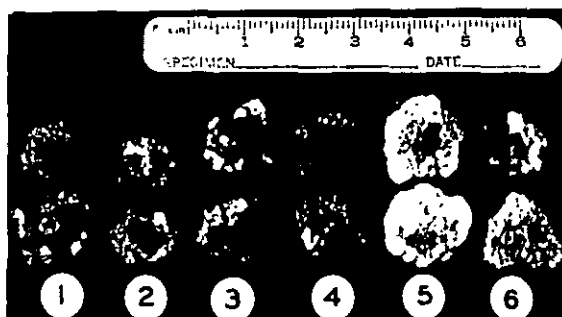


Figure 10. Callus Tissue at the End of the Second Passage in the Dark, on Medium 1, from Triploid Quaking Aspen Clones (Left to Right) T-1-65, T-1-62, T-2-56, T-9-59, T-38-59, and T-71-59

Figure 11 shows callus tissue of Clone T-38-59 only, at the end of the second passage. The top dishes contain callus initiated and grown on the three regular stock media, and the bottom dishes show tissue initiated on Medium 1, then transferred to each of the three stock media when the callus was isolated from the segments. This experiment showed that callus was initiated equally well on Medium 1 and 100, then did best if transferred to either Medium 100 or 10⁴. Callus from Clone T-2-56 usually is initiated on Medium 1, then must be transferred to Medium 10⁴ for continued firm-white tissue growth.

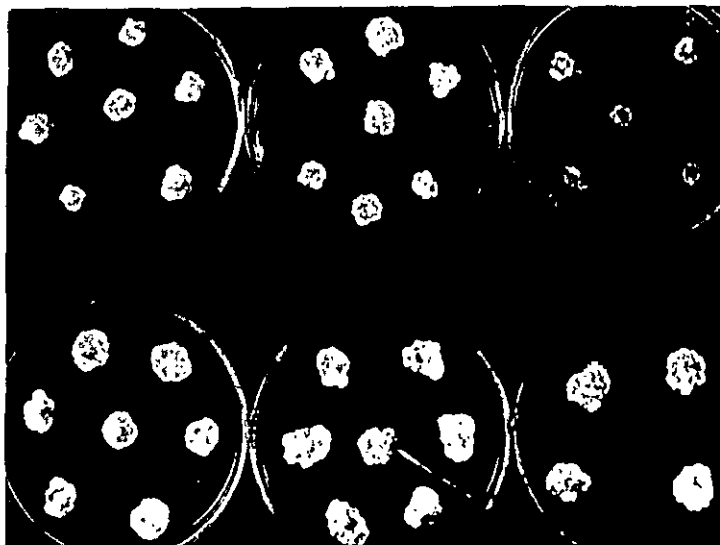


Figure 11. Callus from Clone T-38-59 After Two Passages from Isolation. Callus on Top was Initiated and Subcultured to the Same Medium (Left to Right) 1, 100, and 10⁴, and Callus on the Bottom was Initiated on Medium 1 then Subcultured to (Left to Right) Medium 1, 100, and 10⁴

Stock cultures are now being proliferated, of both Clones T-2-56 and T-38-59, from callus initiated this past winter and spring on Medium 1 or 104. Callus T-38-56 is also growing on Medium IAA-10, after being initiated and isolated on Medium IAA. This is Medium 1 made by substituting 2,4-D with either 1 or 10 mg./liter of indoleacetic acid. Callus on Medium IAA-10 is more yellow than the white tissue on Medium 1 or 104, but both tissues are firm and we hope to induce shoots on callus grown on Medium IAA-10 much sooner than on tissue growing on a medium containing 2,4-D.

TALL HYBRID ASPEN

In the spring of 1969, a number of hybrid crosses were made by Dr. Einspahr and the seed planted in the nearby Greenville Nursery. In the fall, the tallest plant in each seed lot was tagged, and the top 18 inches was used on November 12 to initiate callus tissue for clonal propagation. In order to use fewer, but meaningful seedlings, only tall hybrids were harvested having Populus canescens Clone Ca-2 as the female parent or those with the Swedish tetraploid ($4n$) P. tremula as the male parent. The 14 hybrids are described in Table V.

For each hybrid seedling, sterilized stem segments were distributed among four Petri dishes, 20-mm. deep, at five segments per dish containing Medium C-.04, made by adding 15% deproteinized coconut milk (heating to 60°C., cooling, and filtering) to Medium 1. Two dishes each were placed in the dark and two were left under 300 ft.-c. of light, all at 27°C. After several weeks, firm or soft callus was subcultured to Medium C-.05 or to Medium 104, and some of the faster-growing tissue was transferred to shoot-initiation BAP medium. The best tissue grew in the dark, and all callus was subcultured.

TABLE V

TALL ASPEN HYBRIDS USED FOR CALLUS CULTURES

TH ^a No.	Female Parent	Cross	Male Parent	Height, feet		Total No. Seedlings
				Tallest Hybrid	Av.	
1	Ca-2 (2n)	XCa-T-27-67	T-44-60	4.6	2.3	126
2		XCa-T-28-67	T-46-60	2.9	2.2	131
3		XCa-G-38-67	G-38-67	6.2	2.1	412
4		XCa-G-1-69	G-1-58	6.3	2.6	252
5		XCa-G-2-69	G-1-57	5.9	2.7	607
6		XCa-T-4-69	T-6-67	5.3	2.8	599
7		XCa-TA-6-69 ^b	Ta-10	5.2	2.1	484
7	Ca-2	XCa-Ta-6-69 ^b	Ta-10 (4n)	5.2	2.1	484
8	T-Clone 5	XT-Ta-7-69		5.2	2.0	167
9	T-16-56	XT-Ta-8-69		5.7	1.9	292
10	XT-22-56S-2	XT-Ta-9-69		5.0	2.1	424
11	T-1-58	XT-Ta-10-69		4.9	1.7	557
12	Ta-6-68	XTa-11-69		6.6	2.1	389
13		XG-25-68		4.3	1.8	290
14		XCa-2		5.8	--	--

^aTH = tall hybrid.

^bSame seedling.

At the end of the second passage after isolation, photographs were taken of the best callus of each culture on Medium C-.05 and 10⁴. Differences in growth rates are apparent, as are variations in tissue quality, ranging from firm white to friable tan. All tissues are still in culture, but are all on Medium 10⁴ in passage 10-11. All callus is firm white, and callus from several seedlings has been tested for shoot initiation. Only one hybrid had produced one shoot after eight months in culture.

TH 11, the tallest seedling of Cross XT-Ta-10-69, had exceptionally large pubescent leaves, up to one foot long, and had phenotypic characteristics different from other plants in the same bed. It may be a mutation, or more likely a volunteer seedling blown into the seed bed from a chance wild cross.

INDUCED-PLOIDY SEEDLINGS

In connection with another project (Project 1800, Aspen Tree Improvement Program), haploid, polyploid, aneuploid, and mixaploid seedlings (5) have been produced during the past several years in order to add improved variability to the aspen breeding program. Several of the most promising plants have been cultured for clonal propagation from tissue culture on Medium 1 and C-.04. These cultures are being maintained on Medium 104, along with cultures from the tall hybrids, and in cases where extra callus was available after subculturing, some inocula were placed on shoot-initiation Medium BAP-.10. After 9-10 passages from isolation, none of the tissue has produced shoots. The exception is for the Swedish tetraploid Ta-10, which has produced numerous shoots and two complete plants on callus tissue in the light on Medium BAP-.10. The two tallest plants are about 2-cm. tall, and will probably grow into trees and be the second species of aspen to be reproduced from tissue culture.

A fast-growing tetraploid quaking aspen was produced two years ago, probably from the fertilization of normal quaking aspen female catkins with unreduced pollen from a natural triploid quaking aspen (T-2-56) pollen parent (6). Tree 201 (Cross XT-33-68) is being forced into flowering, and one small side branch was also cultured. Callus is now in its fifteenth passage on Medium 104.

SHOOT INITIATION FROM CALLUS TISSUE

TRIPLOID ASPEN

From August, 1969 to March, 1970, eight shoot-initiation experiments were conducted, with triploid quaking aspen callus subcultured from Clone T-2-56 on January 23, 1967. Tissue was from the same stock cultures discussed earlier in this report, and the purpose was to increase the production of shoots for clonal propagation of aspen. The stock tissue was grown on Medium 1, 100, or 104 for different periods of time from subculture, but the shoot-initiation medium was always Medium without 2,4-D and kinetin, and only the amount of BAP (6-benzylaminopurine) varied. All shoots were initiated in the dark at 27°C., and then were placed in 300 ft.-c. of light on Medium 100 to induce chlorophyll production. Rooting will be discussed separately in a later section.

Tests BAP I and II were two repetitions of the same experiment run concurrently. Six tissues (with different histories) were placed on two concentrations of BAP, and the number of pieces with shoots were counted after 3, 4, and 5 weeks. The percentage of rooting and the six tissues are described in Table VI, for 20 inocula for each treatment, distributed among two dishes for each of the two levels of BAP

The results of this first test were consistent with tests of the past two years, in that more inocula produce shoots with increasing levels of BAP (1, 7). However, the disappointing fact was that only a few vigorous shoots were initiated on Medium BAP-.05, and then only on 4-week-old stock Tissue 104 in the first replication. One explanation for this poor performance could have been that the increase of the tissue had altered the metabolic response to BAP, and perhaps a higher level of BAP was necessary. This would be expected if the tissue was making some of its own auxin.

TABLE VI
THE PERCENTAGE OF ROOTING^a OF SIX TISSUES ON TWO BAP-MEDIA
AFTER THREE, FOUR, AND FIVE WEEKS

Weeks	Tissue ^b	REP I		REP II	
		BAP-.05 ^c	BAP-.15 ^c	BAP-.05	BAP-.15
3	1	20	100	10	70
	2	--	40	--	--
	3	--	--	--	--
	4	--	10	--	--
	5	--	10	--	--
	6	--	44	--	50
4	1	30	100	40	90
	2	--	70	10	10
	3	--	--	--	10
	4	--	10	--	50
	5	--	60	--	30
	6	--	100	20	70
5	1	30	100	40	100
	2	--	90	10	40
	3	--	40	10	20
	4	--	50	--	70
	5	--	60	--	70
	6	--	100	20	80

^aThe number of inocula with one or more shoots, divided by the total number of inocula x 100.

^bTissue 1 = 104 stock 4-weeks old 4 = 1 stock 4 weeks old
 2 = 104 stock 6-weeks old 5 = 1 stock 8 weeks old
 3 = 104 stock 8-weeks old 6 = 100 stock 4-weeks old.

^cBAP media contained 0.05 and 0.15 mg./liter of BAP, respectively.

In Test BAP III, Tissues 1 and 104 were placed on Media BAP-.05, BAP-.10, BAP-.15, or BAP-.20, on three dishes for each BAP medium. After five weeks, the most vigorous shoots were on Medium BAP-.10 for both tissues (Table VII), but the most shoots per inoculum were on BAP-.15 for Tissue 104. Shoot production was poor on both BAP-.05 and BAP-.20. In 1968, vigorous shoots were on BAP-.05 and the most number of (but stunted) shoots were on BAP-.15, so evidently the requirement for BAP has increased with age of the tissue.

TABLE VII
PERCENTAGE OF SHOOTS ON TWO TISSUES

Tissue Source	BAP, mg./liter			
	0.05	0.10	0.15	0.20
1	0	53	20	0
104	33	20	80	60

In Test BAP IV, started October 6, up to 140 inocula of each of the three tissues were put on Medium BAP-.15. After five weeks, pieces with shoots were 21, 88, and 95%, respectively, for Tissues 1, 100, and 104. The following month, in Test BAP V, 111 out of 150 inocula of Tissue 104 had shoots on Medium BAP-.10, for a percentage of 74%. An average of 4.3 shoots per inoculum were initiated on those pieces with shoots.

In Test BAP VI, the three stock tissues were placed on Mediums BAP-.05 and BAP-.10, as well as on BAP-.05 with 10 mg./liter of guanosine-3'-(2')-phosphoric acid added, to make GBAP-.05. In addition to stock Tissues 1, 100, and 104, firm white tissue 1, still on Medium BAP-.15 from Test BAP IV, was also placed on the three BAP media. The percentage of rooting after five weeks is shown in Table VIII.

TABLE VIII
PERCENTAGE OF SHOOT INITIATION IN TEST BAP VI

Tissue Source	Medium			
	BAP-.05	GBAP-.05	BAP-.10	BAP-.15
1 ^a	80	16	0	100
1	20	15	0	0
100	40	70	95	0
104	60	80	100	73

^aFrom Test BAP IV, Medium BAP-.15.

For Tissue 1 from both sources, the addition of guanylic acid apparently inhibited shoot initiation at the same level of 0.05 mg./liter BAP. In the tests conducted last year, guanylic acid was added only to the stock medium and not to the shoot-initiation medium, and shoot production was enhanced. This year, the addition of guanylic acid to the BAP medium only, increased shoot production for Tissues 100 and 104, but not as much as an increase in the level of BAP from 0.05 to 0.10 mg./liter. As in most tests of this series, the best compromise between the number of shoots produced, and their vigor, appears to be Medium BAP-.10 for both Tissues 100 and 104. Shoots produced on Tissue 1 were almost always too small for subsequent rooting experiments.

The two last tests of this series were put out in early 1970, but with tissue only from Stock 104 on Medium BAP-.10. For Test BAP VII, 34 inocula produced 63 shoots from among 150 pieces, for 23% shoot initiation. But there were only 4 vigorous shoots among three inocula. The shoots started appearing later than usual, with three poor ones after three weeks. Also, many of the inocula were necrotic, instead of the usual nongrowing but firm white tissue.

For Test BAP VIII, 40 inocula from Tissue 104 were placed on both Medium BAP-.05 and BAP-.10. After five weeks in the dark, 7 of 33 pieces had shoots (21%) on BAP-.05 and 11 of 26 had shoots on BAP-.10 (42%). In the first case, only one piece had two shoots and the rest had one each. On Medium BAP-.10, there was an average of 1.5 shoots per inoculum for those which had shoots. The shoots from Tissue 104 were of less height and vigor than shoots from concomitant studies with three tissues on BAP-.10, so this series of tests were abandoned.

Starting in December, monthly shoot-initiation tests were put out at the same time that the stock tissues were subcultured, and 15 inocula from each tissue

were placed on Medium BAP-.10. Figure 12 shows the percentage of shoot initiation for the three tissues for the subsequent nine months. Figures 13, 14, and 15 show individual shoot initiation data for Tissues 104, 100, and 1, respectively, along with the growth factors plotted for each tissue for the months represented. The growth factors are from Fig. 2, and in several cases appear to be positively correlated with shoot initiation during the same month.

As stated earlier, in 1967-68 tissue on Medium 104 always seemed to significantly outgrow tissue on Medium 100, and both were much better than tissue on Medium 1. However, Fig. 2 shows that at certain times during the years 1969-70, Tissue 100 grew faster than Tissue 104. During the period of December to August (Fig. 12), shoot initiation from Tissue 104 paralleled, but never exceeded, that of Tissue 100, except for the first month of December. This close similarity indicates a constant difference between the two tissues as far as shoot initiation is concerned and is reasonable because the only difference between the media of the two tissues is the addition of tryptophan to Medium 100 and not to 104. A similar pattern of shoot initiation is also seen in the data for Tissue 1, where the much lower incidence of shoot initiation is consistent with past performance of this tissue.

On the basis of the data presented, one or more factors may be involved in our three stock tissues, not only to cause variations in growth rate during the year, but also to cause seasonal variations in shoot initiation. The supplemented Media 100 and 104 stimulate a more rapid growth of firm white tissue than on Medium 1 alone, and inocula from Tissues 100 and 104 also produce significantly more shoot on BAP medium than does Tissue 1.

The factors responsible for seasonal variation have not yet been identified nor have the apparently independent factors which govern the relative growth and sh

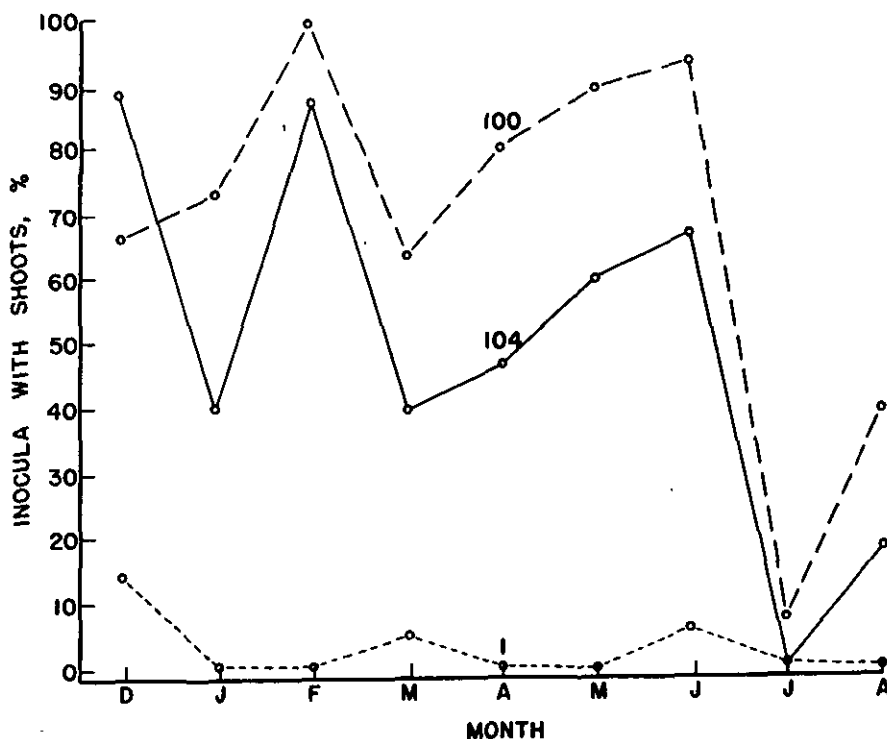


Figure 12. Percentage of Inocula with Shoots, at the End of Five Weeks in the Dark, for Tissue Placed on Medium BAP-.10 After Each Monthly Subculture. The Last Two Points for Each Tissue Were for Shoot Initiation on Medium BAP-1.0 Instead of BAP-.10

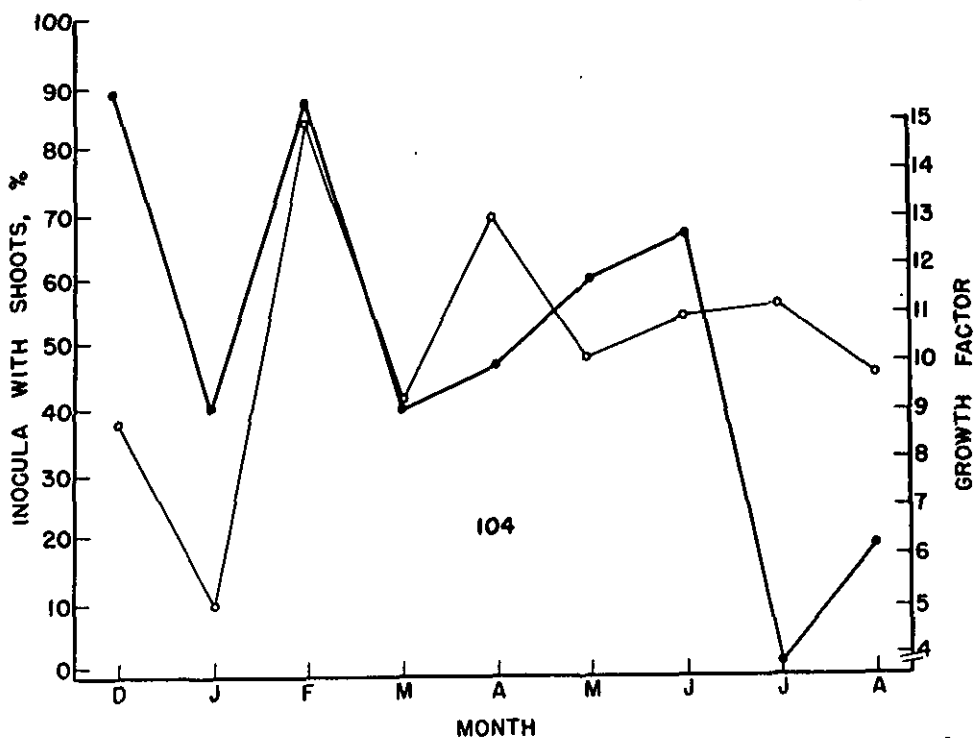


Figure 13. Percentage of Shoot Initiation (Bold Face) from Tissue 104, Compared with the Growth Factor (Light Face) for Each Month

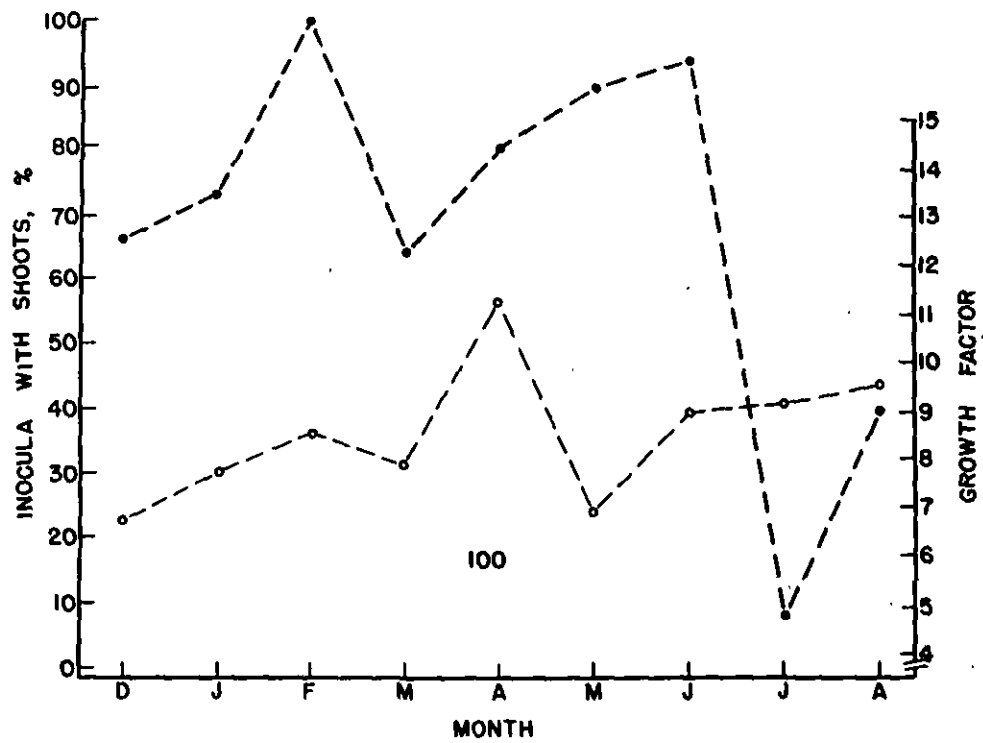


Figure 14. Percentage of Shoot Initiation from Tissue 100, Compared with the Growth Factor for Each Month

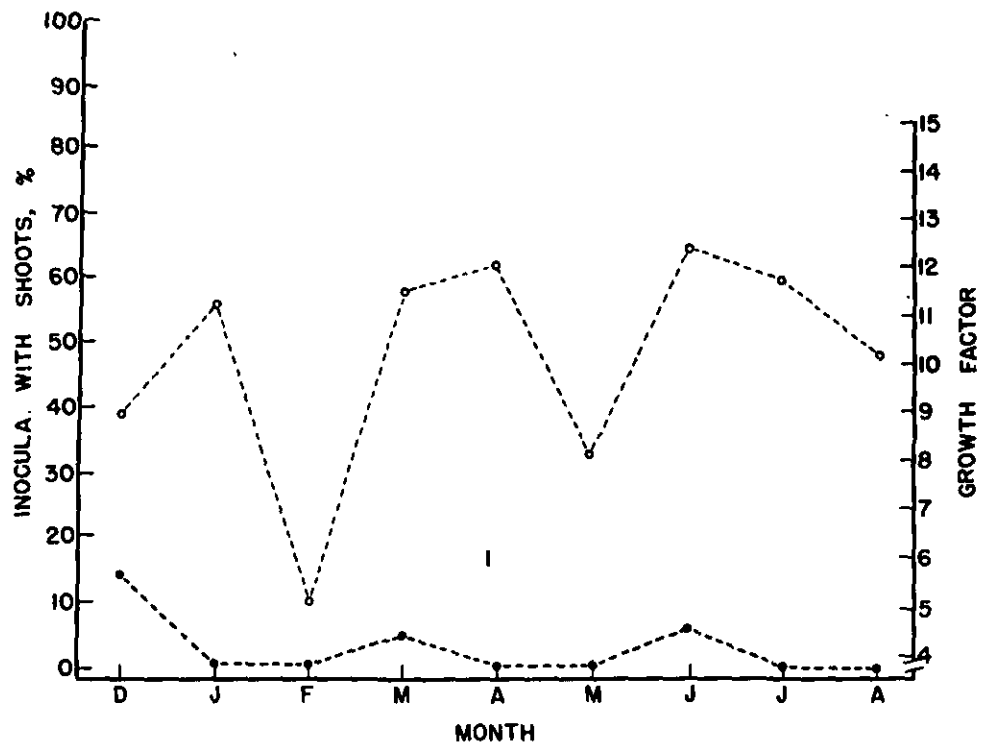


Figure 15. Percentage of Shoot Initiation from Tissue 1, Compared with the Growth Factor for Each Month

production of Tissues 100 and 104. If more time were available, perhaps intensive studies might show that the tryptophan difference between Medium 100 and 104 is related to an endogenous (internal) rhythm of the tissues themselves to produce their own natural auxin IAA. Tryptophan is one in a series of precursors of the auxin IAA, and IAA can act as a growth stimulator, or inhibitor, depending upon the concentration. From an optimum concentration, either increasing or decreasing the level would be inhibiting. This is a problem which could be handled nicely by a student, and will be proposed as an A-291 research problem for the coming academic year.

The sudden decrease in shoot initiation, shown in Fig. 12 for both Tissue 100 and 104 in July and August, 1970, was probably caused by an unintentional tenfold increase in the amount of BAP added to the shoot-initiation medium. A new stock solution of BAP was made in June for another series of tests, and we now know that this stronger solution was used to make BAP medium in August, and was also probably used in July, when the sudden decrease occurred. Thus, Medium BAP-.10 actually was BAP-1.0 for these two months. One interesting aspect of this error was the apparent adaptation of both Tissue 100 and 104 to the high concentration of BAP, as shown by their increased shoot initiation during the second passage on high BAP. When first placed on Medium BAP-1.0, Tissue 104 was completely inhibited to form shoots. Tissue 100, however, still produced one shoot on one of the 15 inocula (7%). This might be explained if the tryptophan in Medium 100 produced a higher level of IAA than in Tissue 104, and the higher level of BAP overcame the high IAA level in Tissue 100 and produced a shoot, but was too high a concentration in Tissue 104, without tryptophan, and inhibited shoot production. At this point we do not know, but careful elucidation of this problem could possibly establish a family of ratio requirements for auxin and cytokinin in aspen tissue for shoot productions under a

variety of conditions. A rapid bioassay for auxin would also permit the addition of the optimum amount of cytokinin, for maximum production of vigorous shoots, for efficient clonal propagation.

OTHER ASPEN SPECIES

Callus was initiated from the tall hybrids in November, 1969, and in some cases there was sufficient callus initiated to place a few inocula on BAP medium when the tissue was isolated from the parent stem segment. For most of the hybrids, however, shoot-initiation tests did not begin until 2-4 passages later.

For TH 14, the self-pollinated female parent (Table V), callus was initiated in both light and dark. In 300 ft.-c. of light, the callus was green on both Medium C.04 and 100 and was isolated and grown independently on both media. No shoots developed on either media, but large red roots grew from tissue on Medium 1 after three weeks in the light. This callus was subcultured and placed on BAP-.10 in the light, and two green shoots developed within a month. Unfortunately, the roots dried and the shoots did not elongate, and these remain the only shoots produced from any of our aspen callus in the light* The points of interest are that shoots did develop in the light, and also during only the second passage after isolation from the callus.

*Except the tetraploid.

PLANTLET FORMATION

European aspen Clone Ca-2 produced a vigorous shoot on firm white callus in 1966, during the first passage from isolation on BAP-.05. The callus piece also grew a long root which had no apparent attachment to the shoot, and the shoot elongated to 3-4 cm. before it died (Fig. 16). At the same time, the same type of "plantlets" were produced from callus of black cottonwood during the first passage after isolation. But none of these survived and grew into trees.

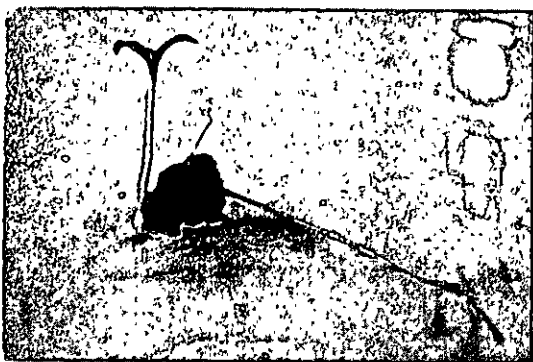


Figure 16. Callus from European Aspen Ca-2, in 1966, with Shoot and Roots. This Shoot Later Died Before Forming a Tree. There was no Visible Connection Between Roots and Shoot

One shoot also grew from the callus of the tall hybrid TH 6, a cross between the European aspen Ca-2 and quaking aspen T-6-67. During the seventh passage from isolation, callus was placed on Medium BAP-.05 in the dark and remains the only tall hybrid (other than the Ca-2) to produce a shoot. This shoot appeared 11 months after callus was initiated, and was excised from the callus on 9-18-70 and placed in semi-sterilized soil in the greenhouse. It is still alive but has not yet rooted.

COMPLETE-PLANT PRODUCTION

A complete plant, as opposed to the erroneous use of the term "plantlet," is a shoot which has rooted directly while still attached to the callus. A complete plant is independent when detached from the callus and usually grows into a tree. A shoot may be excised from the callus, rooted in suitable medium, and also grown into a tree. However, in a "plantlet," there is no apparent connection between the shoot and one or more roots from the same callus piece. The shoot rarely elongates more than a few centimeters because it has no independent root, and hence, is not a true plant. Examples of a complete plant and an excised rooted shoot may be seen in Fig. 17.



Figure 17. Callus Tissue (Center) from Triploid Quaking Aspen, Showing Complete Plant (Right) Detached from Callus with Attached Roots, and Shoot (Left) Excised from the Callus and Rooted in Agar Medium. About 1.5X Actual Size

TETRAPLOID EUROPEAN ASPEN

All of the original four trees reproduced from tissue culture were complete plants separated from callus. Two trees also grew from rooted excised shoots, but no trees have grown from "plantlets." All trees so far have been from the same triploid quaking aspen Clone T-2-56, but now we have a second species which may soon be added to the list of those reproduced from tissue culture.

Graft material was received at the Institute in 1966, from a tetraploid European aspen (P. tremula) produced in Sweden. The Clone Ta-10 has been vegetatively reproduced, brought to flower, and used in crosses with native female aspen to produce a number of interesting triploid hybrids (see tall hybrids). Callus tissue was initiated in late 1967, and was subcultured monthly for 23 passages on Medium 10⁴ in the dark. The rate of growth is about one third that of stock Tissue 1, but is firm and white. In the usual manner, inocula were placed on Medium BAP-.05 in the dark and shoots were produced after eight weeks. After six months in the light on Medium 10⁴, the plant rooted and became a complete plant. However, this first plant died after it was planted in soil. The callus has persisted in growing an extensive root system in the light, both on BAP-.10 and 10⁴, and has put out a dozen or so shoots from the roots, not from the callus.

Most shoots were lost from fungus contamination on the medium, but two shoots on Medium 10⁴ in the light rooted and began elongating in August. The plantlets were photographed in September, while attached to the callus root (Fig. 18), and were then separated (Fig. 19), planted in soil (Fig. 20), and moved to the growth chamber under 3000 ft.-c. of light for 16 hr./day, at 50% relative humidity and 80°F. Some lower leaves died from photography, although leaves were kept wet with water, but new leaves are growing and we hope these plants grow into trees.



Figure 18. Two Complete Plants Initiated from Callus Roots of the Tetraploid European Aspen Ta-10



Figure 19. The Two Plants Separated from Callus Root, Showing Independent Root System. About 1.5 Actual Size



Figure 20. Tetraploid Plants in Soil, Covered with Glass Dishes, Ready to be Placed in the Growth Chamber

TRIPLOID QUAKING ASPEN

During the 1968-69 season, covered in Report Nine last year, our main goal was to produce complete plants on the callus. We recovered seven, and four survived and became trees. Last year, however, during the 1969-70 season, the objective was to remove shoots from the callus and root them in suitable medium. This change of emphasis was dictated by the low efficiency of producing complete plants and the desire to find a method of clonal propagation that was both efficient and relatively simple. The rooting of excised shoots will be reported in the next section of this report. Three additional complete plants were recovered last year (but did not survive), and brings the total number to 14 complete plants produced during the past three years. The low survival rate of 4 of 14 (29%) further strengthened the decision to concentrate on the rooting of excised shoots.

The twelfth complete plant of triploid quaking aspen is shown in Fig. 17 (right). Inocula were subcultured from stock Tissue 100, after its thirtieth passage, and transferred to Medium BAP-.05 in the dark. In June, 1969, a vigorous shoot appeared, rooted, was placed on Medium 100, and removed from the callus after two weeks in the light. The stem was 1.5-cm. tall and the roots about 2-cm. long. The plant was put in soil in the growth chamber, covered with a glass dish, watered with liquid Medium 104 at first and then with distilled water, but the plant died within 5-6 weeks. One cause of death may have been the sudden exposure to the 3200 ft.-c. of light, from the 300 ft.-c. in the laboratory. Another reason could have been that the roots had not had enough time in the laboratory to develop a large enough system to sustain the plant.

Two other complete plants were formed during the extensive rooting study of Test BAP IV. Both shoots were initiated from Tissue 104, after transfer to

Medium BAP-.10 in the dark, and callus pieces with shoots were then placed in light on Medium 10⁴-IBA. This medium was made by adding 0.03% IBA (indolebutyric acid) by weight to Medium 10⁴, or the same amount found in a commercial rooting compound "Hormondin." These plants were also separated from callus and placed in soil in the growth chamber, but both died within two weeks. Evidently, plants should be grown in soil for awhile in low light levels before transfer to the growth chamber, or else they should be partially covered for the first few days in the growth chamber, or possibly both. Unfortunately, we have never had enough complete plants to make a definitive test.

ROOTING OF EXCISED SHOOTS AND THEIR GROWTH INTO TREES

The shoots, which were initiated during the BAP tests I to VIII, were used for the rooting tests. All visible shoots were reported, but only those longer than 5 mm. in length were considered vigorous enough to root after excision from the callus. This conclusion was based on preliminary rooting studies conducted the previous year.

The shoots from Test BAP I and II were mixed, without reference to their origin, but were separated into size groups. Each size group was distributed among two treatments of shoots left intact on the callus and placed in the light, or white shoots excised from the callus before they were placed in the light. The smallest shoots ranged from 1.5-2 cm., the tallest were 4-5 cm., and intermediates were 2-3 or 3-6 cm. tall. Twenty-eight shoots, on callus pieces, were placed on Medium 100 in 300 ft.-c. of fluorescent-tungsten light, in deep Petri dishes 75-mm. deep and sealed with Saran Wrap. The bases of the excised shoots were thrust into the medium. Ten additional shoots were excised and placed in Petri dishes 15-mm. deep, containing CBAP-0.05 or CBAP-.15 media, made by adding 15% coconut milk to regular BAP medium. These were also sealed and placed in light.

At the end of three weeks, 4 excised white shoots had rooted, 12 of 26 were rooted after four weeks, and the total of 14 of 26 rooted by the end of the 6-week test. This was the best rooting (54%) among so many shoots in one test. None of the shoots rooted on CBAP media or while still attached to the callus, and none of the rooted shoots elongated. At this time we were only interested in rooting percentages, so the rooted shoots were not transplanted or great care given to their survival and growth into trees. The result was that all rooted excised-shoots of this test finally died in the medium and were discarded. Evidently, the roots

drowned in the semiliquid agar medium, because from the previous year we knew that the thick red roots had to grow on the surface of the medium, in the air, in order to remain red and viable.

In the rooting Test BAP III, 11 shoots were excised before and 6 after they turned green. The 11 were thrust into Medium BAP-.10 and the 6 into Medium BAP-.05 in the light. After six weeks, none of the shoots had rooted or formed callus and were discarded. From subsequent tests, we learned that these were probably discarded too soon.

Rooting Test BAP IV was perhaps the most successful because excised shoots rooted directly from their base with no callus formation, two complete plants (13 and 14) formed on callus pieces although they later died, and single excised shoots proliferated into bunches of new shoots. For Tissues 1, 100, and 104, respectively, 18 of 85, 106 of 120, and 130 of 140 inocula had shoots, which were all initiated on Medium BAP-.15 in the dark. Three inocula with shoots 2-5 mm. long were selected from Tissue 1, and five inocula with shoots 5-mm. long of Tissue 100 were transferred to Medium 104 in the light. Five inocula with shoots left intact from Tissue 100, and 10 pieces of Tissue 104, were transferred to Medium BAP-.05 in the light. From Tissue 104, 5 and 10 inocula with shoots, were placed on BAP-.10 and BAP-.20, respectively, in the light.

After one week in the light, all shoots had turned green and 10 were excised, dipped in "Hormondin" containing talc and 0.03% IBA, and were thrust into Medium BAP-.10 in the light. After an additional month, the eight most vigorous shoots still attached to callus were excised and four each were thrust into Medium 104 or 104-IBA. Within 28 days, one shoot rooted on Medium 104 and all four rooted on 104-IBA from the second group.

From the first group of excised green shoots, which were dipped in Hormondin and put in BAP-.10 in the light, none rooted. However, most of the 10 shoots proliferated into numerous new shoots without growing callus. Figure 21 shows two masses of proliferated shoots (left) originating from excised shoots in Medium BAP-.10, as well as two inocula with shoots left intact (right) on Medium BAP-.05. The shoots left intact turned green but never rooted or elongated even after several months in the light.



Figure 21. Two Masses of Newly Proliferated Shoots (Left), Each Arising from One Excised Shoot Placed in Medium BAP-.10 in the Light, and Two Callus Pieces (Right) Transferred to BAP-.05 in the Light with the Shoots Left Intact

Six weeks after excised shoots were placed in BAP-.10, some of the proliferated shoots were separated and individually thrust into Medium 10⁴-IBA in the light. In one deep dish, 4 of 13 shoots rooted within eight weeks with thick, red, multiple roots. Figure 22 shows three excised shoots, whose roots remained red and vigorous if the whole plants were occasionally lifted gently from the medium and the roots allowed to grow on the surface of the agar medium.



Figure 22. Shoots Separated from Proliferated Shoot Mass on Medium BAP-.10 in the Light, and Rooted in Medium 10⁴-IBA in the Light

In rooting Test BAP VI, only 10 shoots were vigorous enough to excise, and these were either dipped in Hormondin and placed in soil, or placed in Medium 10⁴-IBA.

All three shoots in Medium 10⁴-IBA rooted, but only one shoot in soil rooted. Figure 23 shows the one shoot which rooted in soil after three weeks in the light. The shoot was initiated from Tissue 100 placed in the dark on Medium GBAP-.05, made by adding 10 mg./liter of the nucleotide guanosine-3'-(2')-phosphoric acid to Medium BAP-.05. This plant was the only rooted shoot that survived this test and is now Tree B. It was 55-cm. (22 inches) tall on September 8, and is still in an 8-inch pot in the greenhouse.



Figure 23. Shoot Initiated on Medium GBAP-.05 from Tissue 100 in the Dark, then Excised from Proliferated Shoot Mass and Rooted in the Light in Semisterilized Soil. This Shoot Rooted December 30, 1969, and is Now Tree B and is 55-cm. Tall

The shoots from Tests BAP VII and VIII were less vigorous than in the previous tests, so were not used for extensive rooting experiments. Occasional vigorous shoots from the monthly shoot-initiation tests for the three tissues were generally excised and placed in Medium 10⁴-IBA, in soil, or else left intact on Medium 10⁴ in the light. From the April test, no shoots were initiated from Tissue 1, but 45 shoots were produced on 13 of the 15 inocula of Tissue 100 placed on Medium BAP-.10 in the dark, and 44 shoots were produced on 7 of 15 inocula of Tissue 10⁴. Most of the shoots on Tissue 10⁴ were approximately 1-mm. tall after five weeks, but three of the Tissue 100 inocula had one vigorous shoot each, the tallest being 8-mm. long. This shoot was excised and thrust into Medium 10⁴-IBA in the light. It was then planted in soil in the greenhouse, but has not yet rooted.

In the shoot-initiation test of June, three tiny shoots were produced on one inoculum of Tissue 1 on BAP-.10. For Tissue 100, 71 shoots were on 14 of the 15 inocula. For Tissue 10⁴, 43 shoots were on 10 of 15 inocula. Only one vigorous

shoot 1.5-cm. tall grew on Tissue 100, but three vigorous shoots over 1-cm. tall were on separate inocula of Tissue 104. The tall shoots were excised and planted in soil, and watered first with liquid Medium 100 and thereafter with distilled water.

The tallest shoot on Tissue 100 rooted and is now Tree A, but another shoot 5-mm. tall was also planted and died. Trees C and D, respectively, grew from shoots excised from Tissue 104, but one of the excised shoots died. Of the five shoots excised, three survived and are now growing rapidly in the growth chamber. During the past month, Trees A, C, and D, respectively, grew from 6 to 24, 6 to 18, and 1 to 12 cm. in height. If all survive, the total number of trees reproduced from triploid quaking aspen tissue, from rooted shoots excised from callus tissue, will be six. This will be two more than have survived from complete plants formed on the callus. However, just from the last rooting test, 60% of the vigorous shoots placed in soil rooted and survived as trees. The low total number of rooted excised shoots reflects the relatively few shoots used for the rooting tests. We believe that most vigorous shoots initiated from callus can be rooted and will survive. The problem now is to increase the number of vigorous shoots produced on the callus, whose height is at least 1-cm. tall. The term "vigorous" in this context means tall enough to excise and handle easily. This should not be confused with the very tall shoots described in Report Nine as "vigorous," which grew to 1-3 cm. after they were initiated on Medium BAP-.05 in the dark. In that study, we found that the very tall shoots would not root, but rather grew callus from their cut bases, and the only shoots to root were those initiated on BAP-.15 and ranged from 0.5 to 1 cm. in height. This was the reason we have adopted Medium BAP-.10 as a compromise between the vigor and the number of shoots produced per inoculum.

FORCING AND ROOTING OF DORMANT VEGETATIVE BUDS

Dormant vegetative buds (axillary buds) can be observed on branches of most tree species, ubiquitous in seedlings but generally confined to the 1-2 year old twigs in mature trees. Auxin, which is produced in the apical bud of the leader and lateral branches, is transported back along the stem and suppresses the growth of vegetative buds until the concentration of the auxin drops significantly. When the apical bud is destroyed or removed, the closest dormant bud is released to take over as the new apical meristem. Logically, all dormant buds along a stem should be capable of growth if separated from inhibiting auxin, yet a lower concentration of auxin is essential for enlargement of the cells after they are produced in the newly-formed leaves and stem which develop from the bud.

Dormant vegetative buds offer a possible alternate method for clonal propagation of difficult-to-root species. In some species there is the possibility of physiological dormancy of the buds that cannot be overcome simply by separation from auxin. This type of dormancy usually is imposed in the fall and is broken by exposure to freezing temperature: the degree being dependent upon the species.

For the past several years, we have been working with dormant buds as a method for clonal propagation in aspen species (Reports Seven and Eight). For many species and hybrids, dormant buds will break and vegetative shoots emerge and elongate if short stem sections, with one bud each, are thrust into agar nutrient medium and placed in the light. Buds will break in many types of nutrient medium, both in light and less in dark (Fig. 24), but the problem has been to root the shoots after they have elongated. Shoots which rooted during past studies did so only after they were excised and left in medium in the light for several months. During the past year, better methods of rooting were investigated.



Figure 24. Leafy Vegetative Shoots Growing from Axillary Buds. Dormancy was Broken by Separating Individual Buds on Stem Sections from Seedlings. This Photo, from Report Eight, Shows Agar-Bottle Vessels After 3-4 Weeks in the Light

When callus was initiated for the induced-ploidy seedlings (reported in an earlier section), vegetative buds from most seedling branches were also isolated and placed in bottles containing Medium C.05 or 104-IBA. For most seedlings, only 1-4 buds were available, and all shoots which elongated were stunted. None of those excised from the stem were able to root, except shoots from seedlings Number 4-68 and 6-68, which were also the most vigorous hybrids between quaking aspen and European aspen, as well as one quaking aspen seedling 126-68. Figure 25 shows two forced shoots from Plant 6-68, which survived from three excised shoots placed in Medium 104-IBA in the light. All rooted shoots were potted and left under 300 ft.-c. of light, because the growth chamber was not then available. However, all plants were lost from fungus or black leaf-spot. The plants may have survived in the growth chamber, but we still lose plants in the growth chamber under the high light intensity if their root system is not well developed before they are potted. We now believe that most rooted shoots can survive, if they are returned to agar medium in the light, until the root system is sufficient to cause the shoot to renew elongation.

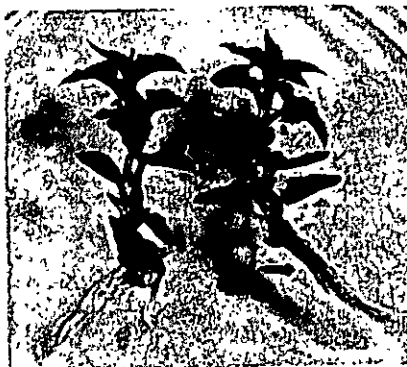


Figure 25. Two Excised Shoots Forced from Dormant Vegetative Buds and Rooted in Medium 104-IBA in the Light. Stems are 2-3 cm. in Length and are Shown About Actual Size. The Roots are Thick and Red, with Agar Medium Held by the Numerous Root Hairs

DISCUSSION AND FUTURE PLANS

The four essential components of successful clonal propagation by tissue culture are (1) rapidly-growing firm white callus tissue, (2) abundant initiation of vigorous shoots at least 1-cm. tall, (3) a high rate of rooting of excised shoots, and (4) survival of the plants and their growth into trees. At this point in the state of the art, there is enough variability in each of these four links to question the use of this technique for the immediate mass propagation of difficult-to-root tree species. Our small measure of success has demonstrated that at least one tree species can be reproduced in this manner, but the main question is whether the route through undifferentiated callus tissue can be perfected to the degree necessary to be usable in the industry as an economic practice. The alternatives open at this time are to either continue to try to pull all of the loose ends together and increase the efficiency of this method, or, to abandon the route of propagation from callus tissue culture and go to liquid cultures, single cells, and embryoid development into complete plants. A compromise, and most appealing at this time, is to continue with fewer large-scale experiments with callus tissue, and at the same time do the preliminary work necessary for large-scale single-cell experiments the following year.

The justification for a compromise is that the single-cell method has the potential to become the most efficient method of clonal propagation. On the other hand, we have reproduced triploid quaking aspen from tissue cultures and now have complete plants growing from a second species, a tetraploid European aspen. Even with the very low efficiency of the tissue culture method, this is presently the best we have, and as far as we know, the best method anyone has. With callus tissue, we at least have a chance to obtain a few propagules from those superior trees which cannot be propagated vegetatively by any other method. So, until the single-cell

method is perfected for tree species, as much as possible should be learned about the callus tissue method.

The single-cell method has been used by other workers to reproduce several herbaceous angiosperms, both dicots and monocots, i.e., flowering plants and grass-type plants. The general technique is to initiate friable callus tissue, which is then shaken in liquid medium in flasks or other vessels, and to provide an optimum nutrient medium so that the single cells which are separated from the callus mass will develop into embryoids, plants, and finally into trees. In some cases, the embryoids of herbaceous plants have developed to a green-plant stage in the agitated liquid medium. In most cases, however, single cells have been isolated with micro-tools under a microscope and grown independently on nurse cultures, or more recently on defined agar medium. The major problem for most species is the specific environment required for cell differentiation, which includes the optimum defined medium, light quality and quantity, temperature, and probably a half-dozen unknowns.

The theory of totipotency states that every newly formed plant cell carries in it all of the genetic information necessary for that cell to form an exact duplicate of the parent plant. A recent amendment suggests that many differentiated cells in leaves and stems can de-differentiate into undifferentiated callus tissue, and the undifferentiated cells can then differentiate into a mature plant.

Unfortunately, we do not yet know the optimum level of the variables for single-cell propagation of trees from aspen. Our supplemented Medium 100 and 104 are significant improvements over the basic Medium 1, but in occasional trials during the past three years, single cells have not differentiated either in liquid or on solid medium, or in light or dark. In another tree species, however, we were able to grow single cells into large enough masses to be seen in slide microculture

with an unaided eye. We feel that the single-cell method is not only desirable, but is essential for the ultimate clonal propagation of most trees.

Our plan, then, should be a continuation of our present course, to try to increase the efficiency of the callus tissue method. In so doing we must improve the medium and the rest of the environment, and when we can efficiently reproduce several tree species from callus tissue, we should then be in a good posture to adapt the new knowledge to single-cell reproduction. But in the meanwhile, some of the mechanical techniques of liquid culture can be practiced and improved.

OTHER RESEARCH PROJECTS

As a direct result of our reproduction of aspen trees from tissue culture (and the subsequent publicity), two cooperative research studies have been started in this laboratory to reproduce trees of interest to the sponsoring companies. One project, concerned with reproducing a hardwood species, was recently renewed for a second year although we have not yet produced any shoots on the callus tissue. In the other project, conifer species are being studied, but callus cultures have only recently been established for one species and no differentiation into shoots has occurred. Should we succeed with the latter project, similar work is promised for more species, as well as studies into the haploidy and polyploidy aspects of tree improvement. With success in either project, we anticipate that other companies and agencies will also become interested in our working with their species of interest.

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