INSTITUTE OF PAPER CHEMISTRY
*Appleton, Wisconsin*

# INITIATION AND GROWTH OF ASPEN TISSUE CULTURES

Project 2351

Report Seven

A Progress Report

to

PIONEERING RESEARCH COMMITTEE

September 18, 1967

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## SUMMARY

One piece of triploid quaking aspen callus was recovered during the past year having both shoots and roots. This is only the second such piece recovered in three years on defined media. Also, some of the problems investigated were of increasing the available callus cultures for differentiation studies, for the eventual growth of single cells to mature trees.

Roots were collected four times during the growing season and callus initiated on root sprouts 2-3 months after each collection. Firm white callus would only grow on an undefined coconut-milk medium early in the season; and then slowly on the defined Medium 1, having a low level of 2,4-D, during the later part of the season. Soft tan callus, grown on high-2,4-D media, became necrotic unless transferred to Medium 1 where it changed to firm white tissue. Cultures of transferred tissue, however, did not maintain firm white tissue as well as cultures initiated for two months then maintained at 3-week subcultures on Medium 1.

In the growth study, soft aspen callus was initiated rapidly on low-2,4-D medium 2. The log phase occurred between 12-18 days and the greatest 4-day increment was between 12-16 days. A subculture period of 18 days was recommended for this type of tissue.

Aspen root-sprout segments, grown under five different light intensities, on a high-2,4-D medium, produced the greatest but most variable amount of soft white callus in absolute darkness. The least amount but most uniform callus was grown on the laboratory bench under weak light. Under continuously strong light, tissue turned

red or green and growth was inhibited. Tissue, grown uncovered in an incubator, grew about 30% faster when given five minutes of strong light every 3-4 days, than if left continuously in the incubator.

A preliminary report was given of a long-range study which will attempt to correlate the rate of seedling callus growth with the final rate of tree growth. Also discussed were the establishment of firm white cultures, as well as cell suspensions of triploid quaking aspen. The recovery is reported of both friable colorless tissue and firm white callus from one clone of *Populus canescens*, on the same medium with different auxin levels.

Future plans include a massive program this fall of tissue initiation and differentiation studies. The immediate objective is to determine the optimum nutrient and light environment to produce plantlets *in vitro* of quaking aspen, bigtooth aspen, and *Populus canescens*. The excellent friable culture of *P. canescens* will also be used for cell-suspension and single-cell-isolation studies.

INTRODUCTION

In Progress Report Six (1966) to the Pioneering Research Committee, several advantages were given for using liquid-grown callus of quaking aspen as a method to study root initiation. Callus was grown for 1, 2, or 3 months uncut in a defined liquid medium having a low auxin level, then subcultured to a similar agar medium supplemented with vitamins. Significantly more pieces of tissue rooted (65%) from large 1/2-sphere explants from the oldest tissue source than from 1/4 and 1/2 explants of the younger one and two-month-old tissue. These results indicated that anatomical differences rather than merely an accumulation of growth chemicals was responsible for the increase in rooting with age of source tissue.

Subsequent microscopic examinations (not included in Report Six) of sections of the source tissue showed that the increased length of columns of cells proliferating from the surface with increasing age of tissue was correlated with increased rooting. A paper on this work has been accepted for publication by the American Journal of Botany and should appear next June.

During the past year, the main emphasis has been an attempt to build up several uniform stock cultures, each originating from single pieces of callus for use in further differentiation experiments. One of our major goals is to produce roots and shoots on callus tissue which will then grow into mature trees. So far we can get good rooting, but only recently there was found the second piece of tissue recovered in the past three years on defined media having both roots and shoots. This tissue piece originated from the triploid quaking aspen strain 3x-f5 grown for nine subcultures on agar medium followed by five subcultures in liquid medium, then nine more passages on agar. One leafy shoot (Fig. 1, left) was found on one of 40 explants individually placed on agar medium 1 in a small baby-food jar in the dark. Two weeks later (3/1/67) the piece was trimmed and placed on fresh medium. The leafy shoot withered, but a new cylindrical shoot 14-mm. tall, as well as two roots 3-4-mm. long grew within two weeks (Fig. 1, right). After four weeks the piece became infected with bacteria and was fixed for later sectioning and study.

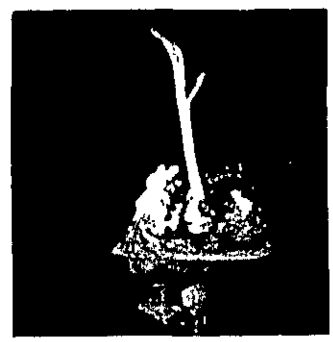

Figure 1. Left--Leafy Shoot on Triploid Quaking Aspen Tissue 3x-f5. Right--Same Piece One Month Later, After Leaf Has Wilted and New Cylindrical Shoot and Two Roots Have Emerged

This example points up one of the problems encountered in a study like this: Only one differentiated piece was recovered from a small study after a series of different consecutive treatments. Determining the trigger for this differentiation would be difficult. Differentiation and the formation of plantlets has been reported elsewhere for several species of lower plants, but each method has been different. So far, no one method will work for all lower plants, let alone tree species.

Major problems attacked during the past year included (1) the decline of tissue quality during prolonged culture, (2) the rate of growth of friable aspen callus, and (3) the effects of different light intensities on callus morphology. Minor studies included the recovery of nondifferentiated embryoids from single cells in suspension, and the establishment of uniform clonal lines of firm white aspen callus. A preliminary report was given of correlating the callus growth of seedlings with their subsequent growth rate as trees. Also, cultures were described of both friable colorless and firm white tissues of Populus canescens grown in the dark, as well as firm green callus of bigtooth aspen grown under strong light.

The main sections of the following report will be submitted to various botanical journals for publication.

## ASPEN CALLUS INITIATED PERIODICALLY

One of the requirements of this program is to grow stock cultures for experimentation on a completely defined nutrient medium. This will allow precise control of quantitative adjustments, in order to achieve differentiation and growth of callus tissue into independent plantlets. Since 1965, when we began using Wolter's (1) defined medium instead of the undefined coconut-milk medium 23 of Mathes (2), callus tissue of triploid quaking aspen has been initiated several times and maintained by subculturing for one to several months. However, after use for one or two experiments, the remaining stock tissue has generally declined in quality from unknown reasons and has had to be discarded.

Prior isolations of callus, made at different times during the year, gave different forms of tissue. With the objective of finding one nutrient medium to support continuous tissue growth, callus was initiated on root-sprout segments four times during 1966-67 from tree roots collected during the 1966 growing season.

### METHODS

Roots were collected in March, June, August, and October, 1966, from trees of a triploid form of normally diploid quaking aspen (*Populus tremuloides* Michx.) growing near Appleton, Wisconsin. These trees were grown vegetatively from clone T-2-56 found near Bruce Crossing, Michigan. Root sections were planted in the greenhouse, and after 2-3 weeks root sprouts were detached and later potted. When 6-12 inches tall, stems were removed above the first node, sterilized 8 minutes in household bleach, rinsed in sterile water, and cut into segments 7-8 mm. long (2). Segments were thrust, base up, half way into three variations (3) of Wolter's agar medium (Media W, 2, and 10) containing a high level of 2,4-D auxin (.5 mg./liter). Segments were also placed in Medium 1, having a low level of 2,4-D, and into the control Medium 23 (coconut-milk) according to the design in Table I. Plates were left in a dark, humid incubator at 27°C. for 2-3 weeks.

TABLE I

ROOT COLLECTIONS AND CALLUS INITIATION OF TRIPLOID QUAKING ASPEN

| Experiment | Roots Collected 1966 | Transfers | | Callus Initiation | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Saucers | Pots | Date 1966-7 | Plates Per Medium | Segments Per Plate | Observed | Media |
| 1 | March 31 | 4/18-5/10 | 5/25 | 6/20 | 4 | 9 | 9/2[a] | 23, W, 2, 10 |
| 2 | June 3 | 6/22-7/11 | 7/5-7/29 | 9/6 | 3 | 10 | 9/22 | 23, W, 1, 2, 10 |
| 3 | August 5 | 8/25-9/12 | 9/30 | 11/15 | 4 | 5 | 11/28 | 23, W, 2, 10 |
| 4 | October 6 | 10/28-11/25 | 11/22-12/12 | 1/23 | 3 | 5 | 2/13 | W, 1, 2, 10 |

[a]Observed after three subcultures on 7/12, 8/8, and 8/19/66. Experiments two and three were observed at the end of two weeks and experiment four after 3 weeks while callus was still attached to its root-sprout segment.

RESULTS

Experiment 1

Firm white callus grew on Medium 23 and was irregular in shape and surrounded by tan-brown tissue. Tissue on all other media was soft, tan, and irregular in shape. The same general type of growth was consistent for three more monthly subcultures, except that tissue on the defined media W, 2, and 10 became generally necrotic and was discarded (Fig. 2).

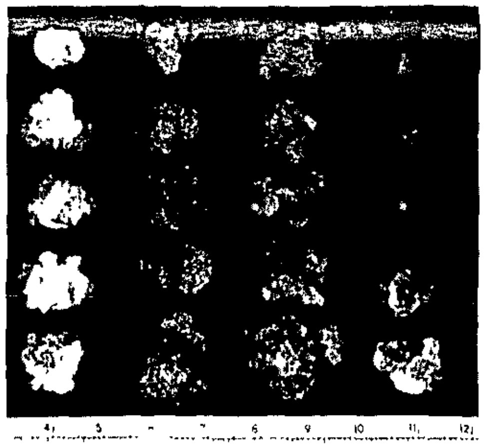

Figure 2. Callus Initiated and Subcultured Three Times on Agar Media 23, W, 2, and 10 (l. to r.). Observed 9/2/66

Experiment 2

Vigorous, firm white tissue was initiated on most segments on Medium 23. Slow-growing firm white tissue grew on Medium 10 and on a few pieces on Medium W (Fig. 3). Soft (friable) light-green tissue grew vigorously on Medium 2 and less rapidly on Media 23, W, and 1. Only two pieces were lost from contamination.

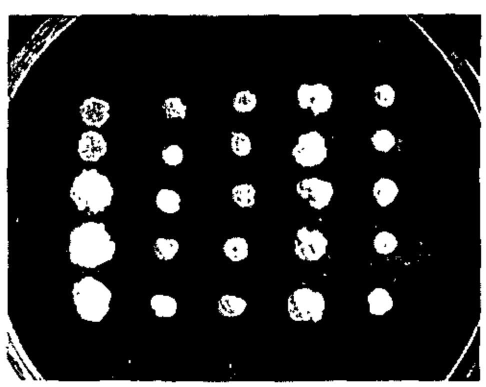

Figure 3. Callus Initiated on Media 23, W, 1, 2, and 10 (l. to r.). Observed 9/23/66

The callus on Medium 2 was subcultured onto Media 23, W, 1, 2, 10, and 12 (Medium 1 plus four vitamins). Firm white tissue grew on Medium 1, but tissue subcultured to Medium 10 eventually turned brown and was discarded.

Experiment 3

Callus initiated in November, on sprout segments from the August root collection, was slow-growing, hard, and yellow on Medium 23. Vigorous tissues on Media W, 2, and 10 was generally very soft (friable) and colorless, although some pieces had subsurface white areas of firmer tissue (Fig. 4).

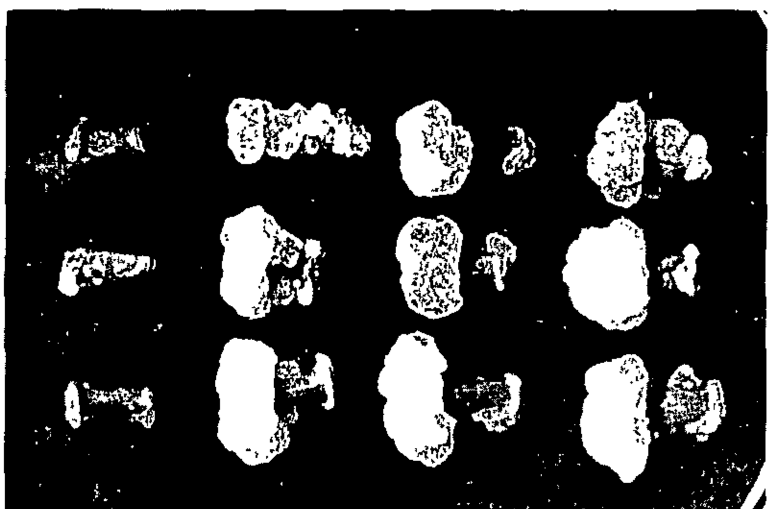

Figure 4. Callus Initiated on Media 23, W, 2, and 10 (l. to r.). Observed 11/28/66

In this experiment, loss was heavy from bacterial contamination, evidently coming from within the sprout segments. The highest survival (65%) was on Medium 2, whose pieces (callus plus segment) averaged 296 mg. The percentage survival and average final weights of other tissues were 15% and 65 mg. on Medium 23, 35% and 235 mg. on Medium W, and 15% and 343 mg. on Medium 10.

Explants of 50-100 mg. were cut from each piece shown in Fig. 4 (except on Medium 23) and placed on fresh medium of the same type as the original culture. After two weeks, each piece weighed 600-700 mg. and was soft yellow-tan. On each medium, 3-5 pieces changed to firm white tissue. All tissue was broken up and divided between two 3000-ml. Erlenmeyer flasks, each containing 500 ml. of liquid Medium 1. After two days in the dark on a gyroshaker, a microscopic examination of the suspension under phase-contrast revealed single cells and cell aggregates.

Several multicell colonies were observed, such as the one shown in Fig. 5 (top), which apparently originated from single cells. This was the first good evidence that aspen cells will form proembryos in liquid culture. In other cells, however, small, dark, spherical bodies were observed in undivided cells, (Fig. 5, left and right). These bodies were moving rapidly inside and outside of the cells and may have been bacteria rather than mitochondria or other normal cell inclusions. This hypothesis is strengthened by the fact that two days later all tissue in both flasks turned black and was discarded. It is still unknown whether the aspen material carried the infection from the roots to the callus or picked up infection during the subculture. A study in 1965 (Progress Report Five, page 8) reported that bacterial infection was possibly transmitted systemically upward into root sprouts from infected roots. However, little infection was found above the first (lowest) node, indicating that the node may act as a natural bacterial trap.

Experiment 4

In an effort to reduce possible systemic infection, root sprouts were harvested in January, 1967 above the first node. Also, segments were cut from each sterilized stem from the top down with one sterile razor blade.

Segments were then placed in agar Media W, 1, 2, and 10. As a control, seven stems were all cut with one razor blade from the base up and placed in Medium 2.

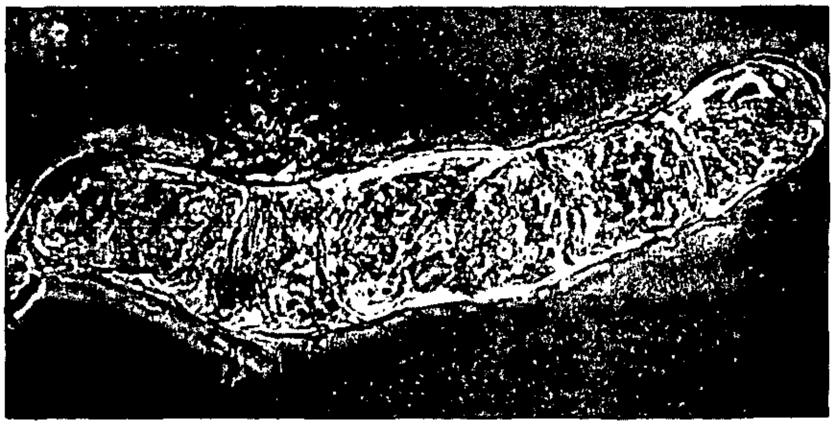

Figure 5. Cells Grown in Liquid Medium 1 from Friable Callus. Top--Seven-Celled Colony from One Cell (875x). Right and Left-Undivided Cells Containing Small, Dark Bodies, Possibly Bacteria (2200x)

No contamination was observed for either cutting treatment by the end of the one-month initiation period. Not until the second subculture did contamination occur, and then from airborne fungal and bacterial spores. For the main treatment (cut from top to bottom), callus did not grow on three segments and was generally firm and white on Medium 1, green and friable on Medium 2, and about half and half on Media W and 10 (Table II).

TABLE II

AVERAGE SIZE, WEIGHT, COLOR, AND TEXTURE OF ASPEN CALLUS INITIATED ON ROOT-SPROUT SEGMENTS ON FOUR AGAR MEDIA AFTER THREE WEEKS

| Medium | Callus Diameter, mm. | Final Weight[a], mg. | Number of Callus Pieces Firm White | Firm Green | Friable Green | Dead |
|---|---|---|---|---|---|---|
| W | 5-9 | 191 | 5 | 1 | 8 | 1 |
| 1 | 4-6 | 87 | 11 | 0 | 4 | 0 |
| 2 | 7-8 | 169 | 1 | 0 | 11 | 3 |
| 10 | 6-11 | 245 | 2 | 5 | 3 | 0 |

[a]Callus plus segment.

The 6-8 best callus pieces were selected from each of the Media W, 2, and 10, and 3-5 explants from each piece were placed on fresh agar medium in separate plates. The firm white callus on Medium 1 was not subcultured because of its small size. Tissue on all four media were then grown for another 3 weeks.

Most of the strains were soft and tan at the end of the passage and were discarded. Some of the better looking cultures were subcultured, however, including one light-yellow strain on Medium W, a tan tissue with white areas on Medium W-2, and a soft yellow strain on Medium 10. Callus on Medium 1 was firm and white and isolated from the root-sprout segments and subcultured clonally.

At the end of the passage, all tissue except that on Medium 1 and one strain on Medium 2 was discarded because of necrosis or contamination. The strain subcultured to Medium 2 also died after one more passage. On the other hand, one strain (originating from one of several pieces of callus left on Medium 1 for two months then subcultured monthly) is still a vigorous, firm white culture after seven passages.

DISCUSSION

The results of these experiments indicate that both the quality and quantity of aspen callus, initiated on root-sprout segments, is influenced by the time of year of root collections, as well as by the composition of the nutrient medium. Vigorous firm and white callus was initiated on Medium 23 in June and September, from material originating with the March and June root collections. Roots collected in August and October, however, produced sprouts which initiated a small amount of hard yellow tissue on Medium 23 in November and January. Some firm white tissue was initiated in later isolations on high-2,4-D defined Media 2 and 10, but did not remain vigorous and alive through continuous subcultures. Most tissue isolated on Media W, 2, or 10 was soft and tan and did not produce good stock cultures.

Friable tissue was produced during the two last isolations on all of the high-2,4-D media. However, tissue used immediately for liquid cell suspensions became infected, and friable tissue subcultured onto fresh Media W, 2, and 10, or onto the low-2,4-D Medium 1, either changed to firm white tissue or became necrotic within 1-2 passages. Callus was initiated the quickest as a soft tan tissue on Medium 2; and, after changing to firm white tissue when subcultured to Medium 1, generally remained more vigorous for a longer time than tissue initiated on Media W or 10.

An important result was that the best firm white tissue for continuous cultures came from callus left on Medium 1 for six weeks (in both September and January) before it was subcultured. In previous isolations, the slow-growing, firm white callus initiated on Medium 1 was usually discarded in favor of the fast-growing soft tissue on Medium 2. This one bit of information alone should now provide uniform cultures grown completely on Medium 1, in contrast to the variable cultures of short viability obtained by transferring soft tan tissue from Medium 2 to Medium 1, then recovering the pieces which change to firm white tissue.

## GROWTH RATE OF INITIATED FRIABLE CALLUS

Characteristically, explants cut from callus tissue and placed on agar medium increase in wet-weight through time in a sigmoidal manner. That is, growth is slow during the first few days, followed by a rapid log phase, then slowing to a senescence phase. Mathes (4) reported that the log phase (most rapid growth) of firm white tissue of quaking aspen occurred between 12 and 16 days after subculturing to the undefined Medium 23. He allowed his cultures to grow for 3-4 weeks, even though both the log phase and increment peaks were greatest after about two weeks. This paper reports the rate of friable callus initiation on material from the same clone of triploid quaking aspen, but grown on a high-2,4-D defined medium.

### METHODS

Root sprouts were harvested December 7 from roots collected August 5. This material remained from the isolation of November 15 reported in the previous section. After sterilization, 145 segments 7-mm. long were distributed among 29 plates, bases up, on agar medium 2. Ten randomly-selected segments were measured, weighed, and grown separately on two plates of Medium 2 in a dark, humid incubator at 27°C. Every four days, the ten numbered pieces were weighed, arranged on one plate and photographed, then returned to their original positions on the incubator plates. During photography, pieces were under strong light for approximately five minutes every four days.

### RESULTS

The initiation of friable callus on root-sprout segments is shown in side view in Fig. 6 (A, B, C) at 4, 12, and 16 days after the beginning of the experiment. A top view of the mature callus after 20 days is seen in Fig. 6 (D).

At the start, segments averaged 8 mm. in length and 2.4 mm. in diameter and weighed 54 mg. After 20 days, the segments had swollen considerably in diameter, but not in length, and averaged 113 mg.

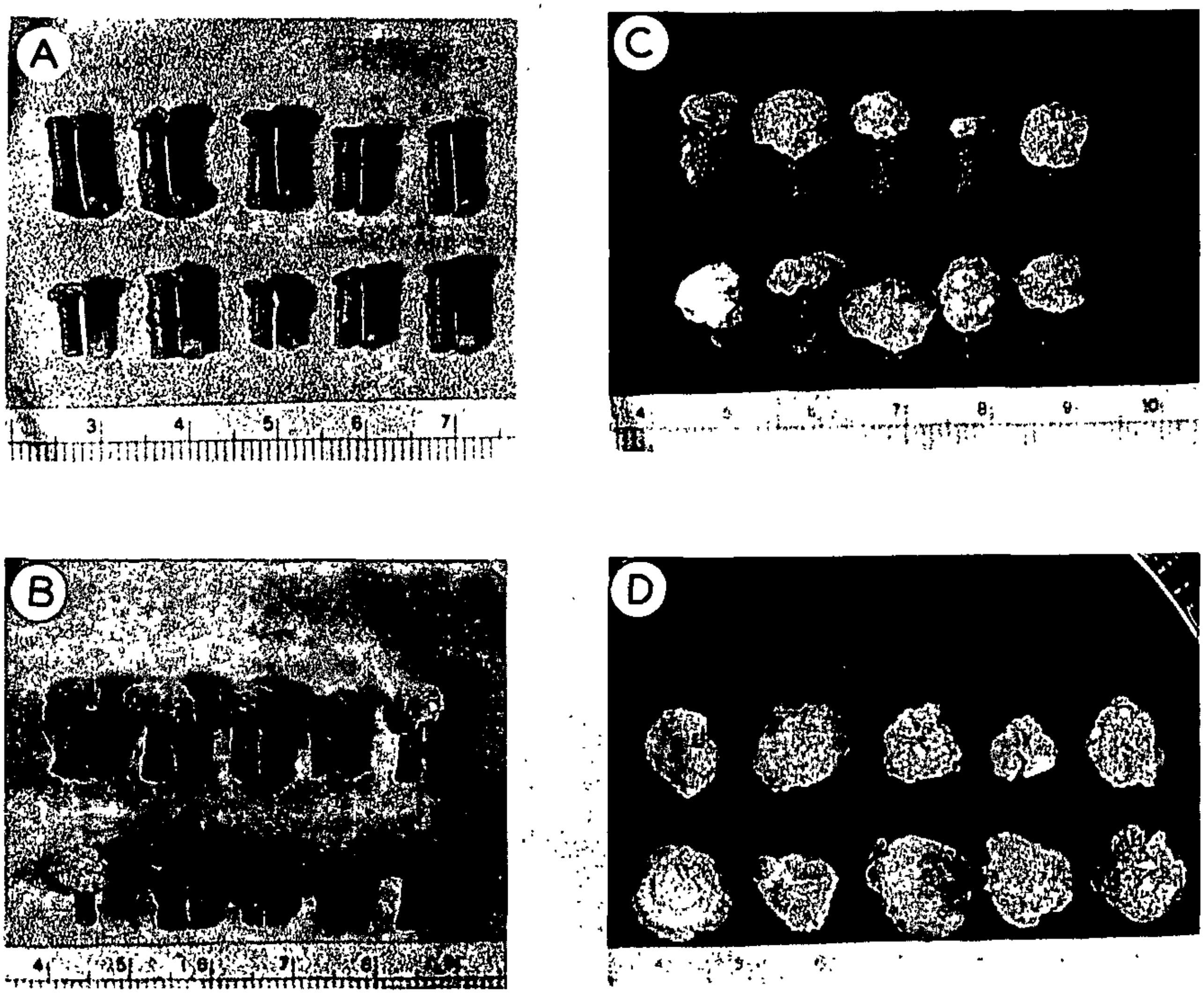


Figure 6. Initiation of Friable Quaking Aspen Callus on Medium 2 After (A) 4 Days, (B) 12 Days, (C) 16 Days, and (D) 20 Days at 27°C. in the Dark

Figure 7 shows the total growth curve plotted on the basis of the average wet weight of callus plus segment. Using the known initial and final weights of the segments, an estimated growth curve of segments alone was constructed, and the net wet-weight of callus estimated for each observation.

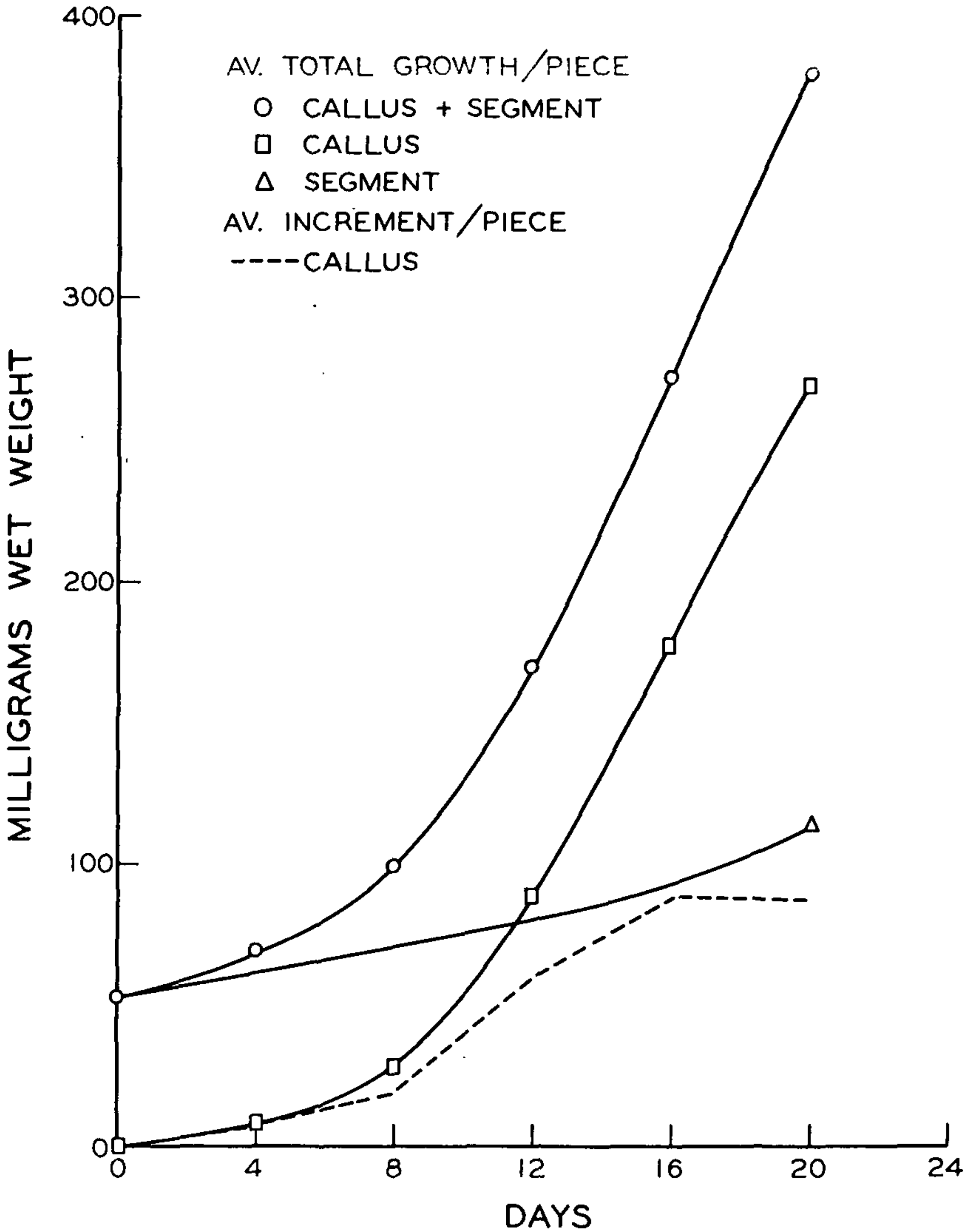


Figure 7. Growth Rate of Friable Aspen Callus Initiated on Medium 2 in the Dark, with Five Minutes of Strong Light Every Four Days

An average increment curve for callus growth for each 4-day period is also included in Fig. 7.

The total growth of callus alone averaged 8, 28, 89, 177, and 264 mg., respectively, at 4, 8, 12, 16, and 20 days. The ratios of callus/callus plus segment during the same period were 12, 29, 53, 65, and 70%. The average increment, per piece of callus alone was 8, 20, 61, 88, and 87 mg. for each of the 4-day periods ending at 4, 8, 12, 16, and 20 days.

DISCUSSION

The data illustrated in Fig. 7 show that the log phase of friable callus initiation occurred during the period of 12-18 days. This is two days longer than the 12-16 days reported by Mathes (4), but probably is not significant. In both studies, the average increment per piece was greatest for the 4-day period ending at 16 days. For firm white callus the increment drops sharply to 20 days but remains at about the same level during the same period for friable callus initiation. This experiment was terminated after 20 days, because the tissue began to show the first signs of quality decline as a tan darkening around the bottom of the otherwise colorless or white callus. On the basis of this study, friable callus should be isolated and subcultured after 16-18 days, but not after 20 days.

In order to determine the effect of light on callus initiation, ten pieces of callus plus segment were weighed after 16 days. These pieces had been kept in the dark incubator and averaged 176 mg., compared to 276 mg. for the pieces photographed every four days. Figure 8 shows that the 5 minutes exposure to bright light at 4-day intervals increased the amount of growth by 36%.

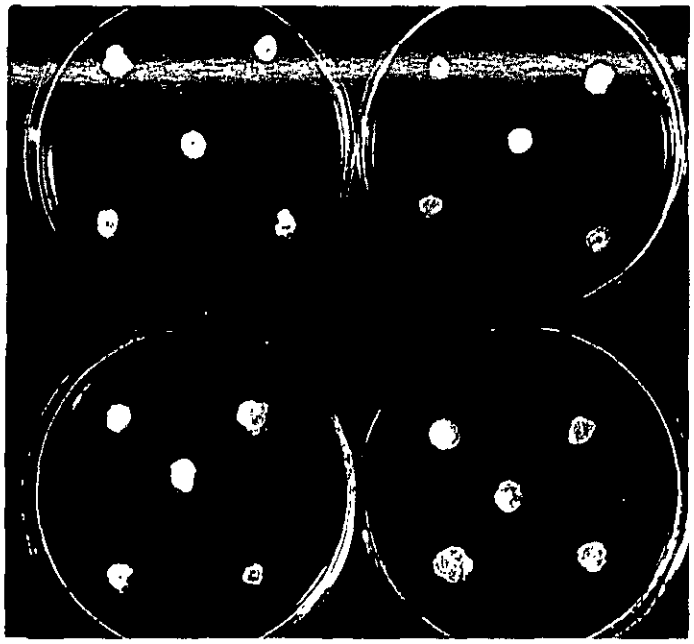

Figure 8. Friable Aspen Callus Initiated on Medium 2 in a Dark Incubator (Top) and in an Incubator but Given Five Minutes of Strong Light Every Four Days (Bottom). Photographed at 16 Days

Past experience has indicated that continuous bright light hinders the growth of aspen callus, and even an exposure of one hour initiates the production of red pigment (2). The friable callus was stimulated by short intermittant exposures to strong light but it seems doubtful whether the whole shape of the growth curve would be influenced by the effects of light. The shape of the curves would probably remain constant, and only the values on the x-axis would increase or decrease. A study to determine the quantitative effects of light on growth is reported in the next section.

## EFFECT OF LIGHT ON CALLUS GROWTH

During the study of the growth rate of initiated callus (see last section) the stimulating effect of occasional, short exposures to strong light was reported for friable aspen callus. In order to further test the effects of light, callus was initiated under five different light environments.

### METHODS

Root sprouts were harvested February 8, 1967, from roots collected in October. The 15 stems averaged 318 mm. in height and were approximately 2.9 mm. in diameter where they were detached 75-100 mm. above the soil surface. After 8 minutes in household bleach, stem sections were rinsed in sterile water and cut into segments 7-8 mm. long. Ten segments were thrust base up into agar Medium 2 in 25 plates. Five plates were grown in each light environment listed in Table III. The occasional strong light for Treatment 5 was supplied by one 500-watt spotlight, at a distance of 2 feet, for 5 minutes alternating every third then fourth day.

TABLE III

FIVE LIGHT INTENSITIES USED TO INITIATE FRIABLE ASPEN CALLUS ON MEDIUM 2

| | Treatment | Location | °C. |
|---|---|---|---|
| 1. | Total darkness | Foil-covered in incubator | 27 |
| 2. | Occasional weak light | Uncovered in incubator with door opened daily | 27 |
| 3. | Lab light | Lab bench | 23-24 |
| 4. | Strong light | Tungsten/fluorescent lights | 27-28 |
| 5. | Occasional strong light | Uncovered in incubator with occasional strong light (see text) | 27 |

RESULTS

After six days, callus averaged 3-4 mm. in diameter on segments under strong light but most tissue was speckled red. Callus initiation was slower for the green tissue in Treatment 3, and the growth of the friable colorless tissue in the incubator was intermediate. Treatment 1 was not uncovered and inspected.

At the end of 19 days, the lightest-color but most variable tissue was from the dark Treatment 1 (Fig. 9). All of this friable culture was colorless, having 17 pieces of callus 10-15 mm. and 21 pieces 3-7 mm. in diameter. *Seven callus pieces in the incubator in* Treatment 2 *were* 5-10 *mm.* in diameter, composed of either green and white friable tissue. All of the firm greenish callus on the laboratory bench in Treatment 3 was 5-6 mm. in diameter and exceptionally uniform. In strong light, most callus development was poor, but a few pieces were 7-8 mm. in diameter and firm green or red. In Treatment 5, with occasional strong light, the friable callus was uniformly larger than in Treatment 2, but not as large as some of the pieces in Treatment 1. The 7-12 mm. callus was white, green, or yellow and generally appeared to be more vigorous than in Treatment 1 which only received occasional weak light. Representative callus pieces were placed in FAE fixative for later sectioning and microscopic examination.

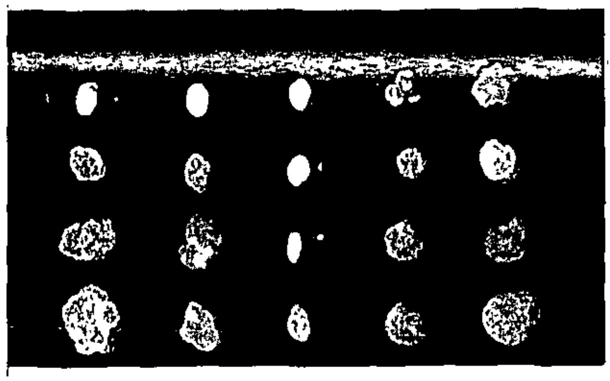

Figure 9. Aspen Callus Initiated After 19 Days on Medium 2 Under Light Treatments 1, 2, 3, 4, and 5 (l. to r.)

A two-way analysis of variance of the growth data showed that neither treatment nor replication differences were significant at the 95% level. The interaction difference was significant, however, revealing a greater difference within these two parameters than between. The practical significance of interaction is that these data cannot be further analyzed statistically on the basis of total weight increment. On the other hand, Fig. 10 supports the subjective observations that the greatest variability was found in Treatment 1, in the dark, as vigorous and colorless friable tissue. The most uniform tissue grew on the laboratory bench as firm white tissue in Treatment 3, but had the smallest average weight. The effect of occasional, short exposures to strong light (Treatment 5) increased the average growth by 22% over the Treatment 2, receiving only occasional weak light. Continuous strong light (Treatment 4) and continuous laboratory light both suppressed growth. However, in the former, variability was high and quality low; but in the latter, quality was high and variability was low.

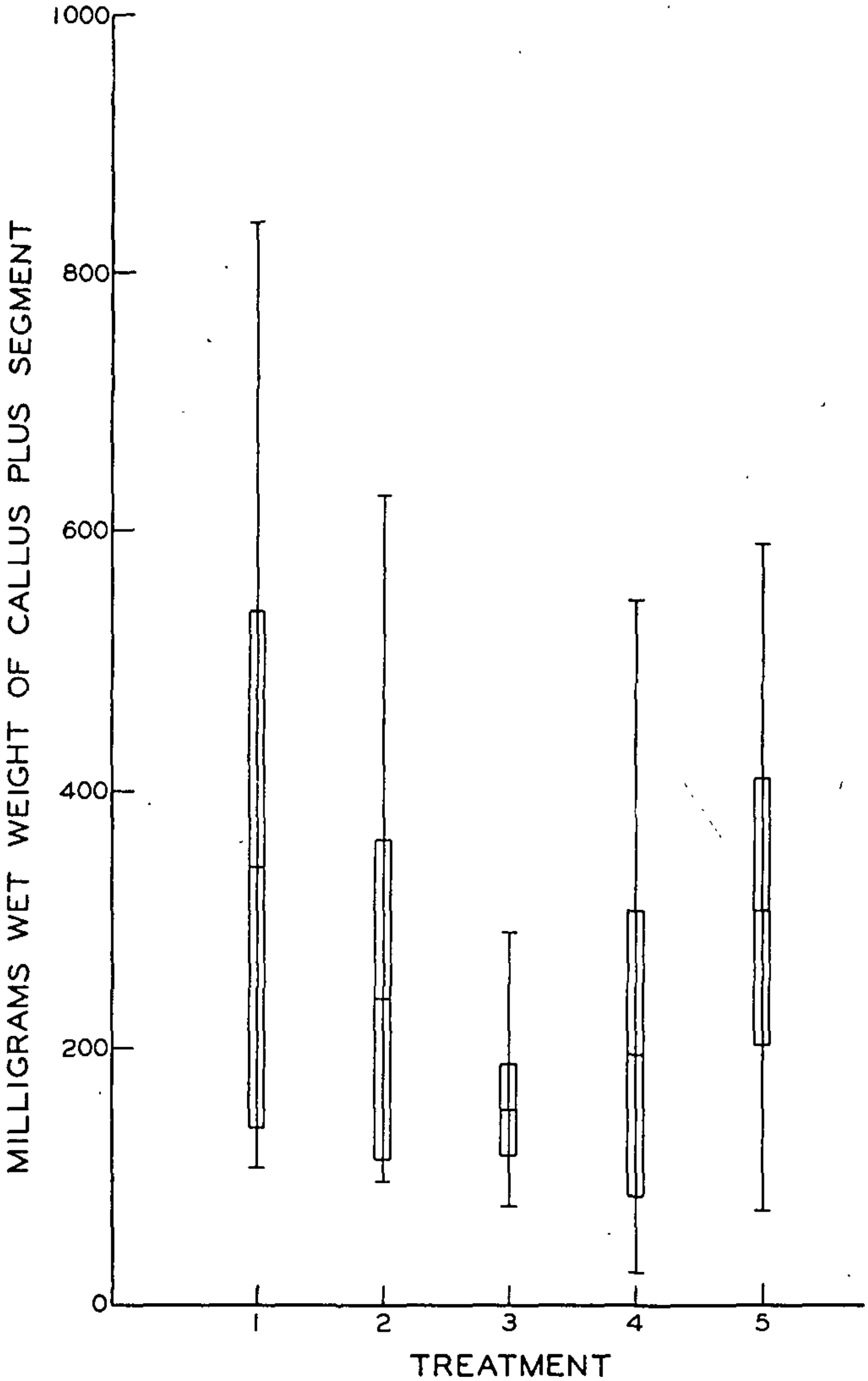


Figure 10. Mean Total Weight of Callus Plus Segment Grown on Medium 2 for 19 Days Under Five Light Intensities Listed in Table III. Rectangles Show Plus or Minus Two Standard Errors and the Lines Represent Ranges

Subculture 1

Explants of 50-150 mg. each were cut from the best 10-20 callus pieces from each treatment and all pieces from each callus placed on either Medium 1 or Medium 2. Callus was then given the same treatment as the original tissue.

After 15 days on Medium 1, about half of the clones in Treatments 1, 2, and 5 were firm and white, and all callus in Treatments 3 and 4 was small and red. On Medium 2, vigorous soft and yellow tissue grew in Treatments 1, 2, and 5, but slow firm and white tissue grew in Treatment 4 and red, green, and white firm tissue was found in Treatment 3.

Subculture 2

Explants from 3-4 of the best clones of Treatments 1, 2, 3, and 5 were placed on the same medium and given the same treatment. After 21 days, callus pieces were 10-15 mm. in diameter on both media for most treatments (Fig. 11), but the best firm white tissue grew on Medium 1 receiving occasional short exposure to strong light (Treatment 5). Callus from Treatments 1, 2, 3, and 5 averaged 339-830 mg. per piece on Medium 1 and 442-1237 mg. on Medium 2.

Figure 11. One Piece Each (l. to r.) From Treatments 1, 2, 3, and 5 Grown on Medium 1 (Top) and Medium 2 (Bottom)

Subculture 3

Only 2-6 of the best clones were subcultured, and then only to Medium 1 for Treatments 1 and 2, to Medium 2 for treatment 3, and to both media for Treatment 5. All of the tissue on the laboratory bench was lost from fungus contamination. Firm white tissue grew only on Medium 1, and was best in Treatment 1, worst in Treatment 2, and intermediate in Treatment 2. The only tissue remaining on Medium 2 (in Treatment 5) was poor in quality and growth. All tissue was discarded because of necrosis and generally declining quality by the end of the sixth subculture.

DISCUSSION

This study shows that various light environments produce quite different growth responses in triploid quaking aspen callus. Generally, all tissue was soft when initiated on the high-2,4-D Medium 2 for all except Treatment 3 on the laboratory bench. In this unusual case, firm white tissue, initiated in continuous weak light on Medium 2, looked quite similar to tissue grown in the incubator on the low-2,4-D Medium 1 reported in the first section of this report. Continuous strong light severely inhibited growth. The vigorous, highly-variable tissue growth in complete darkness was almost equalled in quality by the less vigorous but uniform tissue in the incubator receiving occasional strong light. Less vigorous than these two was the tissue grown continuously in the incubator which was opened occasionally to laboratory light. These relationships indicate a possible auxin-inhibitor system with two or more mechanisms. Hypotheses will not be made until fixed material is examined and correlative data analyzed.

For practical applications to our immediate objectives, callus initiated on Medium 2 under different light conditions may be useful in differentiation studies. Stock cultures can be obtained by transferring tissue grown in the dark from Medium 2 to Medium 1. However, after changing to firm white tissue, the cultures do not appear to be as uniform or of the same high quality as tissue both initiated and subcultured on Medium 1.

# OTHER STUDIES

## CELL SUSPENSIONS

From practically every major callus isolation made last year, cell suspensions were initiated and grown for 1-9 months. Cells of both triploid quaking aspen and <u>Populus canescens</u> averaged 20-40 µm. in diameter, and could be fairly well separated as single cells by pouring cell suspensions through a sterile screen 37 µm. in diameter (400 mesh). Single cells of both species, resuspended in liquid medium, grew into visible cell colonies up to 1-2 mm. long by .5-1.0 mm. in diameter. Growth stopped after six months and differentiation did not occur either in liquid or when embryoids were poured onto agar Medium 1. Differentiation of embryoids from single-cell origin is one of our major objectives.

## CLONAL LINES

From callus initiated during the past season and described earlier in this report, at least 15 clonal cultures were maintained through several passages. Cultures which have remained firm and white were those initiated and subcultured on Medium 1 and left uncovered in an incubator. Most of these strains have since deteriorated and were discarded. Three excellent strains continue, however, and are slowly being built up into usable cultures and are now in their seventh 3-week subculture (Fig. 12).

Our old strain 3x-f5 is also being maintained in closed one-ounce bottles on Medium 1. Three passages have just been completed of 41 days each, testing the growth of one explant per bottle on either a flat or a slant agar surface in the dark. About 13% of the pieces rooted during the second passage, but the growth data have not yet been analyzed. This tissue is now in its twenty-seventh subculture from origin and has remained uniformly firm and white.

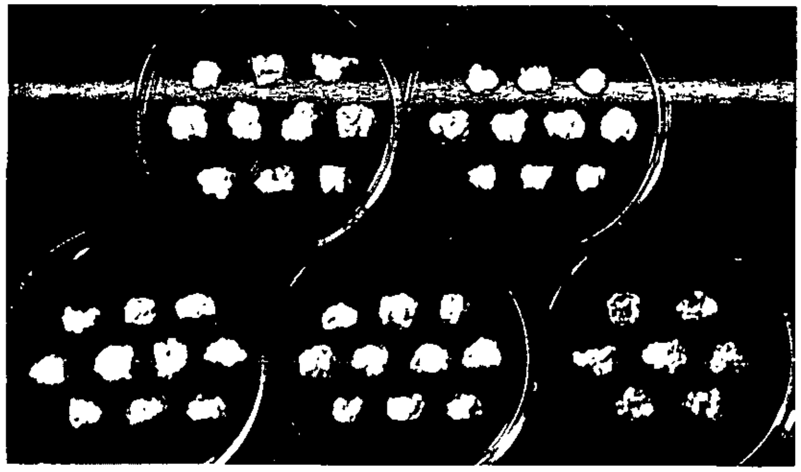

Figure 12. Four Uniform Firm White Strains of Triploid Quaking Aspen and One Necrotic (Lower Right) Variable Strain

FAST-SLOW CALLUS GROWTH

Mathes and Einspahr (5) observed a positive correlation between the rate of callus production on stem sections of diploid quaking aspen grown *in vitro* and the rate of tree growth under natural conditions. A series of tests was begun in 1965 to determine the relationship of callus growth on stem sections of 1-0 seedlings and their subsequent natural growth as trees. A positive correlation from these tests may lead to a useful predictive method for selecting fast-growing trees in the nursery.

During the fall of 1965, the top one-foot sections (Fig. 13) of ten seedlings 2.0 feet tall and ten seedlings 5.3 feet tall were collected, sterilized, cut, and grown on two media (Fig. 14) for 16 days. The second one-foot section was later collected from the same plants and callus grown for 4 weeks (not shown).

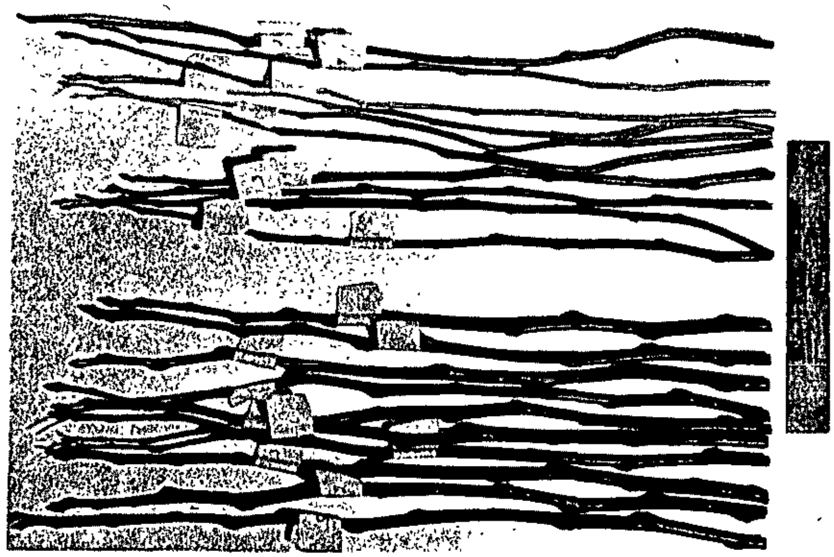

Figure 13. Top One-Foot Sections from Ten Seedlings Each of Quaking Aspen 2.0 (Top) and 5.3 (Bottom) Feet Tall

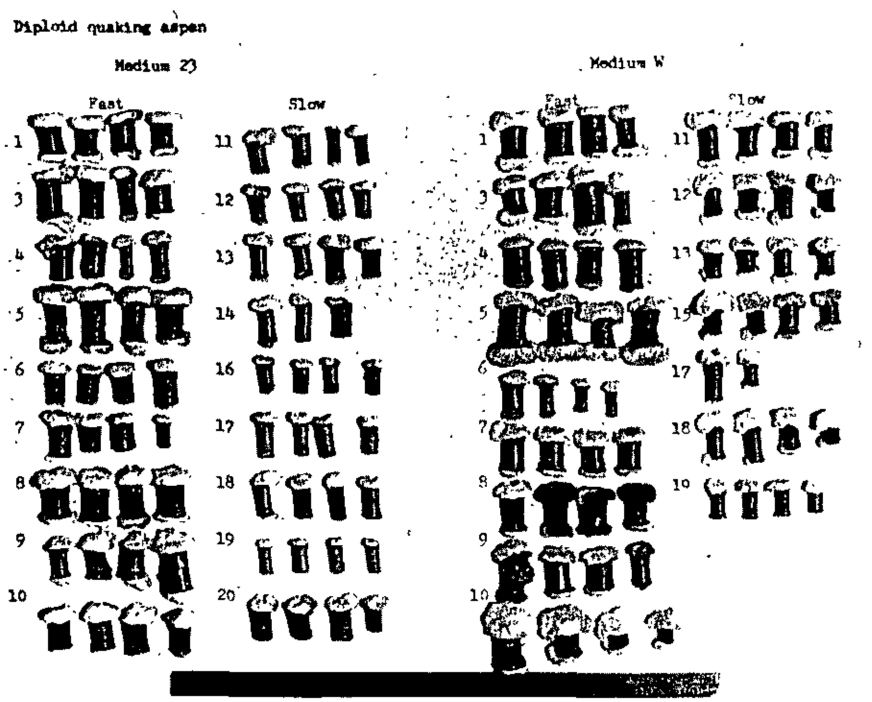


Figure 14. Callus Growth After Two Weeks on Segments From Tall (Fast) and Short (Slow) Seedlings

In 1966, collections were made from 12 tall and 17 short diploid quaking aspen seedlings, 12 sprouts each of two clones of triploid quaking aspen, 12 tall bigtooth seedlings, and 12 each cottonwood and Populus canescens. Each seedling height was measured, and for ten callus pieces per treatment, the callus plus segment was weighed, then the segment alone. All callus was subcultured for extensive growth studies, after each callus weight was estimated by difference and each segment length and diameter were measured.

All original seedlings were outplanted in the nursery and will be measured this fall. Height measurements for the next several years will be correlated with the average ratio of callus/callus plus segment for each species tested. The percentage of callus per segment ranged from 20-66% for tall and short diploid quaking aspen (Fig. 15). Callus growth was more uniform for triploid quaking aspen at 50-60% (Fig. 16).

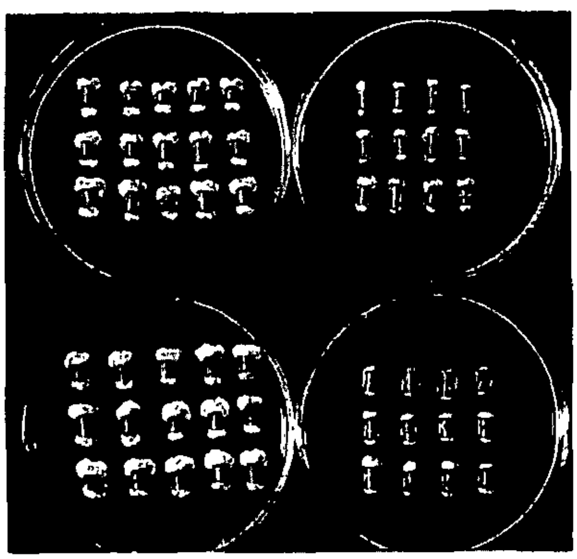

Figure 15. Callus Growth After 13 Days on Segments from Two Short (Top) and Two Tall (Bottom) Diploid Quaking Aspen Seedlings

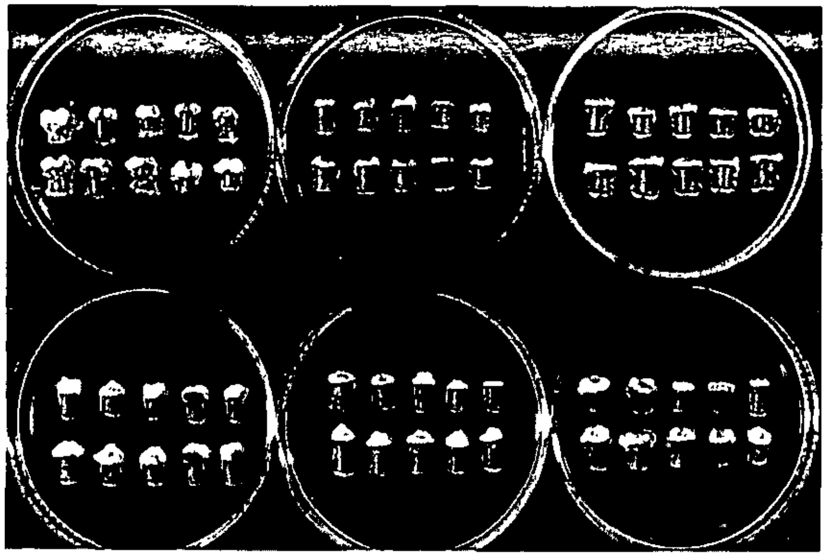

Figure 16. Uniform Callus Growth from Six Triploid Aspen Sprouts

Tree-height data are not yet available so correlations cannot be presented here. Plans for this fall included testing several hybrids between species tested last fall, as well as retesting more of the same species. The long-term nature of this study is evident, but a special report is anticipated after the first height measurements are analyzed with the callus ratios. This method will also be used this fall to evaluate the dozen or more natural clones of triploid quaking aspen growing in the Lake States region.

## POPULUS CANESCENS CULTURES

Twelve seedlings of *Populus canescens* were tested in 1966 for callus growth in the Fast-Slow study discussed in the previous section. Callus pieces from the best five seedlings (Fig. 17) were subcultured to fresh Medium W and grown for 12 days. All tissues were soft, but only the callus from seedling

122 was white. This tissue was subcultured several more times on Media 1 and W, and eventually gave rise to two distinct morphological strains governed entirely by the auxin level of the medium.

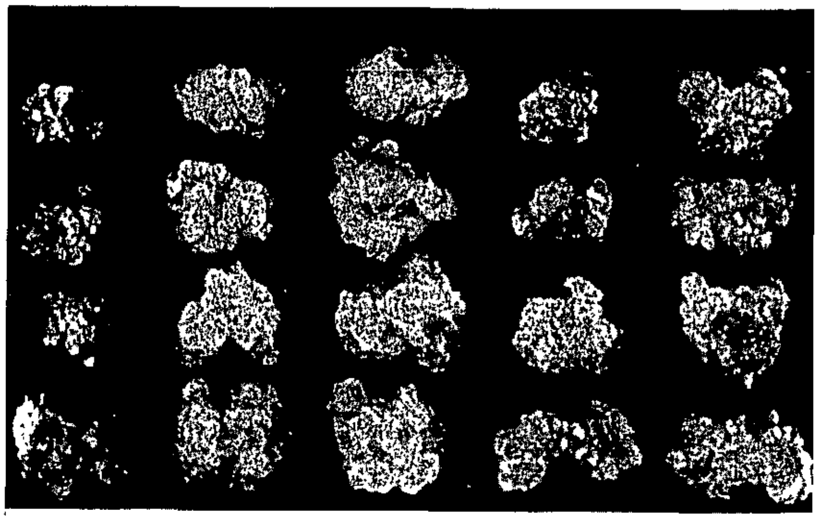

Figure 17. Callus Initiated on Segments from 1-0 Seedlings of Populus canescens. White Callus in Center Column is from Seedling 122 and Gave Rise to the Two Strains Shown in Fig. 18

Tissue grown on the high-2,4-D Medium W was vigorous, colorless, extremely friable, and dispersed well in liquid medium to give good cell suspensions (Fig. 18, left). Callus from the same source, but placed on the low-2,4-D Medium 1, was slow-growing but firm and white (Fig. 18, right). Both strains have been maintained through seventeen subcultures and appear to be constant in growth. Both strains grow slightly better in the dark but can also grow under strong light. In the light, they remain colorless or white and do not turn green.

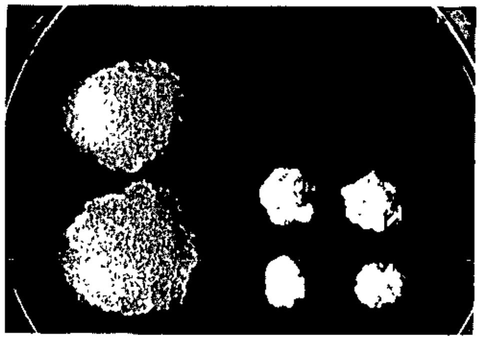

Figure 18. Friable (Left) and Firm White (Right) Callus Tissue, from the Same Source of Populus canescens, Growing Respectively on Media W and 1

BIGTOOTH ASPEN CULTURES

During the Fast-Slow callus initiation studies, bigtooth aspen callus was initiated very slowly in the dark and only slowly under strong light. Firm bright-green tissue has been maintained for almost a year on Medium 2, with subculture periods of 4-6 weeks. This tissue also grows well on Medium 1 in the light but does poorly on both media in the dark. This is the only aspen callus in culture that grows well in the light as a green callus.

## ACCOMPLISHMENTS AND FUTURE PLANS

Significant results obtained during the past year include the knowledge of the nutrient and light environments necessary to initiate and maintain either friable tissue for cell-suspension studies, or firm white callus necessary for differentiation experiments. Minor tests, not discussed in this report, include establishing optimum explant size and some pilot studies on nutrient levels in the medium. We have also found that single cells of Populus canescens and triploid trembling aspen can be filtered, resuspended, and grown to nondifferentiated embryoids. The main block in our program at this point is the lack of reproducible results involving shoot initiation. Emphasis this coming year will be on differentiation studies requiring large amounts of firm white callus.

Ball (6) successfully grew flowering plantlets of Didiscus coerulea on Wolter's (1) medium with no auxin. Our first large-scale experiment will be the growth of callus on (Wolter's) Medium 1 at 0, .001, .01, .1, and .01 mg./liter over a period of several weeks. The addition of auxins and other growth hormones may also be tried. The culture of bigtooth aspen, which grows naturally under strong light as firm green tissue, may possibly react more favorably to differentiation stimulae than tissues of either quaking aspen or P. canescens. Limited work will also continue on cell-suspension differentiation studies.

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Titles of papers by Winton from this report:

Aspen callus initiated periodically.
Growth rate of aspen callus.
Effects of light on aspen callus growth.

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Pioneering Research Committee
Project 2351

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