THE RESPONSE OF ISOLATED STEM SEGMENTS OF *SEQUOIA SEMPERVIRENS* (LAMB.) ENDL. CULTURED *IN VITRO* TO VARIOUS CHEMICAL AND OTHER ENVIRONMENTAL TREATMENTS

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By

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Submitted to the School of Graduate Studies of
Michigan State University in partial
fulfillment of the requirements
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AN ABSTRACT

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ABSTRACT

Stem segments, one centimeter in length, excised from *Sequoia sempervirens* burl shoots, were cultured *in vitro* under the influence of several different environmental conditions.

The basic medium contained Knop's Solution, Berthelot's Solution, three percent sucrose, two percent agar, naphthaleneacetic acid at several levels of concentration, and a number of growth supplementary substances: cysteine, certain B vitamins, ascorbic acid, and in one case, yeast extract.

The addition of three percent yeast extract to the medium along with the supplementary substances resulted in an initial inhibition of growth as well as the killing of some segments. Later, growth was greatly enhanced among the surviving cultures by these substances. At this time, the supplementary substances were more beneficial for the production of fresh weight in darkness than in light. An inhibition of buds occurred in darkness in the presence of the supplements. Yeast extract was omitted thereafter from the basic medium because it furnished unknown constituents to a medium of otherwise known chemical composition.

Naphthaleneacetic acid, at 100 gammas per liter, reduced and sometimes completely inhibited the initiation of buds.

The differentiation and growth of roots occurred in the absence of naphthaleneacetic acid but did not occur in the presence of 50 and 100 gammas per liter. Shoots grew vigorously in the absence of roots.

Maleic hydrazide inhibited all growth of the segments at 10, 100, and 1000 parts per million. No observable differences in growth or bud formation occurred between cultures with and without one part per million of maleic hydrazide.

The original position of the segment on the shoot and the original weight of the segment at the start of the experiment had no effect upon subsequent growth.

Room temperature (about 21°C.) provided a suitable, but not an optimum, temperature for the growth of segments. A controlled temperature of 24°C. allowed better growth than 30°C. At 6°C. buds developed slowly but normally. Buds were formed at 37°C. but they failed to develop and reverted to callus.

The reduction of aeration caused by the submersion of the segments resulted in a complete inhibition of buds and a reduction in the amount of callus produced; the controls, receiving normal aeration, produced numerous buds and a large amount of callus.

Adenine sulfate at 40, 80, and 120 milligrams per liter inhibited bud initiation and fresh weight production by the segments. The inhibition of buds and of fresh weight by adenine sulfate was partially alleviated by the presence of increasing concentrations of naphthaleneacetic acid at 50 and 100 gammas per liter.

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INTRODUCTION

In the study of physiological problems, plant tissue cultures possess an important advantage over entire plants in that the tissue, consisting mainly of masses of undifferentiated cells, accomplishes its growth free from the influence of such organs as shoots, buds, or roots (Ball, 1950). Thus, several factors of plant development, such as the influence of organs upon cellular development, can be eliminated and the excised plant part, or mass of cells, can respond only to a simplified environment. In a medium of known composition, the resulting growth theoretically can be interpreted in terms of the stimulating or inhibiting influence of known physical and chemical conditions. In this manner, the functions of a few cells and of the conditions necessary for their optimum growth can be studied. For these reasons, White (1954) prefers the term "cell culture" to that of "tissue culture".

Often, however, a complicating factor may arise in otherwise uniformly growing tissue cultures by the unexpected differentiation of roots or shoots. This return to organized growth results in the synthesis of unknown quantities of metabolic substances consequently disrupting the carefully prepared balance of the original medium. For example, in a study of growth regulators, the known amount in the medium may be augmented by certain quantities of auxins synthesized in newly developed shoot tips. Furthermore, carbohydrates, vitamins, and other substances may be increased over and above the kind previously added in measured amounts. Ultimately, the culture may produce both shoots and roots becoming, in effect, an entire plant with nearly normal reactions. The response of these cultures to carefully integrated and controlled environments is no longer relatively simple and the advantages of the tissue culture have been lost.

Tissue cultures, from the standpoint of origin and development may be placed into four categories (White, 1946):

1. Embryo
2. Organ
3. Plant fragments
4. Excised cell masses.

Cultures of plant fragments are most often used to obtain an initial mass of cells by proliferation capable of indefinite growth. The addition of certain growth regulators in minute amounts is usually necessary except in the case of tissues derived from plant tumors (White, 1939b) and in the case of certain normal tissues which have spontaneously developed the ability to synthesize the needed auxins after some time in culture (Gautheret, 1946, 1947e).

The basic differences in the response of tissues to environment cannot be foretold with precision, nor can the results obtained with one type of tissue be generalized for all types. The reactions of a great number of species, as well as of organs and tissues must be studied, evaluated, and interpreted before the interrelationships of cell behavior become apparent.

During the course of several attempts to derive a true tissue culture from stem segments of Sequoia burls, the resulting callus, often for no apparent reason, gave rise to shoots and roots. Such cultures, obviously, could not be used for nutritional studies and were discarded because of the lack of control over the influence of the organs upon the development of the culture. The loss of these cultures, in addition to the unpredictable loss from contamination, often reduced the number of successful cultures within a treatment to a point too low for any degree of confidence in the results. In addition, the disconcerting factor existed that some cultures in a series produced organs, whereas others of the same treatment did not. Thus some, but not all segments maintained the capability to produce shoots and roots in nutrient media assumed to be identical in chemical constituents.

The nutrient media had been carefully prepared with known constituents so the only other alternative was to widen the conditions of environment among treatments to determine whether or not certain treatments exerted a preferential effect upon bud or callus production. Suitable variations in environment could lead to a set of conditions whereby the formation of buds could be reduced to a minimum or, at best, to absolute control.

A review of the literature revealed that the spontaneous differentiation of buds and roots in tissue cultures was far from rare. In studies upon the control of buds, three general methods of control had been offered: (1) suppression, by high levels of growth regulators (Thimann and Skoog, 1933), (2) induction, by high levels of certain purines (Skoog and Tsui, 1948, 1949), (3) induction, by low levels of oxygen tension (White, 1939a). Again, the few types of tissues utilized in these studies prevented the formation of generalities applicable to *Sequoia* tissue, but these experimental conditions suggested an excellent starting point for an investigation into the conditions necessary for bud suppression and callus production in *Sequoia* cultures. The shoots from *Sequoia* burls in constant production offered a convenient and uniform year-round source of material.

A series of experiments was devised to seek information concerning the following characteristics of *Sequoia* burl stem segments in culture: (1) The procurement of true tissue cultures of indefinite duration, (2) The experimental control of bud, root, and callus types of proliferation, (3) A comparison of the effects of certain physical and chemical conditions upon organ and callus formation in *Sequoia* cultures with similar effects upon organ and callus formation in the tissues of other species *in vitro*.

REVIEW OF THE LITERATURE

In order to properly appreciate the use of tissue cultures as a method or technique of plant research, it is necessary to return briefly to the work of the latter part of the last century and to trace two separate lines of research which merged in 1939 to make possible the realization of true tissue cultures.

The first line of research began in 1880 with the observation of Charles and Francis Darwin (1885) upon the action of light in the bending of coleoptiles of Phalaris and Avena. They proved by various means that a certain stimulus perceived by the top of the coleoptile was transmitted to the base resulting in a bending of the coleoptile toward the light. They stated: "These results seem to imply the presence of some matter in the upper part which is acted on by light and which transmits its effects to the lower part."

Boysen-Jensen (1910) followed the course of the stimulus and determined that it could pass a wound — as a matter of fact through a separating layer of gelatine inserted between the tip of a coleoptile and the stump.

Paál (1914-1919) repeated the work of Boysen-Jensen and further determined that a growth regulating substance diffused downward from the tip of a coleoptile through the living tissue, exerting an influence on growth in the tissues below. If the distribution of the downward diffusing substance were disturbed on one side, the growth on that side was decreased and a curvature toward the light resulted. Paál concluded that a hormonal substance was involved.

The next steps resulted in the extraction of the active substance into agar and in a method of measuring the concentration of minute amounts of the growth substance (Went, 1928) and the actual identification of definite chemical substances of known structure (Kögl, Haagen-Smit, and Erxleben, 1934). These workers established the identity of indoleacetic acid as a chemical of great activity in the Went test.

Other substances, too numerous to mention, active as growth regulators were discovered. By means of the concept of plant growth regulators the various tropisms, apical dominance, and other correlation phenomena of plants were explained in theory.

Meanwhile, another line of research began slowly with failure, but progressed later to convincing success. Haberlandt (1902) had repeatedly failed to obtain true tissue cultures from cells and tissues of mature plant parts. He is, however, generally accredited with the establishment of the concept of tissue cultures.

One of his students, Kotte (1922) and simultaneously Robbins (1922) developed the idea that the use of meristematic cells held the best chances of success in obtaining tissue cultures. For various reasons, roots were chosen and the resulting cultures in the appropriate media gave rise to growth of the roots and to cell division for limited lengths of time. These, of course, were not true tissue cultures for two reasons: the cultures so derived were of a complete organ and were of limited growth.

White (1934), using yeast extract as a substance containing the then unknown growth factors, was first to obtain unlimited growth of excised roots. The growth factors were soon identified as components of the B vitamins which were shown to be manufactured in the upper portions of plants, transported to the roots where they acted as indispensable factors of growth for the roots. Not all roots of various species were found to require the same vitamins.

Gautheret (1939) Nobécourt (1939) and White (1939b), working independently, finally established what may be called the unlimited proliferation of undifferentiated cells — true tissue cultures. Gautheret and Nobécourt made use of the growth-inciting chemicals naphthaleneacetic acid and indoleacetic acid. The tobacco tumor tissue of White apparently needed no growth substance which was present in sufficient amounts to cause proliferation of the cells. This fact apparently hindered the American workers because up to 1946 no tissue cultures other than of the tumorous type were successful in the United States (White 1946); whereas, the French workers continued to increase the number of species of dicotyledonous plants in tissue culture. Thus two apparently separate lines of research involving growth substances and tissue cultures were brought together. A great number of publications followed along the questions of the action of the growth substances upon morphological and physiological processes. Tissue cultures both in the strict and broad sense were utilized for many studies.

The use of tissue cultures as a tool in the study of the effect of developing buds on subjacent tissues was developed by Camus (1949). By grafting buds on to callus tissue he was able to demonstrate an influence of buds upon the differentation of cells.

The problem of organ formation in tissue cultures is complex and difficult. Not all cultures of the same species or even of the same organ or tissue can be relied upon to react in an exactly similar manner. In the entire plant, the "caline" theory of Went offers an insight into the process of organ formation or, at least, the effect of some organs upon others, but the spontaneous generation of shoots and roots from unorganized tissue seems vague and without correlation. White (1939a), using tobacco callus tissue, made the observation that submerged cultures maintained in the air upon solid media tended to remain in a relatively undifferentiated condition. White attributed the difference in response to differences in the oxygen tension. Thus, organ formation in this case, at least, was apparently tied up with biological systems of anaerobic or low oxygen requirements. Here was the first case of the diversion at will of a long established callus culture from undifferentiation to organ formation.

The concept of the control of bud formation by means of variations in the adenine - auxin ratio received its early impetus from the work of Thimann and Skoog (1933) in which they demonstrated that the inhibition of lateral buds by the terminal shoot was caused by the auxin production of the inhibiting terminal bud. Skoog (1939), using Pisum embryos from which the buds had been excised, showed that growth, as measured by an increase in length of the buds, was inhibited by indoleacetic acid at 0.8 to 10 milligrams per liter. It was clear that auxins could inhibit bud growth at certain concentrations.

Adenine was found to influence leaf size and general vegetative growth by Bonner and Bonner (1940) at concentrations of 0.001 milligrams per liter.

Jones and Baker (1943) found inhibition of seedling growth by adenine at $2x10^{-3}M$ and $1x10^{-3}M$. The activity of adenine, aside from being a part of the nucleotides of nucleic acid, was not clear.

Skoog (1944) using tobacco tissue cultures again was able to completely inhibit differentiation by 0.2 to 10 milligrams of naphthaleneacetic acid and indoleacetic acid and Gautheret (1947a) obtained bud inhibition in cultures of carrot and endive with 10^{-4} grams per liter of indoleacetic acid and with 10^{-5} naphthaleneacetic acid.

Loo (1945) using adenine at the concentration of 0.1 milligrams per liter on asparagus stem tips and deRopp (1945) at the rate of 10 gammas per cubic centimeter on stem tips of rye could find no increase in growth in either tissue.

However, the importance of adenine began to emerge when Skoog and Tsui (1948) suggested that organic complexes containing adenine might be involved in bud formation since high sucrose and high phosphate contents of the medium made necessary high auxin concentrations to prevent the formation of buds and the inhibition of callus growth. They could increase the number of buds per callus piece by the use of adenosine which at 2.0 milligrams per liter with additional phosphate was as effective as 50 milligrams per liter of adenosine alone. They determined further that the inhibitory effects of 0.25 milligrams per liter of naphthaleneacetic acid could be counteracted even though the range of complete inhibition was 0.02 to 0.32 milligrams per liter. They concluded that buds could be induced chemically and extended the previous work to include indoleacetic acid as well as naphthaleneacetic acid. At 40 milligrams per liter, adenine sulfate caused a total of forty buds on a 150 milligram piece of tissue as against no buds on the controls.

Further evidence of the presence of adenine in significant amounts in buds was furnished by Koritz and Skoog (1952) during the course of developing a colorimetric determination of adenine. Buds of pea contained ten times as much adenine as did stems on a fresh weight basis and cotyledons, nodes, and roots contained about three times as much as stems. The adenine content of tobacco was found to be lower, confirming a previous suggestion (Skoog and Tsui 1949) that the concentration would vary with the species.

The true action of adenine was not clear. Silberger and Skoog (1953) studied the interaction of indoleacetic acid with purines and pentoses in organ differentiation and suggested an effect of auxins on nucleic acid metabolism. They used tobacco pith segments. Desoxyribonucleic acid increased most at 0.014 milligrams per liter of indoleacetic acid and pentosenucleic acid at about 1.4 milligrams per liter.

Miller (1953) found that 80 milligrams per liter of adenine sulfate overcame the inhibition of callus and bud formation by 50 milligrams per liter of 2, 6 diamino-purine on tobacco stem segments. This fact indicated the participation of purines, especially adenine, in cell enlargement and cell division in plants.

Miller and Skoog (1953) made an attempt to solve the adenine - indoleacetic acid interaction. Adenine at 40 to 80 milligrams per liter increased the number of buds per segment without any effect upon their size or subsequent development. Although ten gammas per liter of indoleacetic acid gave almost complete inhibition of tobacco buds, seventy-five milligrams per liter of adenine sulfate overcame the inhibition of 5 gammas per liter of indoleacetic acid and 115 milligrams of adenine sulfate overcame the inhibition of 30 gammas per liter. They concluded that the action of adenine was specific to bud formation.

In 1953 Skoog patented a process considered to be essential for the induction of bud formation in plant tissues (U S 2,653,087) utilizing the following medium:

10 - 40 milligrams per liter	KH_2PO_4
1 - 100 milligrams per liter	Amino purine compound. Adenine-sulfate, Adenylic acid Guanine
0.7 - 1.5 per cent	Agar-agar
	p H 4 - 6

Minute amounts of growth regulators.

Wiggans (1943) compared the response of carrot tissue to various growth substances with similar responses of tobacco stem segments. He obtained increased callus production with several growth regulators up to 10 milligrams per liter. Adenine sulfate gave an optimum increase in callus at 40 milligrams per liter but 360 milligrams inhibited callus. Adenine sulfate, furthermore, did not stimulate the initiation and formation of buds. He suggested that, in carrot tissue, auxin is probably naturally present in amounts high enough to prevent the activity of adenine in the range that it can be added without toxicity, and that the same chemicals do not have the same effects upon different species or even tissues.

Howell and Skoog (1955) studied the effect of indoleacetic acid and adenine sulfate on the growth of excised *Pisum* epicotyls. They found that 40 milligrams of adenine sulfate was optimum for growth but that one milligram per liter of indoleacetic acid increased the effectiveness of the 40 milligrams per liter thus giving rise to the opinion that the interaction of the two substances is "not merely a question of competitive inhibition between them."

MATERIALS AND METHODS

General. The plant material chosen for the following experiments consisted of stem segments excised from shoots growing from burls of the Redwood tree, Sequoia sempervirens (Lamb.) Endl. These burls were purchased at various times of the year from a commercial source in California.

Upon arrival at the laboratory, the burls were unwrapped, washed, and placed in a container with enough water to keep the surface of the burl moist. Numerous buds appeared almost immediately, soon giving rise to a lush green growth of shoots. As soon as the shoots had grown to a length of about twelve inches (PLATE 1) they were cut at the base from the burl. All of the leaves were removed. Then the shoots were cut into segments one centimeter long by means of a sharp razor.

In previous work, the standard method for the insertion of the segment in agar required that the segment be inverted in order to take advantage of the proposed basipetal movement of auxins through stem tissues. The means adopted to quickly and accurately identify the physiological apex of the segments consisted of a system of slanting cuts. A fresh green shoot was placed upon a block of wood which had a metric scale in centimeters marked upon its surface. Starting at the base of the shoot, a slanting cut was made at a distance of one centimeter, thus removing a segment, cylindrical in shape, with a slanting cut at the physiological base and a cut, perpendicular to the shoot axis, at the apical end. The slanting cut remaining on the shoot was squared off, resulting in the removal of a small wedge of tissue. The entire process was repeated at one centimeter lengths along the stem. The growing point was discarded. When it was desired to place the true basal end of the segment in agar, as in experiments on polarity, the method was reversed. In this case, the basal end of the segments possessed the slanting cut. In both cases, regardless of which end was placed in agar, the aerial portion always received a cut perpendicular to the shoot axis.

The segments were surface sterilized by an immersion for five minutes in 2.5 percent sodium hypochlorite (Chlorox) followed immediately by a washing in distilled water. All water used in these experiments was distilled twice in pyrex glass. In the latter experiments, it was necessary to keep each segment separate from the others. This separation was accomplished by the use of compartmented refrigerator ice trays for the sterilization and the use of corresponding ice trays for the washing. This system was also used during the weighing procedure.

After washing, each segment was picked up in sterile tweezers and inserted to half its length in the nutrient agar (PLATE 2).

The nutrient medium of Gautheret (1942), adapted by Ball (1950, 1953a) was used as the basic medium for modification in all treatments (Table 1). All components were added before autoclaving. However the mixture was warmed to melt the agar which, as a uniform mixture,was distributed in twenty-five milliliter lots into 22 x 150 milliliter

test tubes. These tubes were plugged with cotton and autoclaved at 20 pounds pressure and 250° F. for twenty minutes. Upon cooling, the tubes containing the solidified nutrient agar were ready to receive the stem segments cut from the burl shoots. The mouth of the tube was flamed, the segment inserted and the mouth reflamed. After the replacement of the cotton plug, the tube was numbered, capped with parafilm, and stored in the appropriate place.

This investigation of the responses of excised segments to environmental conditions was divided into six parts. The basic procedure described above was followed carefully and changes were incorporated into successive experiments only when necessary to meet the special conditions of the experiment.

Room temperature, as used throughout these experiments, was maintained by means of thermostats at approximately 21°C.

Experiment I. The effects of several growth supplements, of light, and of polarity upon the subsequent growth of excised segments were studied. Four hundred cultures were distributed among the treatments shown for Experiment I in Table 1.

A battery of fluorescent lamps, placed two feet above the cultures, furnished continuous light of about five hundred foot-candles. Those cultures receiving a dark treatment were placed in light-proof drawers in the same room.

The segments were placed in culture according to the described procedure. Half of the segments were inverted in the agar, the other half were not. Also, within each treatment of polarity, half of the segments were placed in continuous light and the other half in continuous darkness. Cultures with and without growth supplements were also placed among the treatments.

All cultures received the same medium except those which received, in addition, a number of growth supplements. These supplements consisted of yeast extract, cysteine and certain vitamins as shown in Table 1. The other components of the medium are also shown in Table 1. All cultures received Knop's solution, Berthelot's solution (1934), ascorbic acid, and sucrose.

At intervals of about one week, certain observations were recorded for each individual culture within each treatment. These observations consisted of the weekly progress of successful cultures, the relative formation of shoots, roots and callus, and finally upon the amount of fresh and dry weight produced by the cultures. A certain number of cultures was preserved for future investigation should the growth and proliferation become indefinite.

Experiment II. The effects of maleic hydrazide, of original position on the shoot, and of initial weight upon the relative production of shoots and callus were studied.

The basic medium was modified so as to include 0, 1, 10, 100 and 1000 parts per million of the growth-inhibiting chemical, maleic hydrazide (Table 1). Other changes included a reduction in the amount of Knop's solution, an increase in the amount of Berthelot's solution, the omission of yeast extract and ascorbic acid, and the substitution of glucose for sucrose. Furthermore, naphthaleneacetic acid was added at the rate of 100 gammas per liter to all cultures.

The shoots were selected at random from two burls and cut into lengths of one centimeter. The terminal three centimeters portion of each shoot containing the growing point was discarded. Each segment was marked to preserve the apical direction. In order to introduce the five treatments of maleic hydrazide, groups of five segments, in sequence from the apical end, were given the five treatments of maleic hydrazide at random within the groups (Figure 6). The purpose of this method was to maintain a record of each individual segment in regard to its original position on the stem as well as to its original weight and treatment with maleic hydrazide. Each segment was individually weighed, sterilized, and inserted with its physiological apex in the nutrient agar. The cultures were placed randomly on a bench under continuous light of about 500 foot candles.

Observations were made at three week intervals on the number of buds and shoots, the presence or absence of callus cells, and of the general condition of the cultures. The segments, however, were not weighed at the end of the experiment because of the unavailability of a balance at the critical time. The toxicity of maleic hydrazide at various concentrations was also observed.

<u>Experiment III</u>. The effects of several differences in temperature upon growth of cultures were studied in order to determine whether or not the usually selected room temperature, with its lack of absolute control, was suitable for optimum growth of the tissues.

The selection of constant temperatures for this experiment depended more upon availability than upon choice. Cold rooms and incubators not in use by other agencies were available at temperatures of 6°, 24°, 30° and 37° Centigrade. Several laboratories with an average of 21° Centigrade as a room temperature were utilized to store cultures for comparison with the growth obtained from cultures in the several controlled temperatures. All cultures were maintained in darkness except one group at room temperature in diffuse daylight.

The medium selected varied very little from that of Experiment I (Table 1) except that biotin was added at the rate of one milligram per liter and naphthaleneacetic acid was added at the rate of 100 gammas per liter in an attempt to inhibit the formation of buds. The medium for Experiment III became basic for all subsequent work.

The original intention in this experiment involved constant additions of cultures to the temperature treatments and the termination of these cultures at various lengths of time. However, circumstances caused the initiation of only two sets of cultures which were allowed to run for three time lengths (Tables 14 and 15).

The segments, prepared from fresh shoots, as previously outlined, were inserted with the distal end in the agar. The two sets of cultures were started five months apart.

At the termination of the time chosen, the cultures were weighed individually and the final weight of shoots and callus was determined as well as the increment of growth as expressed by gain in weight of each culture averaged for the treatment (Table 14). In addition, information on the number of buds and the total length of shoots was compiled (Table 15).

Experiment IV. The effect of reduced aeration upon the production of buds on excised segments was studied.

The nutrient medium was not changed in any way from that of the two previous experiments.

The segments, prepared in the usual manner, were placed in the agar in an inverted position. In half the cultures, reduced aeration as compared to the controls, was achieved by adding five milliliters of distilled water by means of a syringe to the completed culture. This treatment completely submerged the culture in most cases (PLATE 7). Both sets of cultures were stored in daylight at room temperature. Each segment was weighed before and after the experiment.

Observations were made upon increase in weight and number of buds produced at the end of the experiment.

Experiment V. The effect of inversion of the polarity upon the subsequent growth of excised segments was studied.

The basic medium of Experiment III (Table 1) was used in its entirety. In the attempt to block the movement of growth regulator from the nutrient, through the segment, to the aerial portion of the segment, the segment was placed in the agar with the basal end downward. An equal number were inverted in the agar so that the growth regulator would have the normal path of translocation.

The segments were weighed before and after the experiment and the dry weight and percentage dry weight for each individual culture was determined.

Experiment VI. The effect of combinations of adenine sulfate and naphthaleneacetic acid upon the production of buds by excised segments was studied.

The procedures outlined in the three previous experiments, both for the preparation of segments and for the integration of the medium, were not changed except to accomodate several concentrations of the two chemicals (Table 1). Adenine sulfate was added at the rates of 0, 40, 80 and 120 milligrams per liter to three sets of cultures containing naphthaleneacetic acid at the rates of 0, 50 and 100 gammas per liter.

Each individual culture was weighed before and after the experiment. Observations were made upon the increment of growth, the percentage dry weight and the number of buds and shoots produced.

EXPERIMENTAL RESULTS

Experiment I

Light, Growth Supplements, Polarity

Bud and Callus Growth. Two weeks after the implantation of the stem segments in the nutrient medium, small nodule-like masses of cells appeared on the sides of the segments. The difference between bud and callus development could not be determined with accuracy until the fifth week; so, initially, the percentages of cultures that produced any type of proliferation were computed and recorded for the weekly intervals (Table 2). Once started, the computation of these percentages was continued biweekly beyond the fifth week until the end of the experiment at the tenth week.

Such very small differences existed between treatments of the normal and inverted placement of the segment in agar (Figures 1 and 2), that the data for these two treatments were combined and reproduced graphically in Figure 3. The curves of Figures 1, 2, and 3 all indicate that the segments cultured in the light without growth supplements began to display external growth at an early and rapid rate. The other treatments of light and growth supplements show nothing conclusive except that those segments in darkness, without growth supplements, were less successful, at the end, in producing external proliferations. It is apparent that continuous light in the absence of growth supplements exerted a great influence upon the early ability of the cells to increase.

After the fifth week, the growth pattern of the cultures, in regard to the formation of buds, began to appear. Buds could be distinguished from the nodules of callus with certainty. An attempt then was made to determine which treatments had the greatest tendency to produce buds and, conversely, callus as expressed by percentages of the total number of cultures over a period of time. Accordingly, at intervals of two weeks until the end of the experiment, these calculations were made (Table 3).

Analysis of the figures shown in Table 3 indicates significant differences between the supplement treatments in darkness. The percentage of cultures producing buds over a five week period of time was much less in darkness with supplements than without supplements.

Other differences between treatments, such as between normal and inverted segments, appear to be present but, since the observations are expressed as percentages, and not of number of buds, the differences may not actually be significant. By plotting the data on Figures 4, 5, and 6, an inhibition of the ability of cultures to produce buds, as expressed by percentages of the total, can be observed in cultures maintained in darkness and furnished with the certain growth supplements. Conversely, this treatment enhanced the ability of the cultures to form callus.

No treatment completely prevented the initiation of buds. The results are, therefore, a matter of degree of control, rather than of absolute control. According to the limits set up in this experiment, about fifteen percent of the cultures grown in the dark with growth supplements produced buds; whereas, about eighty-five percent of those grown in the dark without growth supplements produced buds (Figure 6). Thus, some tendency toward the inhibition of buds appears to be possible in the light depending upon whether or not the growth supplements were added to the medium.

Data regarding the number of buds per segment offer a better basis for conclusions regarding the ability of the segments to initiate buds than the previous percentage data.

Records of the number of buds per culture were taken on the eighth and tenth weeks of the experiment. The data for the two periods showed no differences, therefore, only the data for the tenth week are shown in Table 4. These data show significant differences between light and darkness in the number of buds produced per segment. A greater number of buds per segment is produced in the light than in the dark. This condition was always obvious when the periodic observations were made. The cultures in the light that possessed shoots almost invariably had clusters of short shoots. Nearly all cultures in the dark had long, etiolated shoots, very few in the dark with supplements and a few more in the dark without supplements. The influence of developing shoots upon the growth of other shoots or upon the callus itself was not studied.

<u>Fresh and Dry Weights</u>. Eighty-eight days after the start of the experiment, the cultures were opened and the tissues were weighed individually in the fresh condition and collectively for each treatment in the dry condition (Tables 6 and 7). The rather large probable error

associated with each mean average weight for each treatment indicates considerable variability among segments. But the figures show clearly that much more fresh weight was produced in the light than in the dark. There were no significant differences between supplement treatments in the light nor between inverted or normal segments. In darkness, however, a significant difference existed between the supplement treatments. A greater amount of tissue was produced in the dark as a result of the addition of the supplement. The same general conclusions can be drawn for the final dry weight.

In regard to the figures for the percentage dry weight, (Table 8) no significant difference can be found. The conclusion must be reached, therefore, that within the limits of the experiment, no differences exist in regard to the relative production of dry matter among the treatments.

Although no significant differences are apparent in the fresh weight data in regard to those cultures in the light with and without growth supplements (Table 6), the actual appearance of these cultures, during the latter stages of the experiment showed great differences. The cultures maintained in the light with supplements formed vigorous, dark green, thick growths of shoots and callus; whereas, the segments in the light without supplements appeared to lack vigor and color. Had the experiment progressed longer, the segments given growth supplements would have surpassed, no doubt, the others in wet weight. Evidence to this effect is brought out in Table 5. The segments which had been set aside for another investigation were weighed one year after the end of this experiment. The differences in weight are significant and the conclusion can be reached that the supplementary substances greatly enhanced growth over a long period of time.

Dead Cultures. Some of the segments receiving the supplementary substances began to die early in the experiment. At first it appeared that the supplements, probably yeast extract, possessed an inhibitory action upon growth, but, when surviving segments showed excellent growth, the early deaths of these segments became of interest. The percentage of cultures that failed to grow (disregarding contamination) are tabulated in Table 9. Those in the light with supplement show a high percentage of deaths. No other relationships are apparent. Thus, throughout the whole experiment, fewer segments survived in the light with supplements than in any other treatment. These results indicate an early toxic effect of the growth supplements. The toxic property gradually disappeared until at the end of the experiment the growth supplements actually enhanced growth among surviving segments.

Roots. Toward the end of the experiment roots began to appear, although the initiation of roots was not expected at so low a level of auxin. The roots did not show any specific inclination to grow into the agar. A count of the total number of roots showed 31 growing into the agar and 47 into the air above the agar. Another observation regarding roots revealed that roots were initiated following the production of shoots in twenty-five cultures and appeared before shoots in only seven. On the other hand, shoots grew in the complete absence of roots in 139 cultures.

Downward Growing Shoots. In fifty-three cultures, the buds gave rise to shoots which grew downward into the agar (PLATE 3). There was a slight indication that most of these shoots occurred in the cultures not furnished with growth supplements but they were so few in number that no conclusion can be drawn. As a matter of interest, a few shoot tips normally discarded were inverted in nutrient agar with the result shown in PLATE 4. These tips showed excellent growth into the agar without the formation of roots at any time. The growth of these excised shoots can be observed by the amount of penetration into the agar.

Table 10 represents a balance sheet concerning the dispositions of the four hundred cultures at the end of the experiment. Observations on growth were made with the totals shown at the top of the table; whereas, observations on final weight were made on somewhat fewer samples because some cultures were saved for future experimentation.

Experiment II

Maleic Hydrazide, Initial Weight, Position

In previous work, all shoots and segments were removed from the parent material without consideration as to position, relative age, or initial weight. The segments were excised as uniformly as possible. The variability of final wet weight within treatments in Experiment I led to the suggestion that the original location of the segment on the shoot might have an influence upon subsequent growth, and that variations in original weight might be responsible for the observed variations in the amount of callus produced. An experiment accordingly was set up to test this hypothesis within the usual limits of the technique utilized for the cultures. The experimental design is shown diagrammatically in Figure 7. A study of the action of maleic hydrazide as a growth inhibitor was superimposed upon the study of the effects of position and original weight.

Examination of Table 11 shows that the original weights of segments given the several treatments of maleic hydrazide were remarkably uniform in the various distances from the apex of the segment.

Maleic Hydrazide. The action of maleic hydrazide as a growth inhibitor is very clearly shown in Figure 8 in which maleic hydrazide at 10, 100, and 1000 parts per million gradually killed cultures until the twelfth week at which time all of these cultures, except a few at ten parts per million, were dead. The cultures receiving no maleic hydrazide and those receiving one part per million progressed equally well until the twelfth week at which time these cultures began to die. The deaths of these cultures, after the twelfth week, was caused by the development of an unknown deficiency in the medium as shown by the fact that the rate was approximately equal for the controls receiving no maleic hydrazide and for the one part per million treatment.

In spite of the toxicity of 10, 100, and 1000 parts per million of maleic hydrazide to the *Sequoia* segments, several cultures in each series became contaminated with bacteria and fungi of undetermined identity. Apparently the toxicity of maleic hydrazide at this range did not affect the growth of these microorganisms.

Shoot and Callus Growth. As in Experiment I, the time course of the treatments upon the cultures, as expressed by the production of external proliferations, was followed. The 100 and 1000 parts per million treatments of maleic hydrazide produced no external growth and the 10 parts per million treatments produced a negligible amount of growth. These treatments, therefore, receive no further consideration. In regard to the controls and the 1 part per million series (Figure 9), no difference in the production of external proliferations can be observed. After the twelfth week, a necrosis of tissues is evident.

The effect of maleic hydrazide as a growth inhibitor was followed in the controls and in the cultures receiving 1 part per million of maleic hydrazide. Figure 10 shows the nearly equal amount of growth by shoots in both series. Thus, maleic hydrazide, at 1 part per million, did not inhibit the elongation of shoots. It must, however, be remembered that no shoots were formed at the 10, 100 and 1000 parts per million maleic hydrazide treatments.

The number of shoots and buds produced by each treatment is shown in Table 12. Again no significant differences occur between the controls and the 1 part per million treatment.

Table 13 also shows the parallel ability of both treatments to produce callus. The reduction in percentage of cultures after the twelfth week is included to show that an unknown factor other than maleic hydrazide had become effective in killing the tissues.

Position and Initial Weight. In spite of the fact that 10, 100 and 1000 parts per million of maleic hydrazide had completely killed all tissues by the twelfth week, the characteristics of the experimental design permitted a consideration of the effects of position and initial weight upon the ability of the segments to proliferate. The data shown in Figure 11 portrays clearly the fact that the proliferation of callus and shoots was not confined to any position or weight group within the limits of the experiment. At the twelfth week, almost all the cultures receiving the two treatments were growing satisfactorily. Four percent of the losses which occurred among the total number of segments was caused by death of the segment and fifteen percent was caused by contamination of the culture.

The growth of the surviving cultures of this experiment was inferior to that of the first experiment. Terminal wet weights of the cultures were not taken.

Although roots occurred in Experiment I without naphthaleneacetic acid, no roots appeared in this experiment with naphthaleneacetic acid at a concentration of 100 gammas per liter.

Experiment III

Temperature

Shoot and Callus Growth. This experiment consisted of two trials as nearly alike as possible. However, some minor differences in the treatments existed. The first, begun in April, contained cultures with segments weighing initially about 160 milligrams; the second, begun in September from another burl, contained segments weighing about 80 milligrams (Table 14). The original segments all measured one centimeter in length. Furthermore, in the interpretation of the effects of temperature in this experiment, three different time lengths must be considered.

In regard to the production of shoots and buds, the principal difference between the two trials lies in the fact that, although some buds occurred in the first trial, none occurred in the second trial (PLATE 6). Naphthaleneacetic acid at 100 gammas per liter completely inhibited the formation of buds in one case and not in the other although the number of buds produced was small (Table 15). The shorter time length of the second trial does not account for the absence of buds which always appeared within thirty days in other experiments.

The presence of a few buds and shoots in the first trial, however, made possible a comparison of the effects of temperature upon their relative number and subsequent growth. According to the data of Table 14, the maximum production and growth occurred at 24°C. and at room temperature both in light and in darkness. A low, constant temperature of 6°C. permitted the slow growth of both shoots and callus (Table 14 and 15). Although growth was seriously retarded at 6°C., no damage to the tissue was apparent. The shoots were long and etiolated because of the darkness.

A completely different condition existed in the 37°C. treatment. These cultures also showed retardation of growth but considerable necrosis and browning of the tissues was evident. In the surviving cultures, seven buds had appeared early in the experiment. The development of these organs was immediately diverted from the organized growth of normal buds to the undifferentiated growth of callus. At the end of the experiment, these buds were reduced to a mass of callus-like cells.

Fresh and Dry Weights. Table 14 includes all the data on initial and final weights per segment for the controlled temperatures and for the room temperature series. Each individual segment was weighed before and after the experiment. The low production of fresh weight in the 6°C. and 37°C. cultures obviously resulted from temperature extremes. The best growth occurred at 24°C. and at room temperature both in light and in darkness. The experimental differences between treatments do not allow any close comparisons except between the 24°C. and the 30°C. components of the second trial. In this trial, a temperature of 30°C. resulted in the formation of significantly less tissue than the 24°C. temperature (Figure 12). Thus, it can be concluded that room temperature is a suitable temperature, but not necessarily the optimum temperature, for the production of cells of *Sequoia* in culture.

Experiment IV

Aeration

Shoot and Callus Growth. The effects of reduced aeration in submerged cultures (PLATES 8 and 10) gave wholly unexpected results in view of the findings of White (1939a). The submerged cultures, instead of initiating buds, produced only callus; whereas, the controls produced numerous buds although some of these produced callus only. The data of Table 16 clearly indicate that a decided difference existed in the two treatments in regard to the ability of the cultures to produce buds.

The actual production of buds by the controls indicates the inherent ability of all the segments to form buds. The submerged cultures, therefore, completely inhibited the initiation of buds.

PLATES 7, 8, 9, and 10 represent a photographic record of this experiment. PLATE 7 illustrates the method of submersion of the culture by insertion of the segment in agar followed by the addition of five milliliters of distilled water. PLATE 8 shows the type of callus masses produced in the submerged cultures. The water had been removed for photographic purposes. PLATE 9 presents an example of the type of callus produced by the aerated cultures. The excellent shoot growth of many of the aerated cultures is shown in PLATE 10.

Wet and Dry Weight. Although a reduction in aeration destroyed the ability of the segments to form buds, it did not actually enhance the production of callus cells as measured by final wet weight (Table 16). The aerated cultures produced six times the callus produced by the submerged cultures. The aerated segments showed a higher percentage of dry matter.

Experiment V

Polarity of Segments in Medium

Shoot and Callus Growth. Too few shoots were produced by either treatment to reach any conclusions about the relative production by inverted and normal segments. The callus formed by each treatment appeared at the base of the segment and on the sides at the surface of the agar. Both series of cultures exhibited remarkable similarities in rate and appearance of growth.

Fresh and Dry Weight. The two treatments produced similar amounts of fresh and dry material (Table 17). No significant difference could be found in the increments of fresh weight formed during the experiment.

Experiment VI

Adenine Sulfate - Naphthaleneacetic Acid

Bud and Callus Growth. The level of naphthaleneacetic acid that inhibited bud formation according to Experiment IV consisted of about 100 gammas per liter. This amount of the growth regulator was used in the controls of this experiment with the idea that adenine sulfate, at 40, 80, and 120 milligrams per liter, would overcome the bud inhibition according to its concentration. Examination of Table 18 shows that 100 gammas of naphthaleneacetic acid permitted the formation of an average of only 1.3 buds per culture when no adenine sulfate was present in the medium. The presence of 50 gammas per liter of naphthaleneacetic acid permitted the formation of 0.3 buds per culture. These figures are not sufficiently different to allow conclusive estimates of variability; nevertheless, they are low enough to act as controls for measurement of the bud-inducing effects of adenine sulfate. The table shows that adenine sulfate at the rate of 40, 80, and 120 milligrams per liter did not, in any case, cause an increase in the number of buds per culture. In fact, a decrease seems to be in effect. The total length of the shoots produced by the various treatments (Table 18) offers no insight into the effect of adenine sulfate upon the growth of shoots inasmuch as the growth for all treatments averaged 2.3 to 4.4 centimeters per shoot and no individual record was kept of the time of the initiation of each bud.

Fresh and Dry Weight. The data for the initial weight, the final fresh weight, and the final dry weight are included in Table 19. The growth of the cultures is expressed as the average amount of fresh matter produced per culture in each treatment during the course of the experiment. A gradual decrease in the amount of fresh matter produced is apparent with increases in the concentration of adenine sulfate in the medium. (Figure 14).

A decrease occurs also in the treatments receiving 50 gammas per liter of naphthaleneacetic acid in which case the fresh weight dropped from 60 milligrams to 19 as a result of the addition of 120 milligrams of adenine sulfate to the medium.

That no growth occurred in the series receiving no naphthaleneacetic acid seems to indicate that, in this experiment, the growth regulator was necessary for the proliferation of callus and of shoots. No roots occurred in any treatment.

The results obtained at the end of this experiment in regard to the production of buds (Table 18), and more specifically to the production of fresh matter (Table 19), all indicate that the adenine sulfate exerted an inhibitory effect which was partially overcome by increasing amounts of naphthaleneacetic acid.

The effect of shoots, when present in the culture, upon the amount of callus produced was not studied. However, since some of the growth increment consisted of shoots, this amount was subtracted from the means shown in Figure 13 and the result shown in Figure 14. This procedure is justified on the grounds that the shoots after initiation acted more under their own organismal influence than under the influence of the medium. Admittedly, the effects of shoots upon callus growth can not be resolved by this experiment; nevertheless, the growth of callus shows a progressive drop with increased concentration of adenine sulfate.

General Observations

Cultures Maintained at Room Temperature

The results obtained from preliminary investigation as well as the results from the six previously described experiments indicated considerable variation in response to environment among the segments from different burls. For instance, 100 gammas per liter naphthaleneacetic acid did not always completely inhibit the initiation of buds. In most experiments, a few buds always occurred. Furthermore, the rate of growth between experiments generally varied in amounts not always attributable to the environment. These variations in response could be attributed to differences existing in the responses of different burls, since each experiment was prepared from a freshly sprouted burl at different seasons of the year. In order to exercise some sort of control over the interpretation of data between experiments, a group of cultures from each experiment were subjected to the conditions of room temperature in the type of light available.

The data obtained from the cultures maintained at room temperature was subjected to light of several intensities according to the experiment. The light ranged from continuous at high intensity to the weak, diffused daylight entering the laboratory during the winter. In addition the room temperature varied somewhat from artificial heat of winter to the air conditioning of summer. These data are shown graphically in Figure 15. A group is shown for each experiment except Experiment II which utilized a medium too different to be compared to the others (Table 1).

Experiment I, cultivated at a high continuous light intensity, produced rapid and abundant growth. Groups from Experiments III, IV, and VI although of different time lengths, were all maintained in similar environments of room temperature and daylight. Two groups from Experiment V stored for 192 days in very weak daylight produced little, but similar, amounts of tissues.

Some general considerations are possible in regard to the inhibition of buds by several treatments. Table 20 portrays, in diagrammatic form, the treatments utilized throughout the investigation and their comparative effect upon the inhibition of shoot growth and the production of callus by the segments. As indicated, a certain degree of bud inhibition was manifest in Experiment I in a treatment utilizing darkness and growth supplements. In the temperature series, the buds formed were inhibited from further development in darkness at 37°C. Buds were completely inhibited at all temperatures in another trial of the same series by 100 gammas of naphthaleneacetic acid from a fresh supply.

In this treatment, growth of callus was somewhat less than in other experiments. In Experiment IV, reduced aeration completely inhibited the initiation of buds although the controls produced them. In Experiment V, the production of buds was inhibited but not completely suppressed by 100 gammas of naphthaleneacetic acid, again from the fresh supply.

The matter of the breakdown of the older naphthaleneacetic acid into a chemical of less activity received attention. Chemical analysis by two methods showed the older crystals to be 99.6 percent pure and the fresh supply to be 98.8 percent pure. These differences are hardly significant and the conclusion must be drawn that individual differences in burls caused differences in response to higher levels of the growth regulator.

DISCUSSION

Medium. The establishment of the proper nutrient medium for any type of tissue culture involves a large number of trials with combinations of nutrients, vitamins, amino acids, and growth regulators at a great expense of time and effort. However, the importance of the establishment of the proper medium cannot be minimized. Once the optimum nutrient balance has been determined, experiments can be devised regarding the response of the tissues to various chemicals simply by maintaining the tissue in the optimum basic medium at the same time varying the concentration of the substance in question. The problem is complicated somewhat by the fact that tissue cultures fluctuate greatly in response to environmental conditions (de Capite, 1955) because of their delicate nature. At any rate, tissue culture, at best, is a technique capable of eliminating some, but not all, variables inherent to the work with whole plants.

Fortunately, Ball has worked with Sequoia tissue and has determined that a basic medium of Knop's Solution, three percent sucrose and $1 x 10^{-6}$ grams per liter of indoleacetic acid gave excellent results in terms of growth in his studies on differentiation of tissues and movement of radioactive chemicals (Ball, 1950, 1953a, b, c, d, e). In many preliminary tests, Ball's medium proved to be entirely satisfactory substituting alpha naphthaleneacetic acid for indoleacetic acid because of its greater stability in solution. In addition, the B vitamins, cysteine, yeast extract, and coconut milk often enhanced the results of other investigators.

Early in the study of roots in culture (Bonner, 1937; Robbins and Bartley, 1937; White 1937), the effective agent in yeast extract for the indefinite growth of most excised roots was found to be thiamin. Gautheret (1937) also included thiamin in the media for the growth of tissue cultures of cambial origin. However, Nobecourt (1940) did not include thiamin in his original formula for carrot tissues which synthesized as much thiamin as did normal tissue. This vitamin was found necessary for tomato roots by Rabideau and Whaley (1950). The reports of beneficial activities for cultures other than roots is not so clear. It was deemed unnecessary for the culture of excised asparagus tips (Loo, 1945) and for the growth of the first leaf of isolated stem tips of rye (deRopp, 1946). The beneficial results in stem cultures of normal tissues (Gautheret, 1937; Henderson, Durrell, and Bonner, 1952) and of atypical tissues (Burkholder and Nickel, 1949; Skoog and Tsui, 1948) probably indicate that thiamin is synthesized in most tissues of stem origin but sometimes not at optimum amounts.

Pyridoxine also gave favorable results for root cultures (Robbins and Schmidt, 1939; Day, 1941), for sunflower tissue (Hildebrandt, Riker, and Duggar, 1946), and for virus tumors of Rumex (Burkholder and Nickel, 1949).

Pantothenic acid was found to be occasionally beneficial for the growth of excised corn root tips (Robbins and White, 1936) and indispensable for cambial cultures of hawthorne (Morel, 1946a). Pantothenic acid reduced the inhibiting effects of tannins in the formation of callus by cambial cultures of Quercus (Jacquoit, 1947) which, up to that time had not been cultured.

Cysteine was used for carrot root cultures by Nobecourt (1937) and for cambial cultures by Gautheret (1937). Nobécourt considered cysteine unnecessary for carrot root cultures (1942) but later included it in the medium for rose stem cultures (Nobécourt and Köfler, 1945; Nobécourt, 1946).

The effects of the various growth supplements upon other tissues were evaluated and the decision was made to include them in the basic medium, inasmuch as no harmful effects, at the low concentrations used, were found in any case. The results of Experiment I showed that growth was definitely enhanced by the addition of these supplements. The decision to include biotin and thiamin received further justification from the fact that traces of these substances have been found in Difco agar (Robbins, 1939; Day, 1942). White (1953), in a criticism of the techniques currently in use, objected to the use of agar in critical studies because of its "unknown nutritive properties".

The inclusion of ascorbic acid received attention, not especially for its metabolic effects, but for the fact that its presence in the medium reduced the necrosis and browning of cut surfaces. Wetmore and Morel (1949) made tissue cultures of horsetails and ferns in which growth ceased as soon as the medium became dark brown. They suggested that ascorbic acid reduced the action of the polyphenol oxidase system at the injured edges of the fragments. This treatment eliminated the browning and diffusion of the substance into the agar with the result that growth progressed. Another beneficial effect of ascorbic acid was demonstrated by Waygood (1949) in which hydrogen was transferred from the malic dehydrogenase - Coenzyme I system to molecular oxygen by way of an ascorbic acid oxidase. At any rate, ascorbic acid always exerted a decidedly beneficial effect upon the early growth of Sequoia stem segments especially in regard to the control of the browning and necrosis of cut surfaces.

Several sources of carbon received attention. Sucrose invariably gave the best results and subsequently was used in all experiments except the second in which glucose was used. Examination of Figure 8 reveals that after the twelfth week all cultures, regardless of treatment, began to die at an increasing rate. These cultures seemed unable to derive some substance from the agar. On sucrose, however, the cultures of the other experiments always progressed considerably beyond this point with no decrease in growth or vigor. Sucrose, no doubt, remains the best carbon source for Sequoia, a result also found for this tissue by Ball (1953a). White (1940) obtained ten times the growth of excised tomato roots in two percent sucrose than in two percent glucose. Each tissue according to species has its particular optimum carbon source as well as a certain optimum concentration. A comprehensive work on carbohydrates as sources of energy for tissues is that of Hildebrandt and Riker (1949, 1953). They concluded that many basic differences in carbohydrate utilization exist among species and among types of tissue. By way of explanation they offered the theory that these tissues may have missing or incomplete enzyme systems, may be impermeable to certain carbohydrates, or may be suppressed by the formation of inhibiting metabolites.

The problem of the adjustment of the p H to the optimum was met by the addition of appropriate amounts of 0.1 normal sodium hydroxide before autoclaving. The tissue grew well at a p H of 5.6. Gautheret (1947b, c, d) found that for carrot tissues, the tissue had a tendency to change the p H of the substratum to a value slightly higher than the proper p H of 5.5, but that growth proceeded normally between p H 6 and 9. He found a buffer action by the tissue which, if not immediately killed, neutralized a high or low p H and destroyed its toxicity. de Capite (1955) influenced by these findings of Gautheret found a p H of 6 to be entirely satisfactory for the growth <u>in vitro</u> of three species of plants. In the case of <u>Sequoia</u> tissue, the final p H did not differ from the initial by more than one log.

Experiment I consisted partly of a test as to the enhancement of growth by the addition of certain vitamins, cysteine, and yeast extract. Several points are obvious. In the first place, the addition of these growth supplements initially retarded growth and killed a large number of segments (Table 9). Secondly, after a certain length of time, the surviving cultures in the treated series became greener and more compact until, at the end of the experiment, they had completely surpassed the controls in general vigor.

Thus, the time length of an experiment may have some effect upon the interpretation of the results, especially in regard to tissue cultures which, unlike intact plants, do not possess normal physiological periods of vegetative or reproductive growth. A tissue culture dependent upon a medium for minerals and carbohydrates without the presence of roots and leaves either becomes adjusted to its environment or expires.

The balance of enzyme systems within the original segment originally may not be able to cope with the demands of a certain environment. Later, the adjustment of already present systems, or of new systems, may enable the segment to shift its biological functions to a more favorable balance. This phenomenon has been observed many times but especially in regard to the "habituation" of certain tissues to indoleacetic acid (Gautheret, 1946, 1947e) and to naphthaleneacetic acid (Morel, 1946b). These cultures adjusted themselves to the absence of growth substances by the gradual assumption of the ability to synthesize them while growing. White (1949) refers to cellular adaptation as the boosting of a subcritical physiological system or of the shifting in the relative amounts of pre-existent systems.

Plant Material. Statistics regarding the Redwood tree in its native habitat are well known. Generally the tree is considered to be capable of living between 500 and 1300 years and to grow to a height of 360 feet with a circumference of forty-seven feet (Hollister, 1953).

Very little information, however, is available regarding the origin and development of the burls. According to Betts (1945) burls are irregular swellings or growths near the base of the trees consisting of closely clustered groups of twisted fibers through which adventitious buds are scattered.

All of the burls received from a commercial source in California sprouted when placed in water. Those burls received during the winter sprouted at a slower rate than those received during summer. Although originally the burls were considered to be uniform in growth and condition of the shoots, a remarkable dissimilarity existed between them in the number, color, texture, and rate of growth of the sprouts produced.

These differences between burls are probably reflected in some of the variability within experiments in regard to subsequent development and final wet weights.

The effect of seasons of the year upon the sprouting of burls probably changes the response of the shoot segments to treatments because of the differences in auxin content among burls from different positions on the tree. In addition the shoots may have variable abilities to produce auxin especially after excision. Burls resemble most the tumor growths of plants. According to Kulescha and Gautheret (1948) differences in the normal tissue, gall tissue, and habituated tissue of black salsify contain different amounts of indoleacetic acid, a fact which accounts for their relative differences in growth and response to added growth regulators. They also determined (1947) that tissue cultures of Jerusalem artichoke taken at several seasons grew in proportion to the amount of growth substance normally present for that time of year. Kulescha (1949) found the proliferating capacity of Jerusalem artichoke to be related to the quantities of contained auxin depending upon the season of the year. Differences in the auxin content of the excised segments of Sequoia burl shoots may account for the fact that 100 gammas of naphthaleneacetic acid did not always inhibit the initiation of buds. Another alternative is that this concentration of naphthaleneacetic acid represents a threshold value at which buds may or may not be entirely inhibited depending upon the amount of natural auxin in the plant material.

Another disturbing difference between the growths from different burls existed in the texture of the sprouts or shoots. Some, depending upon the burl, were thick, soft, and fleshy; whereas, occasionally, a burl gave rise to hard, brittle shoots. Ball (1950), using the same species, makes no mention of the texture of the shoots other than to state: "Thick green shoots from a burl were used".

Light. In Experiment I, the use of continuous light of rather high intensity resulted in the production of greater amounts of tissue than in darkness. The results of the other experiments in diffuse daylight of variable intensities are not strictly comparable but much less tissue was produced than in continuous light. These results, of course, were not unexpected. de Capite (1955) studied the effects of light intensity upon three types of tissue in culture and determined that all three grew best at 350 foot candles, with less growth at 150 and 750 foot candles. His data consistently shows a greater wet weight in continuous artificial light than in either natural light or continuous darkness.

The presence of sucrose in the medium eliminated the necessity for photosynthesis and any benefit from light probably resulted from the higher temperature caused by the light (de Capite, 1955) or from the formation of substances dependent upon photochemical reactions.

Since shoots appeared in some cultures and not in others, a method is suggested whereby the effect of shoots upon the growth of callus might be studied by means of the final wet weights obtained from a great number of cultures.

The data (Table 4) revealed that more shoots are formed in light than in darkness. Again an unanswered question arises as to the effect of shoots upon the initiation of buds as expressed by apical dominance. Refinements in the present technique may offer information on this subject.

Temperature. The growth of cells in culture depends greatly upon an optimum temperature according to the findings of de Capite (1955). He suspected that tissues in culture, because of the delicate structure and of the lack of an epidermis, might be much more sensitive than the intact plant to external factors.

Likewise, in the growth of Sequoia tissue the question arose whether or not room temperature, with its lack of control, represented a suitable temperature for growth as opposed to a constant optimum temperature.

The results of Experiment III show that the room temperature of about 21°C. provides almost as good an environment as that of a controlled temperature of 24°C. Lack of facilities prevented a more exhaustive study of temperature optimums but the findings indicate that as long as the entire experiment is conducted under the same conditions of temperature, consistent results within the experiment may be expected.

However, as de Capite (1955) demonstrated that changes of a few degrees between experiments cause differences in wet weights that would completely invalidate the results of experiments compared at greatly differing room temperatures. de Capite using three different species of plants found that the growth of tissues *in vitro* was strongly influenced by temperature with a rapid drop in final wet weight on each side of the optimum temperature. In addition, he found that high light intensities have a tendency to raise the temperatures of the tissue. Optimum temperatures for the three species ranged between 23 and 26°C. and he concluded that optimum temperatures for the growth of tissue cultures are higher than those observed for intact plants of the same species.

The principal difference between the segments used in these experiments and the cuttings used for propagation of plants lies in the comparative sizes and sources of carbohydrates. In cuttings, the carbohydrates are stored in the tissues; the segments derive carbohydrates from the medium by diffusion. Both, however, form callus to varying degrees. Shippy (1930), studying the callusing of apple cuttings, found that the complete range for the formation of callus occurred between 0° and 40°C. with injury to the tissues above 32°C. He found that variable temperatures did not change the relationship in which callusing was accelerated or retarded according to the degree and duration of the temperature.

Skoog (1944) placed tobacco tissue cultures in temperatures ranging from 5°C. to 33°C. The amount of growth increased with the temperature but the formation of buds was greatly suppressed at 33°C. This result is in agreement with the suppression of bud growth found at 37°C. in Experiment III. Skoog further found that at 5°C. and 12°C. the growth rate was so slow that very little differentiation occurred. This condition also existed at 6°C. in Experiment III. Hildebrandt, Riker, and Duggar (1945) found an optimum temperature of 26°-32°C. for tobacco and 24°-28°C. for sunflower cultures. In using final wet weight as the measure of growth, they found considerable variation between individual pieces.

The culture of root tips presents a different problem from the culture of stem tissues. However, a parallel condition may exist in a supposed seasonal fluctuation in growth rates. White (1937), while investigating the seasonal fluctuation in growth rates of excised tomato root tips, utilized controlled temperatures in the range from 5°C. to 40°C. Growth was practically nil below 10°C. and at 40°C. and slow at 15°C. and 35°C. Optimum growth was best, and very sharply so, at about 30°C. He concluded that the temperature range of a laboratory probably explained the seasonal variations and that, for root tips at least, the temperature conditions must be rigidly controlled.

Aeration. The inhibition of buds by the submerged cultures in Experiment IV is directly opposite to the results obtained by White (1939a). White proposed the theory that low oxygen gradients in the tissue may be important factors in the differentiation of organs. He found that, in the case of tobacco tissue cultures, the mere submersion of cultures resulted in the production of stems and roots even with cultures which had long produced only callus.

Several important differences exist between the tobacco and redwood tissues, however. White's tobacco tissue was derived directly from neoplastic growths on the stem; whereas, the redwood segments, although originally from a burl, were derived from normally appearing stems. A difference between the media of the two tissues lies in the fact that tobacco tumor tissues can grow indefinitely without the addition of growth substances and in the case of the redwood tissues, 100 gammas per liter were added. In the case of the redwood tissue, this amount was utilized to control or greatly inhibit the production of buds. Thus, the relationship between aeration and control of bud formation requires further investigation especially in regard to the interaction of oxygen tension and amount of growth regulator furnished. Skoog and Tsui (1948) confirmed the work of White (1939a) on tobacco tissue but also found that the addition of indoleacetic acid or naphthaleneacetic acid prevented bud formation in submerged cultures. Other evidence from their experiments also led to the conclusion that oxygen tension is not the only factor active in organ formation. They noted that shoots removed from the liquid continued to produce shoots and leaves rather than callus, and that a severed shoot placed on agar generally reverted to callus at any point on its surface.

The amount of growth as expressed by the increase fresh weight at the end of Experiment IV revealed that the submersion of the cultures greatly inhibited growth. Gautheret (1950) also found a suppression of growth in submerged cambial explants of Salix caprea.

Polarity. In order to take advantage of the polar movement of auxins, the shoot segments were normally inverted in the agar. This procedure necessitated an accurate and time consuming marking of the apical or basal end of the segment so that it might be inverted in the agar without error. Any differences between normal and inverted segments in regard to growth were tested in Experiments I and V.

In both experiments, the production of fresh material was uniform between the two treatments. Apparently, within the limits of these experiments, the polarity of the segment had little effect upon its ability to absorb naphthaleneacetic acid from the medium. However, this result cannot be conclusive for a number of reasons. The possibility exists that certain amounts of naphthaleneacetic acid could have moved up the outside of the segment by diffusion on the moist surface. The concentration may have been high enough for the growth regulator to move upward against the gradient in the vascular bundles as found by Snow (1936). The polarity may have been reversed by the formation of new tissues of opposite polarity as found in inverted Tagetes cuttings by Went (1941).

Thus, the sensitivity of the experiments was not conducive to conclusive results in regard to the polar movement except that, within the limits of the experiment, no effect of reversal of polarity upon growth was evident.

Several other results, closely related to the movement of auxins, have a certain amount of interest. Went (1937) proposed a number of hormone-like factors called "calines" which are formed in various organs, transported to other organs where they produce growth effects. For instance, caulocaline, produced in roots specifically causes the elongation of stem and buds. Rhizocaline must be furnished by cotyledons and leaves before roots can be formed. Likewise phyllocaline must be present and necessary for leaf growth. Normal growth of the plant thus depends upon the relative distribution of the calines in conjunction with auxin.

Some evidence against this theory is discernable in the work of Loo in which he cultivated excised stem tips of asparagus (1945, 1946b) and of dodder (1946a). In both cultures of stem tips, growth progressed satisfactorily in the complete absence of roots although in the case of asparagus the growth was slower among non-rooted tips than among rooted tips. The appearance of cladophylls also enhanced the growth. These results, however, do not exclude a partial need by the stem-tips for caulocalene in the growth of shoots.

de Ropp (1946), on the other hand, working on the influence of roots upon the growth of rye showed that, in stems that failed to regenerate roots, growth was confined to the first leaf of the stem. The amount of growth increased with increased root system.

Galston (1948) found that roots developed in the dark on asparagus stem tips when exposed to one to ten gammas per cubic centimeter of indoleacetic acid in the medium, but that no roots developed in the light in the presence of the same concentrations. He assumed the presence of a material formed in the light other than auxin essential for root initiation.

The stem-tips shown in PLATE 3 were excised from the tips of <u>Sequoia</u> shoots and allowed to grow for eighty days. They grew well, as demonstrated by the amounts of penetration into the agar, without the formation of roots. In fact, most of the cultures of these experiments produced shoots and callus without the formation of a single root. Furthermore, by actual count of the cultures, the formation of what roots occurred nearly always was preceded by the initiation and growth of shoots. In *Sequoia* cultures, therefore, there seems to be no specific need for a substance formed in roots in order to allow the formation and growth of shoots.

Maleic Hydrazide. In order to investigate a condition which might lead to the production of callus cells only, the growth inhibiting chemical, maleic hydrazide (Schoene and Hoffman, 1949), was incorporated into the nutrient medium at several concentrations with the idea that the maleic hydrazide might act as a selective inhibitor permitting the growth of unorganized callus at the same time suppressing the initiation of buds and shoots.

The quick deaths of the segments in the 10, 100, and 1000 parts per million maleic hydrazide concentrations indicates that this chemical moves rapidly in all directions in the shoot, a condition found by Linder (1951) in stems. The possibility exists, however, that the maleic hydrazide could have killed the tissues of the segment at the surface, thus blocking the absorption of nutrients from the agar. At any rate, the two treatments remaining gave several comparisons between cultures growing with and without one part per million of maleic hydrazide.

A complete parallelism existed in the growth responses of *Sequoia* tissue to media with and without one part per million of maleic hydrazide but some instances of the stimulatory effect of small dosages have been reported. Greulach and Atchison (1950) claim a stimulation of the production of onion roots at the one part per million level. In their experiment ten parts per million showed extreme growth inhibiting properties.

Another example of a stimulating effect is found in the work of Brown (1952) who determined residual toxicities in the soil. In the case of flax, the original amount of maleic hydrazide stimulated growth in a crop after the first was completely inhibited. Nickel (1953) found, however, that maleic hydrazide inhibited the growth of Rumex tissue directly in proportion to the concentration. In regard to Sequoia tissue, the fact that one part per million did not inhibit growth in eighty days indicates that this amount can be tolerated. The range of concentration between one and ten parts per million could possibly contain a concentration at which stimulation of growth might occur.

Some anti-auxin (antagonistic) effects of maleic hydrazide also have been reported. According to Hitchcock and Zimmermann (1951), combinations of 2, 4 - dichlorophenoxyacetic acid and maleic hydrazide produced additive and antagonistic effects on several plants. Johnson and Buchholz (1951) report that proliferation and rooting induced by 2, 4 - dichlorophenoxyacetic acid could be eliminated in combination with maleic hydrazide. Leopold and Klein (1951) concluded that the inhibition of growth by low concentrations of maleic hydrazide is completely relieved by the addition of auxin and that maleic hydrazide is not a growth regulator because it cannot promote growth in the absence of auxin. The results of the treatment of Sequoia tissue with one part per million of maleic hydrazide do not eliminate the possibility of an antagonistic effect of the two chemicals inasmuch as the naphthaleneacetic acid at 100 gammas per liter may have been sufficient to overcome the inhibition exerted by one part per million of maleic hydrazide but not of ten parts per million.

Evidence that some organisms can withstand large concentrations of maleic hydrazide was obtained from the fact that cultures became contaminated even at the concentration of 1000 parts per million. Since all of the *Sequoia* tissue died in concentrations above ten parts per million, the survival of microorganisms seemed contradictory to conclusions drawn concerning the toxicity of the chemical. Laste (1952) observed that, although maleic hydrazide was strongly inhibitory to *Cytophaga*, it had little effect upon *Azotobacter* in the soil. Nickel and English (1953) studied the effect of maleic hydrazide on soil bacteria and other microorganisms. They found that 100 parts per million had no activity against many of them and that 280 parts per million had no effect against *Rhizobium*. Some withstood concentrations up to 1000 parts per million, the same concentration used in this experiment.

Thus, in this experiment, maleic hydrazide failed to act as a selective inhibitor in the suppression of organized growth. Toxicity was rapid and general at ten parts per million and above. Segments grew equally well whether or not one part per million had been added to the medium.

Adenine Sulfate. The review of the literature indicated that the formation of buds in tissue culture could be inhibited by high auxin concentrations and enhanced by high adenine concentrations (Skoog and Tsui 1948). In regard to tobacco tissue the optimum concentrations for bud formation appear to be in the vicinity of 100 milligrams per liter for adenine sulfate (Skoog, 1953) and for complete inhibition of buds, 10 gammas per liter of indoleacetic acid (Miller and Skoog 1953).

Carrot callus responded well in increased callus production up to 10 milligrams per liter of naphthaleneacetic acid (Wiggans 1954). The above investigators established the amount of adenine sulfate which would overcome the bud inhibiting properties of auxin as somewhat near 100 milligrams per liter. Wiggans (1954) pointed out that the ratio probably differed among species, organs and tissues.

In regard to Sequoia tissue, a concentration of 100 milligrams per liter of naphthaleneacetic acid was found capable, in most cases, to inhibit bud formation. If this were true, then a concentration of adenine sulfate should exist that would counteract the inhibition caused by the naphthaleneacetic acid. As in the experiment of Wiggans (1954) with carrot tissue, no apparent increase in bud formation resulted from 40, 80, 100, and 120 milligrams of adenine sulfate used to counteract 50 and 100 gammas per liter of naphthaleneacetic acid (Table 18). As a matter of fact, any amount of adenine sulfate above forty milligrams appears to be toxic to growth of the segments. Since forty milligrams is the lowest amount used, it follows that the inhibition of buds by 50 and 100 gammas per liter of naphthaleneacetic acid was not overcome within the toxic range of adenine sulfate, a condition similar to that noted by Wiggans (1954) in the case of carrot tissue.

The inhibition of buds by the naphthaleneacetic acid is somewhat overshadowed by the general toxicity of the adenine sulfate. Examination of Table 18 reveals from little data, to be sure, that the higher concentration of naphthaleneacetic acid actually overcame some of the toxicity of the adenine sulfate and allowed a few more buds to be produced.

Howell and Skoog (1955) point out a stimulating effect of 1 milligram per liter of adenine sulfate in the growth of excised pea epicotyls.

The inhibiting action of adenine sulfate upon the production of fresh weight is shown in Table 19. Fresh weight rapidly increases with concentration of naphthaleneacetic acid in the presence of all concentrations of adenine sulfate and again the toxicity of the adenine sulfate appears to be lessened by the increased amount of naphthaleneacetic acid. The same results are apparent in regard to final dry weight (Table 19). The differences are not so obvious in regard to percentage dry weight but the same results apply.

Thus the evidence of this experiment points toward a toxicity to the tissues exerted by the adenine sulfate. This toxicity is reflected by the decreased production of buds, and of fresh and dry weight. Very little data exists in the literature on the effect of adenine upon fresh and dry weights, since the emphasis is usually directed toward organogenesis. However, Skoog and Tsui (1948) using adenosine show progressive decreases in the mean fresh weight of callus tissues from 48.4 milligrams at 0.5 milligrams per liter of adenosine to 28.5 milligrams at 50 milligrams per liter. The dry weight dropped from 4.5 to 2.8 milligrams.

The effect of adenine sulfate upon the initiation of buds and upon the production of callus cells apparently varies widely among species and tissues. The findings of Wiggans (1954) that the concentration of auxin within the tissue may be high enough to prevent the expression of the adenine sulfate in the range in which it can be added without toxicity also can be applied to Sequoia stem segments in culture.

SUMMARY AND CONCLUSIONS

Stem segments, excised from shoots of Redwood burls (*Sequoia sempervirens*), were cultured *in vitro* under various conditions of chemical (maleic hydrazide and adenine sulfate) and other environmental treatments (growth supplements, light, polarity, temperature, reduced aeration). The effect of the original position of the segment on the stem and of original weight were studied. The effects of the above environmental treatments were expressed mainly in the relative control over the initiation of buds and shoots and in the relative amounts of fresh weights produced by each treatment.

The basic medium consisted of one adapted from Gautheret containing the mineral nutrients of Knop's Solution, Berthelot's oligodynamic elements, three percent sucrose, two percent agar, naphthaleneacetic acid, and certain growth supplements: cysteine, thiamin, pantothenic acid, biotin, inositol, pyridoxine, and ascorbic acid.

The segments responded in the following manner to the various treatments:

1. The ability of the segments in culture to form cell masses was initially greater and more rapid in the light in the absence of growth supplements (and yeast extract). In darkness, however, the presence of growth supplements (and yeast extract) enhanced the power of the segments cultured in darkness without supplementary substances. (FIGURE 3).

2. The percentage of proliferating cultures that produced buds was highest in darkness without the growth supplements (and yeast extract) and lowest in darkness with growth supplements. The percentages of cultures that produced buds in the light with and without growth supplements (and yeast extract) gradually approached the high percentage shown for darkness without supplements (FIGURE 6).

3. A greater number of buds per culture occurred in the light than in the dark (TABLE 4).

4. Cultures maintained in light produced a greater final fresh weight than those in darkness. Among segments in darkness the presence of growth supplements (and yeast extract) enhanced the production of fresh matter over that produced in the absence of the supplements (TABLE 6). Although the presence of supplements initially inhibited growth (FIGURE 3) or killed segments (TABLE 9), the surviving cultures overtook and surpassed the cultures without supplements in the production of fresh weight (TABLES 5 and 6) and in general appearance and vigor.

5. The initiation and growth of roots occurred in the absence of naphthaleneacetic acid (EXPERIMENT I). Roots were completely inhibited in the presence of 50 and 100 gammas per liter of naphthaleneacetic acid (EXPERIMENTS II, III, IV, V and VI).

6. Shoots grew vigorously without the presence of roots in most cultures of all experiments.

7. Maleic hydrazide, at concentrations of 10, 100, and 1000 parts per million, killed all segments (FIGURE 8). No differences in the relative production of shoots or callus were observed between cultures with and without one part per million of maleic hydrazide (FIGURE 9).

Certain microorganisms maintained growth and contaminated cultures containing 100 parts per million of maleic hydrazide in the medium.

8. The effect of position on the shoot and of the original weight of the segments upon subsequent growth gave no significant differences within the limits of the experiment (FIGURE 11).

9. Naphthaleneacetic acid at a concentration of 100 gammas per liter reduced and sometimes completely inhibited the initiation of buds (EXPERIMENT III).

10. The optimum temperature for the production of fresh weight occurred in a controlled temperature of 24°C. and less than 30°C. Cultures maintained at room temperature grew almost as well as those at the controlled temperature of 24°C. Buds developed slowly, but normally, at 6°C. At 37°C. buds were initiated but failed to develop (TABLE 15).

11. Submerged cultures failed to produce buds. However control cultures receiving the normal aeration produced numerous buds. The submerged cultures produced less callus than the controls (TABLE 16).

12. Adenine sulfate, at concentrations of 40, 80, and 120 milligrams per liter, progressively inhibited, rather than enhanced, bud initiation and fresh weight production by the cultures in treatments of naphthaleneacetic acid at 0, 50, and 100 gammas per liter. The inhibition of buds and fresh weight by adenine sulfate was partially alleviated by the increasing concentrations of naphthaleneacetic acid (TABLE 18, FIGURE 13).

13. Within the limits of the technique, no differences between the normal or inverted polarity of the segments in agar could be determined with respect to the production of buds or fresh weight.

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TABLE 1. COMPONENTS OF NUTRIENT SOLUTIONS USED IN EACH EXPERIMENT. INGREDIENTS USED IN SEVERAL CONCENTRATIONS WITHIN AN EXPERIMENT ARE SHOWN IN THE AMOUNTS USED SEPARATED BY DASHES.

	TYPE OF EXPERIMENT					
	I	II	III	IV	V	VI
COMPONENT (Amount per liter)	Light, Vitamins, Polarity	Maleic-Hydrazide, Position. Init. Wt.	Temperature	Aeration	Polarity	Adenine-Auxin Ratio
Knop's Solution*	500 ml	100	500	500	500	500
Berthelot's Solution**	0.5 ml	1	0.5	0.5	0.5	0.5
l(+)Cysteine-HCl	0-10 mg	10	10	10	10	10
Thiamin HCl	0-1 mg	1	1	1	1	1
Ca-d-pantothenate	0-1 mg	1	1	1	1	1
Biotin	0 mg	0	1	1	1	1
i-Inositol	0-100 mg	100	100	100	100	100
Pyridoxine-HCl	0-1 mg	1	1	1	1	1
Ascorbic Acid	400 mg	0	400	400	400	400
Yeast Extract	0-4 gm	0	0	0	0	0
Sucrose	30 gm	0	30	30	30	30
Glucose	0 gm	30	0	0	0	0
Agar	10 gm	6	10	10	10	10
Naphthaleneacetic Acid	0	100	100	100	100	0-50-100
Adenine Sulfate $2H_2O$	0 mg	0	0	0	0	0-40-80-120
Maleic Hydrazide Diethanolamine Salt	0 mg	0-1-10 100-1000	0	0	0	0

* Knop's Solution: Components per liter of stock (twice strength)

1.0	gm $Ca(NO_3)_2 \cdot 2H_2O$
.25	KNO_3
.25	$MgSO_4 \cdot 7H_2O$
.25	KH_2PO_4

** Berthelot's Solution: Components per liter of stock

50.0	gm $Fe_2(SO_4)_3 \cdot nH_2O$	0.10 gm	$ZnSO_4 \cdot 7H_2O$
2.0	$MnSO_4 \cdot H_2O$	.05	$CuSO_4 \cdot 5H_2O$
.5	KI	.10	$BeCl_2$
.05	$NiCl_2 \cdot 6H_2O$	.05	H_3BO_3
.05	$CoCl_2 \cdot 6H_2O$	1.00 ml	H_2SO_4
.20	$TiCl_4$		

TABLE 2. PERCENTAGES OF CULTURES IN EACH TREATMENT IN WHICH PROLIFERATIONS APPEARED ON THE ORIGINAL SEGMENT.

Weeks after start of Experiment	Light				Darkness			
	With Supplement		Without Supplement		With Supplement		Without Supplement	
	Inverted	Normal	Inverted	Normal	Inverted	Normal	Inverted	Normal
2	27	36	72	75	15	18	10	22
3	52	49	85	80	38	33	32	49
4	58	59	92	93	49	44	53	60
5	73	83	97	98	79	55	58	60
6	75	80	100	98	80	78	70	63
8	100	100	100	100	95	89	72	68
10	100	100	100	100	100	100	81	72

TABLE 3. PERCENTAGES OF CULTURES IN EACH TREATMENT IN WHICH BUDS OR SHOOTS APPEARED ON THE ORIGINAL SEGMENT.

Weeks after Start of Experiment	Light				Darkness			
	With Supplement		Without Supplement		With Supplement		Without Supplement	
	Inverted	Normal	Inverted	Normal	Inverted	Normal	Inverted	Normal
5	30	39	59	59	16	15	68	83
6	37	48	62	64	16	13	65	88
8	48	73	70	70	14	13	69	88
10	56	84	67	74	17	11	65	92

TABLE 4. EFFECT OF SEVERAL ENVIRONMENTAL CONDITIONS UPON THE PRODUCTION OF BUDS AND SHOOTS PER CULTURE IN EACH TREATMENT.

Polarity of Segment	Light		Darkness	
	With Supplement	Without Supplement	With Supplement	Without Supplement
Inverted	1.5	3.1	0.1	0.3
Normal	3.2	4.0	0.1	0.5

TABLE 5. FINAL FRESH WEIGHTS (MG.) OF TWO SETS OF SEGMENTS MAINTAINED ONE YEAR AFTER TERMINATION OF EXPERIMENT I. INITIAL WT. 40 MG.

	With Supplement	Without Supplement
No. of Cultures	12	14
Mean Final Fresh Wt. (mg)	1874	1174

TABLE 6. FINAL FRESH WEIGHT (MG.) OF SEGMENTS. EACH SEGMENT WEIGHED INDIVIDUALLY.

Polarity of Segment	Light		Darkness	
	With Supplement	Without Supplement	With Supplement	Without Supplement
Inverted	475 ± 125	517 ± 126	141 ± 35	96 ± 17
Normal	544 ± 139	523 ± 103	125 ± 72	97 ± 14

TABLE 7. FINAL DRY WEIGHT (MG.) OF SEGMENTS. SEGMENTS WEIGHED COLLECTIVELY FOR EACH TREATMENT.

Polarity of Segment	Light		Darkness	
	With Supplement	Without Supplement	With Supplement	Without Supplement
Inverted	65	76	20	11
Normal	70	80	19	12

TABLE 8. PERCENTAGE DRY WEIGHT OF SEGMENTS.

Polarity of Segment	Light		Darkness	
	With Supplement	Without Supplement	With Supplement	Without Supplement
Inverted	13.7	14.7	14.2	11.5
Normal	12.9	15.3	15.2	12.6

TABLE 9. PERCENTAGE OF DEAD SEGMENTS IN EACH TREATMENT AT END OF EXPERIMENT I.

Polarity of Segment	Light		Darkness	
	With Supplement	Without Supplement	With Supplement	Without Supplement
Inverted	39	8	5	11
Normal	46	12	8	13

TABLE 10. INITIAL AND FINAL DISPOSITION OF CULTURES IN EACH TREATMENT OF EXPERIMENT I.

Disposition of Cultures	Light				Darkness			
	With Supplement		Without Supplement		With Supplement		Without Supplement	
	Invert	Norm	Invert	Norm	Invert	Norm	Invert	Norm
Cultures with Shoots & Callus & Living at end of Experiment	36	32	49	50	38	36	32	35
Cultures Dead at end of Experiment	23	27	4	7	2	3	4	5
Cultures Contaminated	1	1	7	3	0	1	4	0
Total	60	60	60	60	40	40	40	40
Cultures Weighed at end of Experiment	28	24	35	38	30	30	24	27
Cultures Saved for Future Experiment	8	8	14	12	8	6	8	8
Total	36	32	49	50	38	36	32	35

TABLE 11. AVERAGE WEIGHT (MG.) OF SEGMENTS FOR THE VARIOUS TREATMENTS OF MALEIC HYDRAZIDE, ACCORDING TO DISTANCE FROM THE APEX.

		Maleic hydrazide (ppm)				
Group	Distance from Apex	0	1	10	100	1000
1	1-5 cm.	55	60	55	62	63
2	6-10	83	95	92	80	87
3	11-15	99	104	101	100	100
4	16-20	121	132	135	115	129
5	21-25	120	150	160	128	150
6	26-25	165	125	140	155	130
	Average	89	96	94	88	92

TABLE 12. NUMBER OF SHOOTS (AND BUDS) PRODUCED BY THE CULTURES WITH INCREASING AMOUNTS OF MALEIC HYDRAZIDE.

	Weeks after start of Experiment				
Maleic hydrazide	6	9	12	15	18
0 ppm	19	19	25	34	34
1	18	27	28	31	31
10	6	6	6	6	6
100	0	0	0	0	0
1000	0	0	0	0	0

TABLE 13. PERCENTAGE OF CULTURES IN WHICH THE CALLUS TYPE OF CELLS WAS PRODUCED (IN LIVING CONDITION AT THE TIME INDICATED).

	Weeks after start of Experiment				
Maleic hydrazide	6	9	12	15	18
0 ppm	16	70	92	49	3
1 ppm	19	45	83	43	26

TABLE 14. EFFECT OF TEMPERATURE UPON THE PRODUCTION OF FRESH AND DRY MATTER IN CULTURE.

Treatment	Age Days	Init. Wt. of Segment	Final Wet Wt.	Wt. of Shoots	Wt. of Callus	Final Dry Wt.	Perc. Dry Wt.	Increment of Growth
Part 1								
6°C Darkness	262	152	251	26	73	43	17.3	99
Room Temp. Light	197	156	572	46	371	88	15.3	416
Room Temp. Darkness	197	170	697	120	408	94	13.5	527
24°C Darkness	197	161	508	67	280	72	14.2	347
37°C Darkness	262	179	331	0	152	40	12.2	152
Part 2								
Room Temp. Light	137	74	301	0	227	51	16.8	227
Room Temp. Darkness	137	58	471	0	413	67	14.2	413
24°C Darkness	137	82	494	0	412	79	15.9	412
30°C Darkness	137	74	373	0	299	47	12.8	299
37°C Darkness	137	85	156	0	71	18	11.4	71

TABLE 15. EFFECT OF TEMPERATURE UPON THE PRODUCTION OF SHOOTS, BUDS, AND ROOTS.

Treatment	No. of Cultures	Age in Days	Total No. of Buds	Total No. of Shoots	Total Length of Shoots (cm.)	Total No. of Roots
Part 1						
6°C Darkness	14	262	4	6	10	0
Room Temp. Light	13	197	5	15	47	0
Room Temp. Darkness	9	197	4	8	38	0
24°C Darkness	19	197	6	12	43	0
37°C Darkness	12	262	7*	0	0	0
Part 2						
Room Temp. Light	12	137	0	0	0	1
Room Temp. Darkness	5	137	0	0	0	0
24°C Darkness	7	137	0	0	0	0
30°C Darkness	7	137	0	0	0	0
37°C Darkness	9	137	0	0	0	0

* 7 buds quickly degenerated to callus.

TABLE 16. EFFECT OF REDUCED AERATION UPON THE AVERAGE FRESH AND DRY WEIGHTS (MG.) OF CULTURES.

Treatment 260 days	No. of Cultures	Initial Wt. of Segment	Final Wet Wt.	Wt. of Shoots	Wt. of Callus	Final Dry Wt.	Pct. Dry Wt.	Incre. of Growth
Submerged	14	93	154	0	61	24	15.8	61 ± 12
Control	17	92	592	101*	379	105	18.4	480 ± 100

* Consisted of two shoots per culture; total length 104 cm.

TABLE 17. EFFECT OF INVERTION OF SEGMENT IN AGAR MEDIUM UPON THE AVERAGE FRESH AND DRY WEIGHTS (MG.) OF CULTURES.

Treatment 192 days	No. of Cultures	Initial Wt. of Segment	Final Wet Wt.	Wt. of Shoots	Wt. of Callus	Final Dry Wt.	Pct. Dry Wt.	Incre. of Growth
Inverted	9	62	247	4	181	38	15.5	185 ± 53
Normal	14	54	224	27	143	32	14.3	170 ± 44

TABLE 18. RELATIVE PRODUCTION OF BUDS AND SHOOTS AT VARIOUS RATIOS OF ADENINE SULFATE AND NAPHTHALENEACETIC ACID.

Treatment						
Adenine Sulfate mg/l	Naphthalene-acetic acid gammas/l	No. of Cultures	Total No. of Buds	Total No. of Shoots	Total Length of Shoots (cm.)	No. of Shoots and Buds per Culture
0	100	43	35	22	50	1.3
40	100	39	14	7	22	0.5
80	100	43	1	4	13	0.1
120	100	36	2	11	48	0.4
0	50	18	0	5	10	0.3
40	50	19	0	1	3	0.1
80	50	14	0	0	0	0
120	50	10	1	0	0	0.1
0	0	22	0	0	0	0
40	0	22	0	0	0	0
80	0	23	0	0	0	0
120	0	23	0	0	0	0

TABLE 19. EFFECT OF ADENINE SULFATE AND NAPHTHALENEACETIC ACID UPON FRESH AND DRY WEIGHTS (MG.) OF CULTURES.

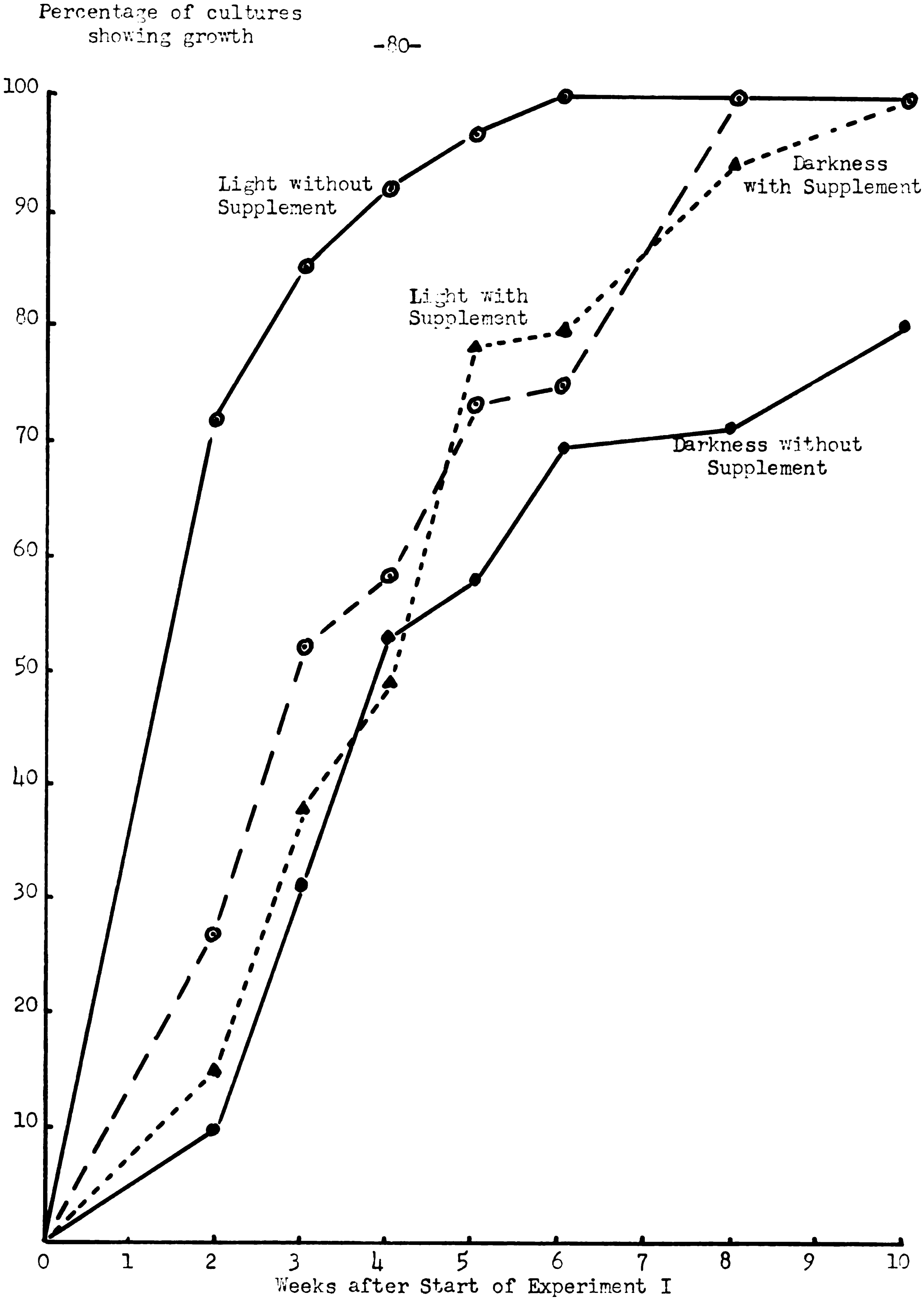
Treatment									
Adenine Sulfate mg/1	Naphthalene-acetic acid gammas /1	No. of Cultures	Initial Wt. of Segment	Final Wet Wt.	Weight of Shoots	Weight of Callus	Final Dry Wt.	Perc. Dry Wt.	Incre. of Growth
0	100	43	53	171	18	100	23	13.7	118
40	100	39	54	151	9	88	20	13.6	97
80	100	43	52	134	6	76	16	11.9	82
120	100	36	55	139	26	58	17	12.3	84
0	50	18	49	109	10	50	16	14.3	60
40	50	19	46	88	6	36	11	12.8	42
80	50	14	51	73	0	22	9	12.0	22
120	50	10	43	62	0	19	7	11.8	19
0	0	22	55	63	0	8	8	12.0	8
40	0	22	57	60	0	3	6	10.7	3
80	0	23	54	61	0	7	7	11.0	7
120	0	23	55	61	0	6	6	10.3	6

TABLE 20. SEMIDIAGRAMMATIC REPRESENTATION OF CULTURES OF SEGMENTS SHOWING TREATMENTS WHICH PARTIALLY OR ENTIRELY INHIBITED BUD PRODUCTION. PRESENCE OF BUDS AND CALLUS IS INDICATED BY THE SYMBOL B+C. TREATMENTS WHICH CONTROLLED BUD FORMATION ARE INDICATED BY OVALS. FIGURES REFER TO APPROXIMATE NUMBER OF BUDS PER CULTURE.

	Number and Type of Experiment						
	I	II	IIIa	IIIb	IV	V	VI
Treatment	Light Vitamins Polarity	Maleic Hydrazide Position Weight 100 r/1 NAA	Temp. Part 1 100r/1 NAA	Temp. Part 2 100 r/1 NAA*	Aeration 100 r/1 NAA	Polarity 100 r/1 NAA*	Adenine Sulfate 100 r/1 NAA*
6°C Darkness	- - -	- - -	B+C 0.7	- - -	- - -	- - -	- - -
24°C Darkness	- - -	- - -	B+C 1.0	(C 0)	- - -	- - -	- - -
30°C Darkness	- - -	- - -	- - -	(C 0)	- - -	- - -	- - -
37°C Darkness	- - -	- - -	(C** 0)	(C 0)	- - -	- - -	- - -
Room Temp. Light	B+C 2.4	B+C 1.6	B+C 0.7	(C 0)	B+C 2.0	(C 0.4)	B+C 1.3
Room Temp. Darkness	(C 0.1)		B+C 1.3	(C 0)	- - -	- - -	- - -
Room Temp. Low Aeration	- - -		- - -	- - -	(C 0)	- - -	- - -
Room Temp. 1 ppm Maleic Hydrazide	- - -	B+C 1.7	- - -	- - -	- - -	- - -	- - -

* Fresh Supply of Naphthaleneacetic Acid.

** Buds Degenerated to Callus.



<u>FIGURE 1.</u> EFFECT OF ILLUMINATION AND OF CERTAIN GROWTH SUPPLEMENTS UPON THE INITIATION OF GROWTH AS EXPRESSED BY THE APPEARANCE OF BUDS AND CALLI UPON SEGMENTS INVERTED IN THE AGAR.

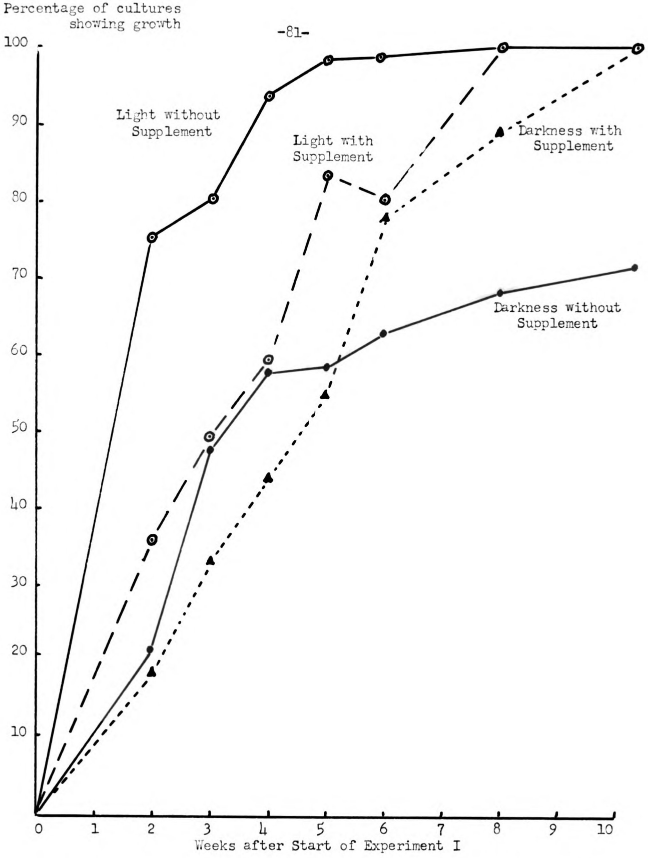


FIGURE 2. EFFECT OF ILLUMINATION AND OF CERTAIN GROWTH SUPPLEMENTS UPON THE INITIATION OF GROWTH AS EXPRESSED BY THE APPEARANCE OF BUDS AND CALLI UPON SEGMENTS NOT INVERTED IN THE AGAR.

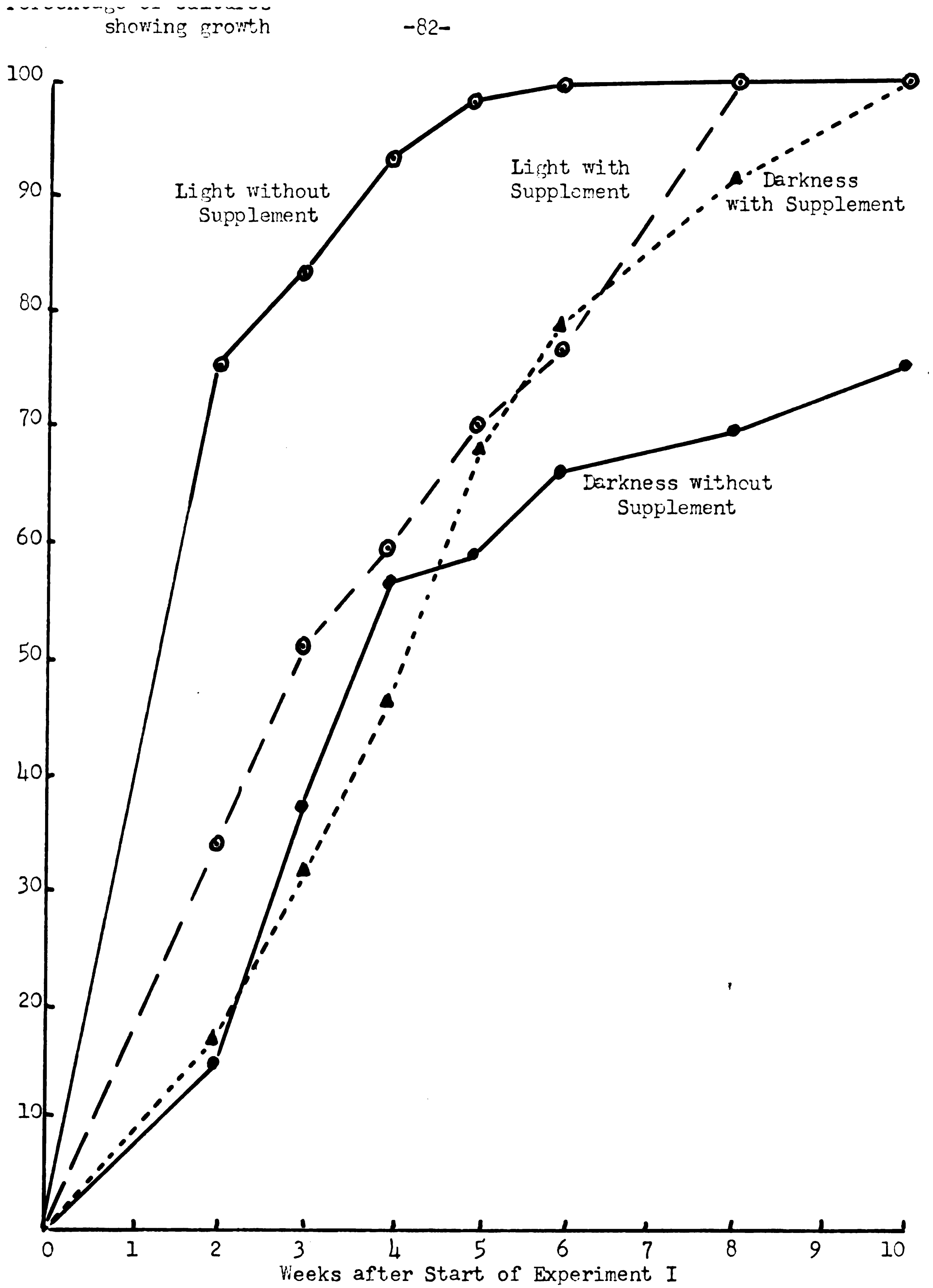


FIGURE 3. EFFECT OF ILLUMINATION AND OF CERTAIN GROWTH SUPPLEMENTS UPON THE INITIATION OF GROWTH AS EXPRESSED BY THE APPEARANCE OF BUDS AND CALLI UPON SEGMENTS REGARDLESS OF POLARITY.

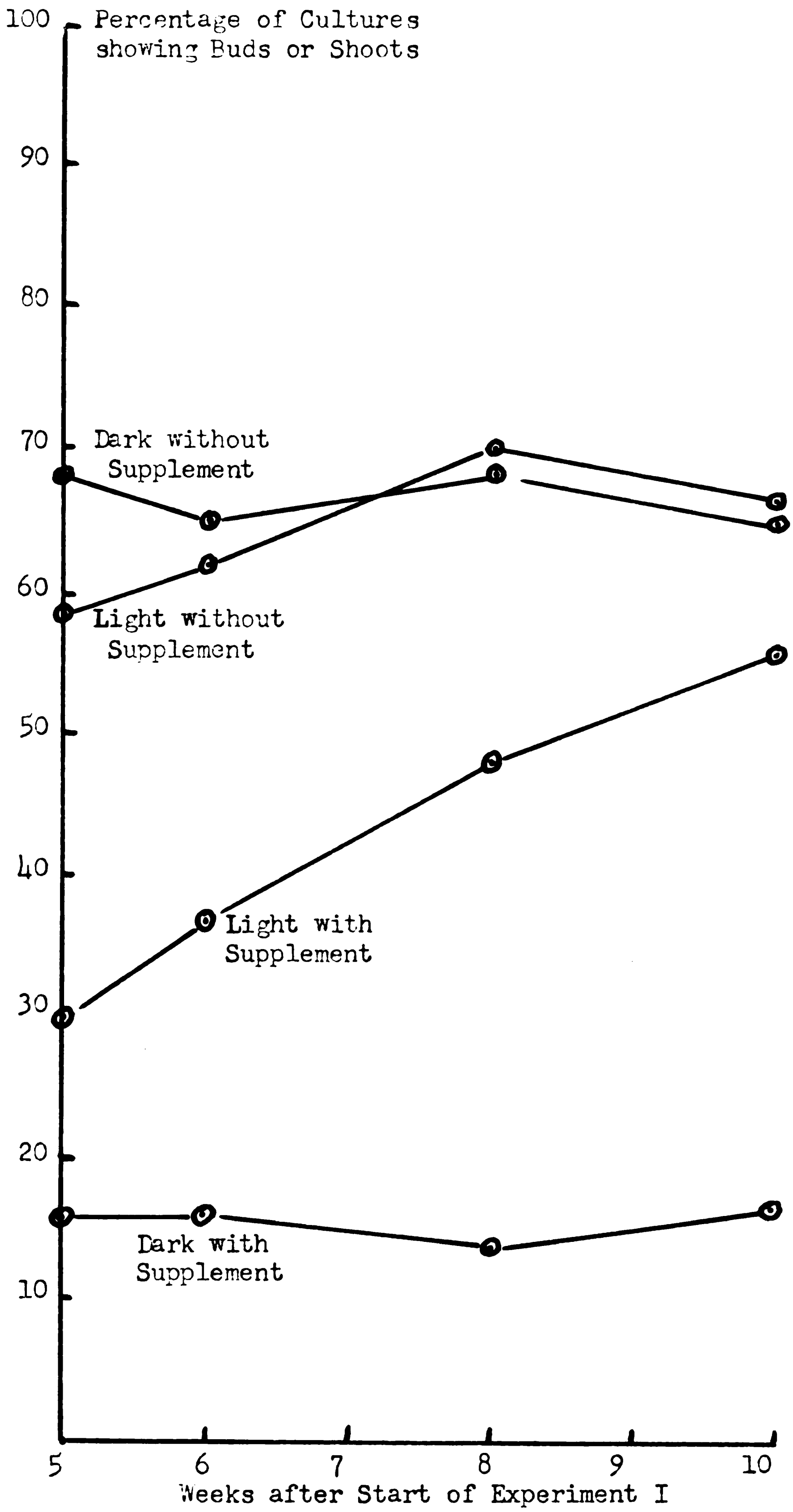


FIGURE 4. EFFECT OF ILLUMINATION AND OF CERTAIN GROWTH SUPPLEMENTS UPON THE INITIATION OF BUDS AND SHOOTS AS EXPRESSED BY THE APPEARANCE OF BUDS AND SHOOTS UPON SEGMENTS INVERTED IN AGAR.

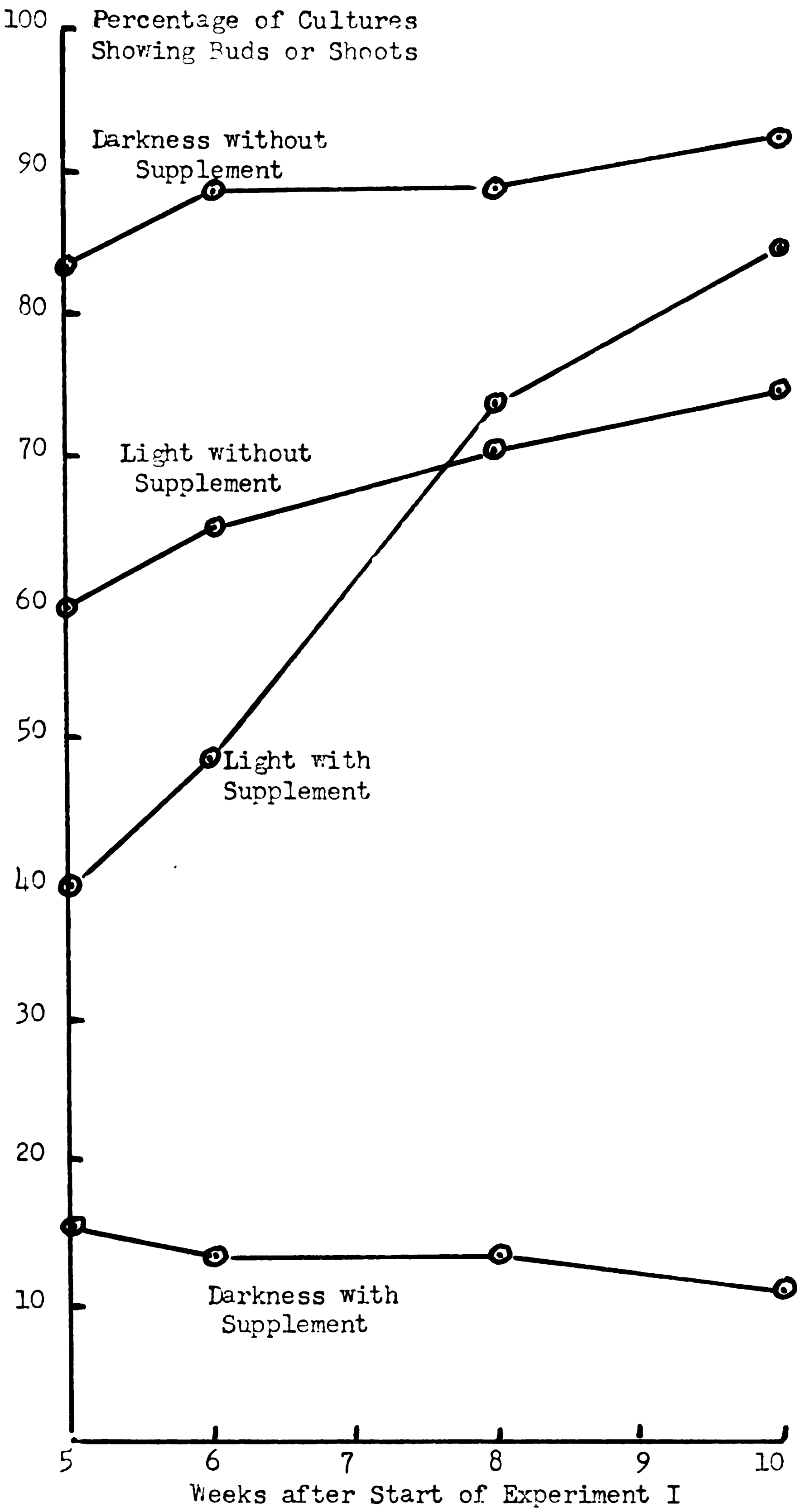


FIGURE 5. EFFECT OF ILLUMINATION AND OF CERTAIN GROWTH SUPPLEMENTS UPON THE INITIATION OF BUDS AND SHOOTS AS EXPRESSED BY THE APPEARANCE OF BUDS AND SHOOTS UPON SEGMENTS NOT INVERTED IN AGAR.

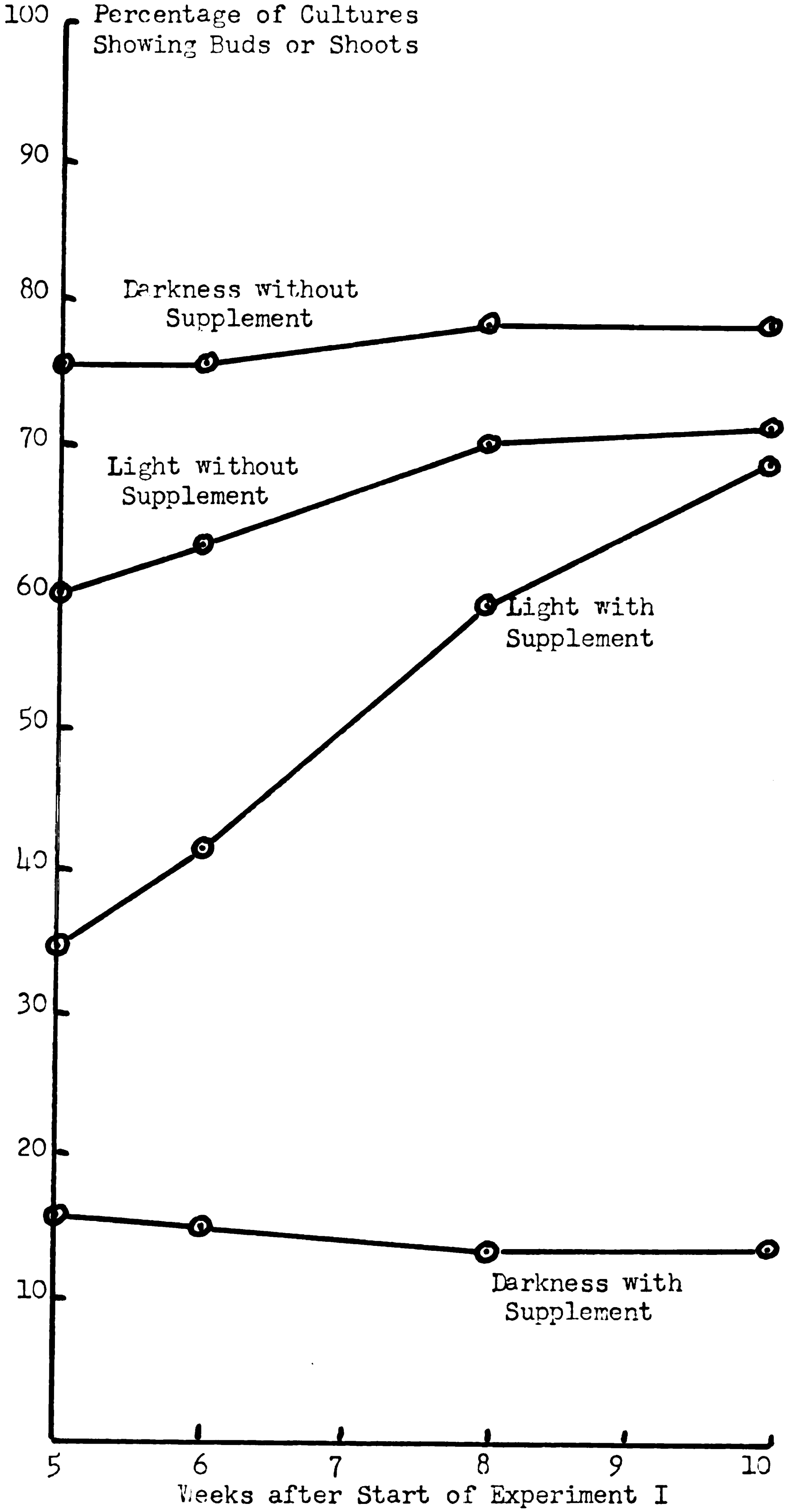


FIGURE 6. EFFECT OF ILLUMINATION AND OF CERTAIN GROWTH SUPPLEMENTS UPON THE INITIATION OF BUDS AND SHOOTS AS EXPRESSED BY THE APPEARANCE OF BUDS AND SHOOTS UPON SEGMENTS REGARDLESS OF POLARITY.

	1		2		3		4		5		6		7	
	85	0	40	10	60	1000	60	0	45	0	35	10	35	10
	85	1	50	100	75	10	60	1000	45	1	40	0	45	1000
	90	100	65	0	80	100	60	1	55	10	70	1000	60	1
	90	1000	75	1	85	0	80	10	75	100	65	1	50	100
5 cm	95	10	75	1000	85	1	100	100	115	1000	55	100	45	0
	125	100	80	10	120	1	120	1000	90	10	65	0	50	100
	140	10	65	0	125	0	110	0	85	1	55	1	65	1000
	135	1000	85	100	115	10	115	1	80	1000	70	100	130	10
	100	0	105	1000	100	1000	95	10	70	100	75	10	140	1
10 cm	150	1	100	1	110	100	115	100	85	0	90	1000	90	0
	140	0	110	0	110	1000	125	0	80	10	85	100	110	1
	120	1	100	1	120	10	105	100	95	100	85	10	100	100
	120	100	105	100	110	0	110	1000	90	1000	80	0	100	1000
	135	1000	85	10	125	1	135	1	85	1	95	1000	110	0
15 cm	155	10	100	1000	125	100	125	10	85	0	100	1	105	10
	160	1000	95	100	120	0	135	10	80		100	10	45	1000
	145	1	110	1	145	1000	135	100	85		85	100	135	1
	175	0	95	1000	140	1	160	0	95		110	1	120	10
	225	10	100	10	165	10	140	1000	85		100	0	110	0
20 cm			110	0			200	1	110				140	100
			110	0										
			125	10										

Averages

130	90	111	114	87	77	91

Averages for First 15 Centimeters

118	83	103	101	79	71	82

FIGURE 7. DIAGRAMMATIC REPRESENTATION OF THE SHOOTS AND SEGMENTS OF EXPERIMENT II SHOWING INITIAL WEIGHTS (MG.) INSIDE EACH SEGMENT AND TREATMENT OF MALEIC HYDRAZIDE (PARTS PER MILLION) AT THE RIGHT OF EACH SEGMENT.

Burl No. 2

	7a		8		9		10		1		2		Ave
	25	1	50	10	50	1	30	100	30	0	50	1	46
	45	0	50	100	55	1000	35	0	35	10	45	100	51
	55	10	70	0	55	10	55	1000	30	1000	45	1000	61
	60	1000	65	1000	60	100	65	1	30	1	55	10	67
	75	100	85	1	60	0	55	10	45	100	50	0	71
	75		95	1	55	1	60	1000	55	100	75	100	82
	70	1000	85	10	60	0	60	1	55	0	70	10	80
			85	100	65	10	70	0	65	1	85	0	92
			100	1000	55	100	65	100	55	1000	80	1000	87
10 cm			95	0	75	1000	75	10	85	10	100	1	98
			160	0	70	1	80	0	105	1	85	0	100
			95	1000	80	100	85	1000	90	10	95	10	98
			105	100	75	10	80	10	95	1000	95	1	97
			100	1	80	0	105	100	85	0	90	100	103
15 cm			115	10	90	1000			80	100	100	1000	107
									110	0			112
									130	1			121
									150	10			131
									125	100			131
20 cm									170	1000			146
									130	100			120
									130	0			148
									160	10			
									150	1000			
25 cm									150	1			
									140	10			
									165	0			
									125	1			
									130	1000			
30 cm									155	100			

Average

7a	8	9	10	1	2	Ave
58	86	66	69	102	75	92

Average for first 15 cm

7a	8	9	10	1	2	Ave
	86	66	69	63	75	83

FIGURE 7 CONTINUED.

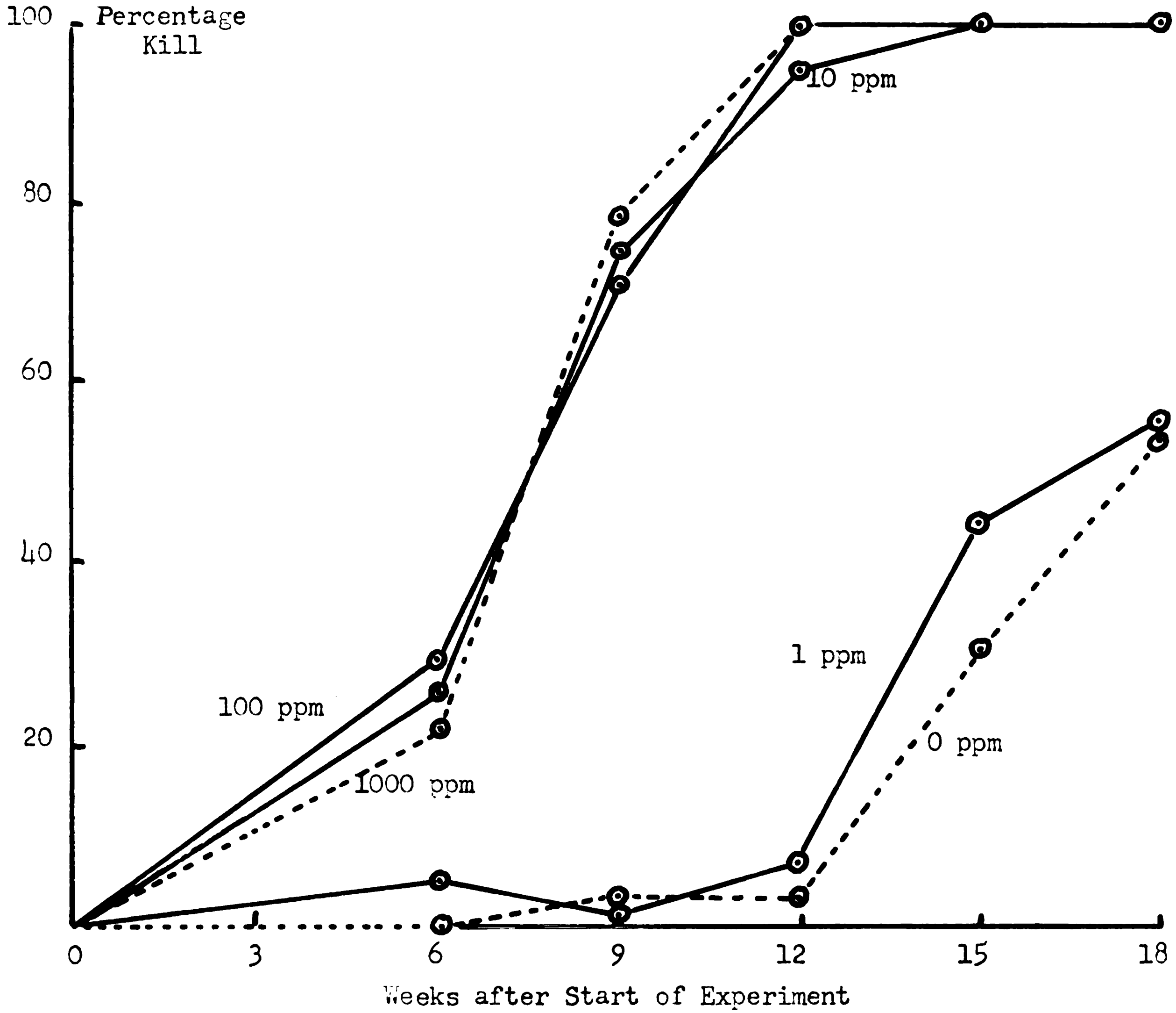


FIGURE 8. PERCENTAGE OF CULTURES KILLED AS A RESULT OF TREATMENT WITH MALEIC HYDRAZIDE.

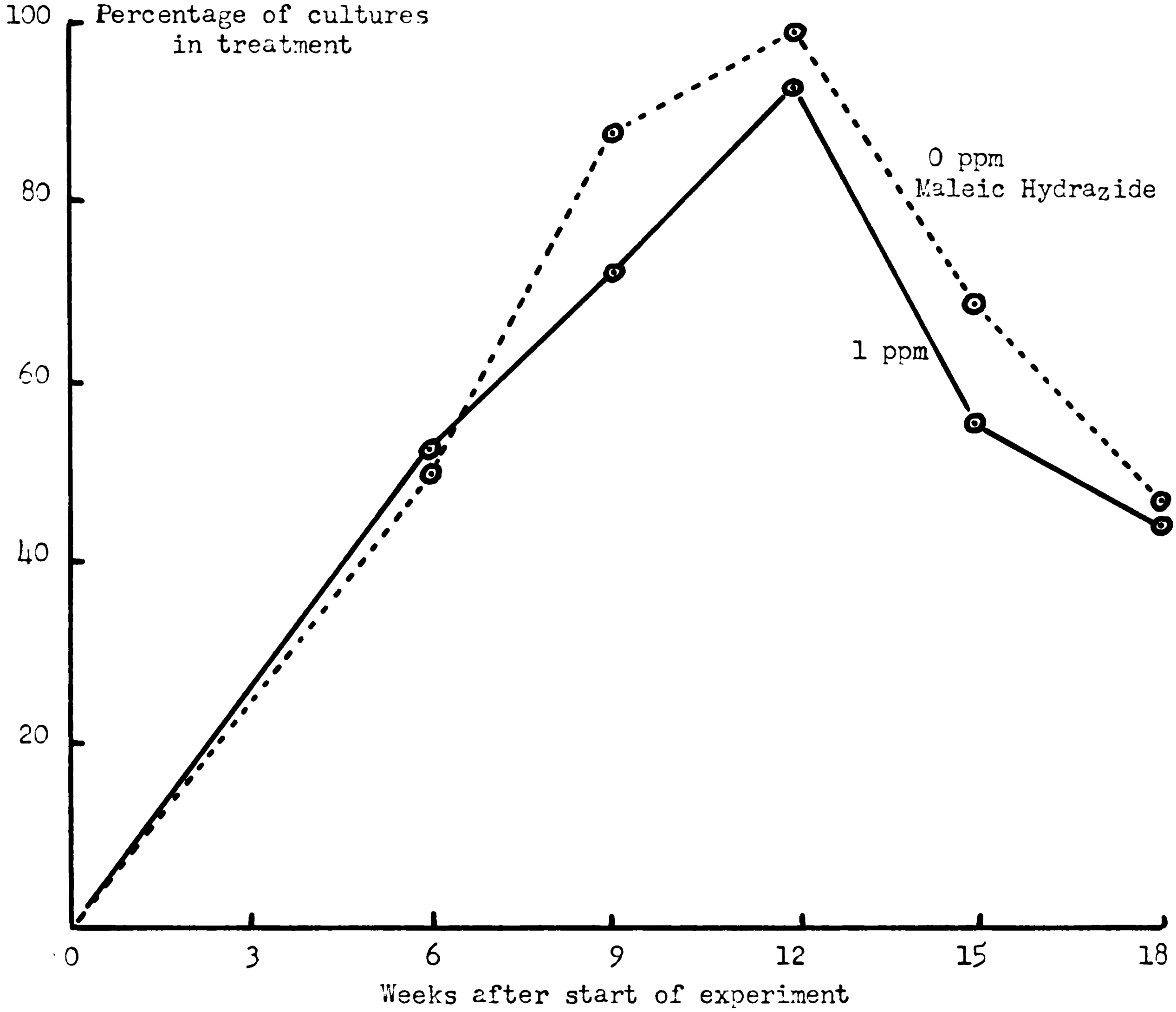


FIGURE 9. PERCENTAGE OF CULTURES SHOWING ANY TYPE OF GROWTH (CALLUS, SHOOTS, OR BUDS).

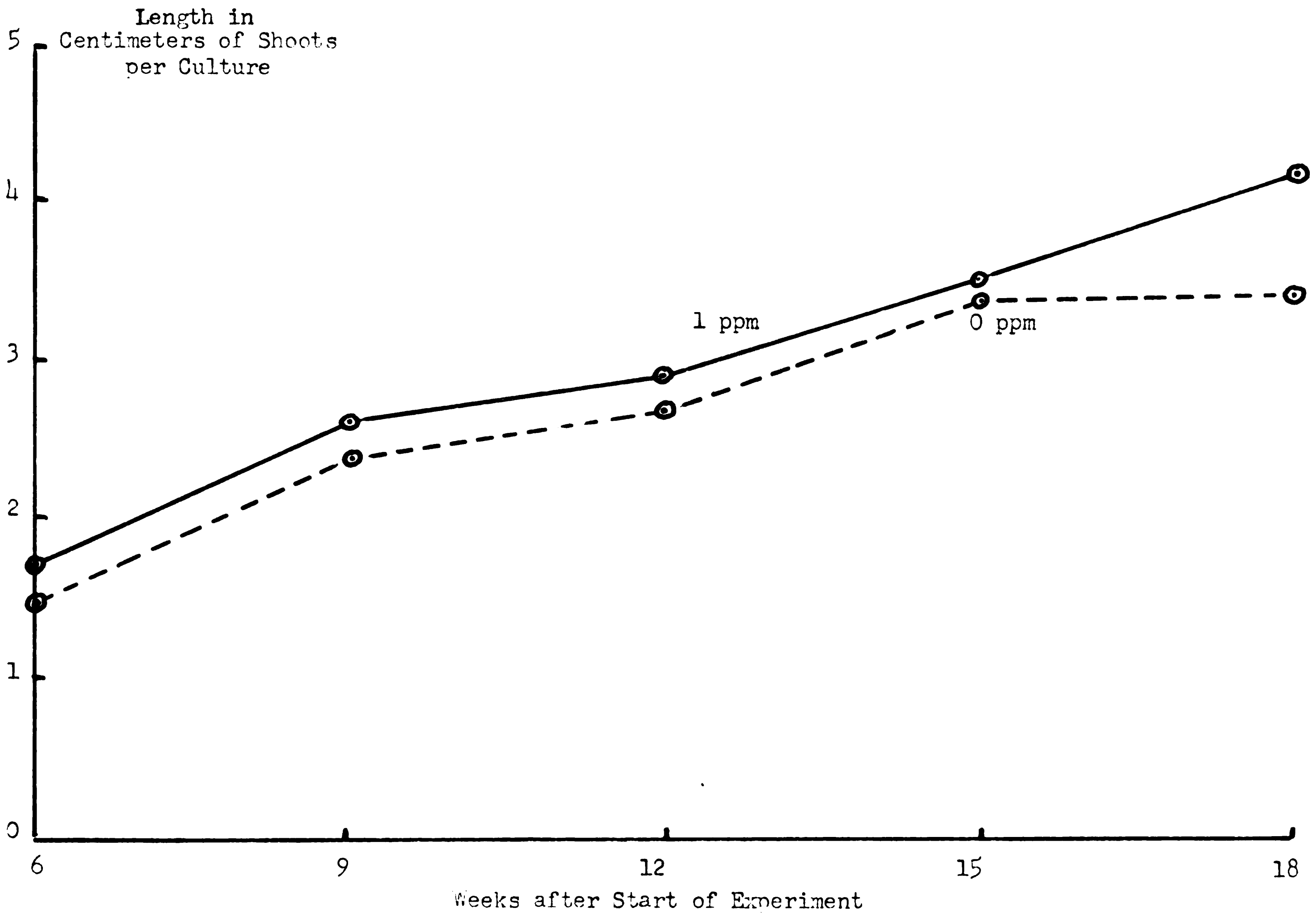


FIGURE 10. COMPARATIVE GROWTH OF SHOOTS WITH AND WITHOUT MALEIC HYDRAZIDE IN THE MEDIUM.

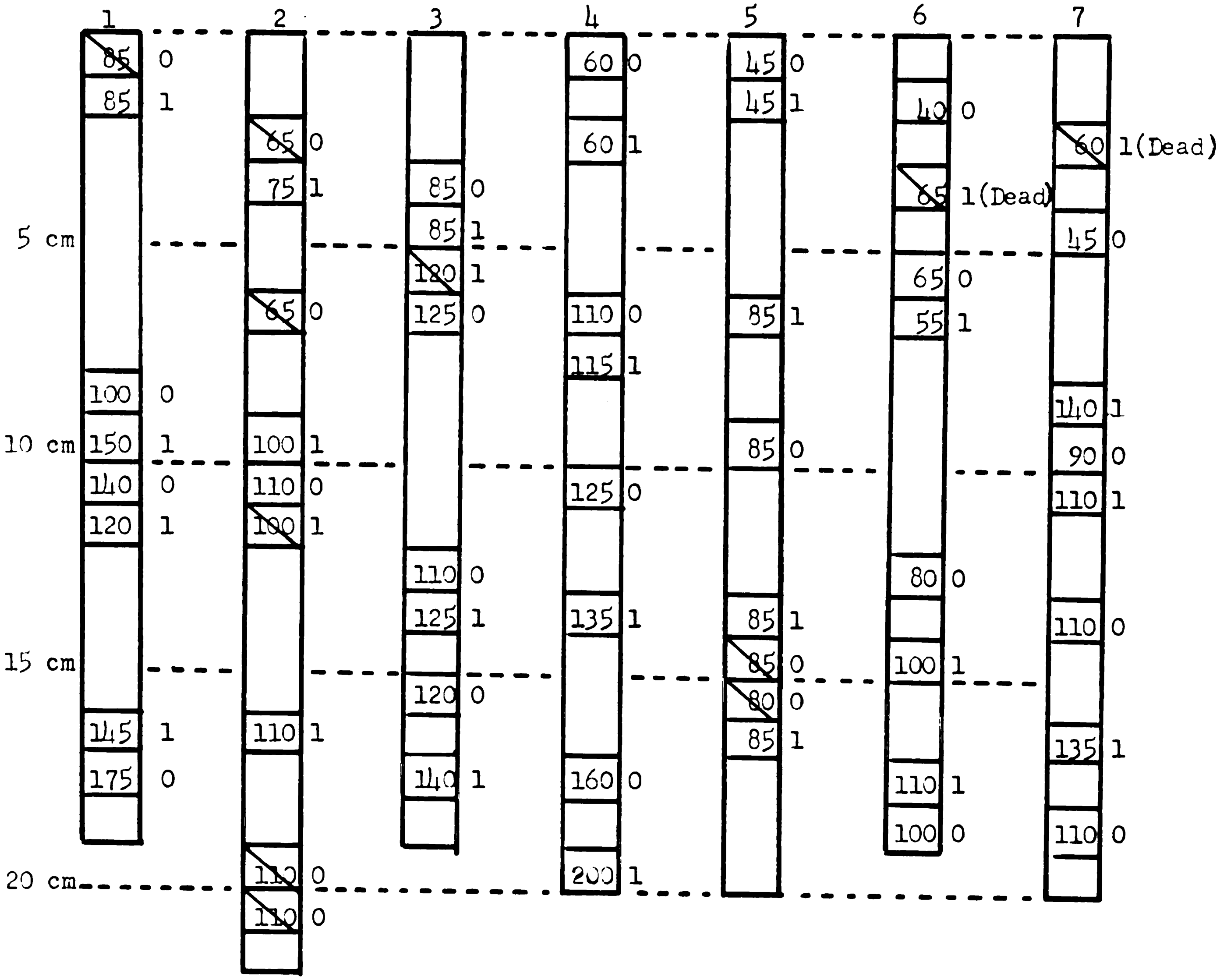


FIGURE 11. POSITION, ORIGINAL WEIGHT, AND TREATMENT OF SEGMENTS WHICH PRODUCED PROLIFERATIONS IN CULTURE. SEGMENTS LOST BECAUSE OF CONTAMINATION ARE LINED OUT. DEAD SEGMENTS ARE INDICATED.

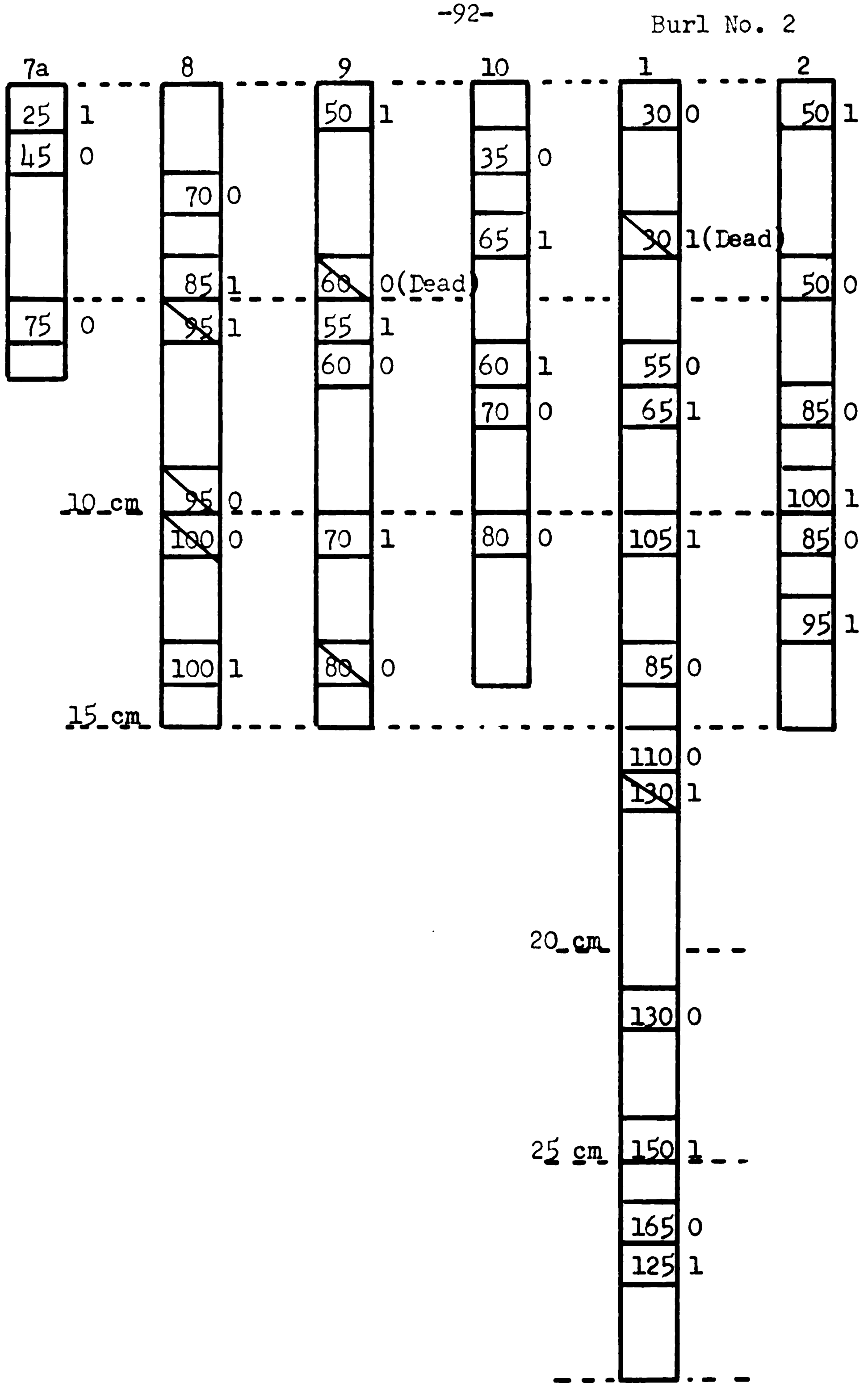


FIGURE 11, (CONTINUED)

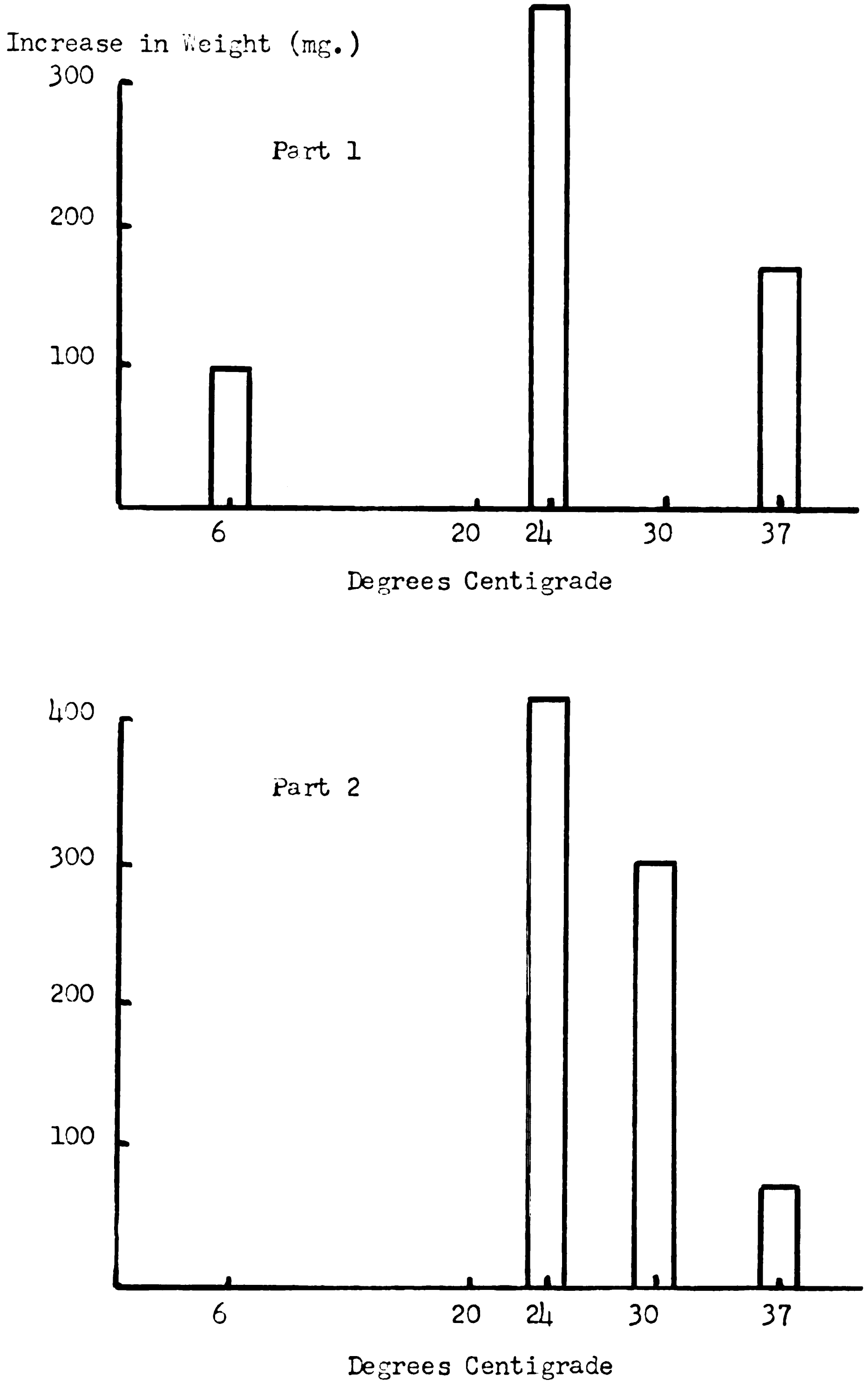


FIGURE 12. TERMINAL INCREMENTS OF FRESH WEIGHT (MG.) PRODUCED BY CULTURES MAINTAINED IN CONTROLLED TEMPERATURES.

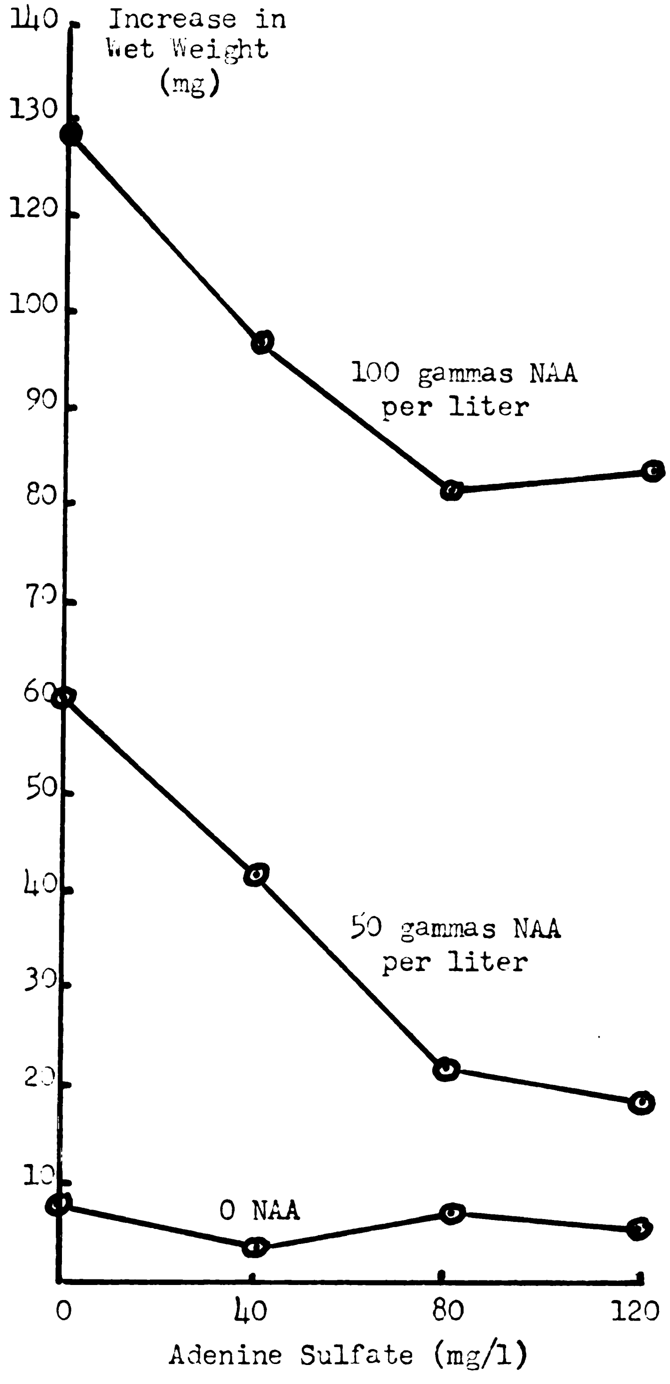


FIGURE 13. EFFECT OF ADENINE SULFATE UPON THE TERMINAL INCREMENTS OF FRESH WEIGHT (MG.) PRODUCED BY SEGMENTS CULTURED AT SEVERAL LEVELS OF NAPHTHALENEACETIC ACID (NAA).

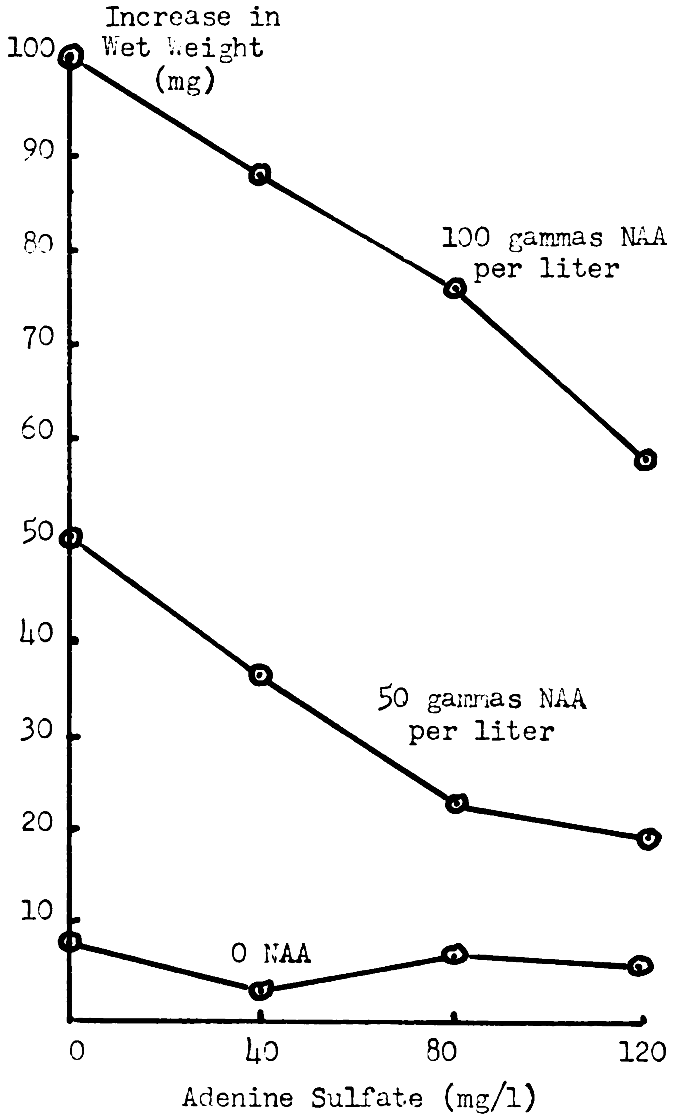


FIGURE 14. EFFECT OF ADENINE SULFATE UPON THE TERMINAL INCREMENTS OF FRESH WEIGHT (MG.) OF CALLUS PRODUCED BY SEGMENTS CULTURED AT SEVERAL LEVELS OF NAPHTHALENEACETIC ACID (NAA).

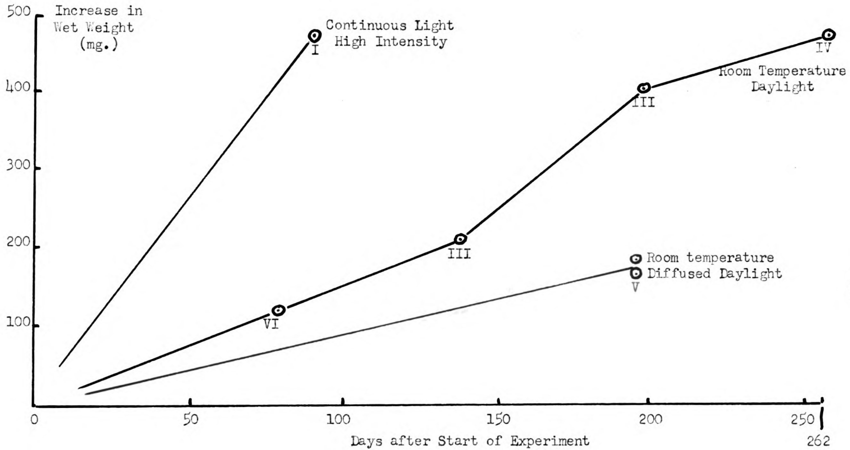


FIGURE 15. INCREASES IN FRESH WEIGHT PRODUCED BY THE SEGMENTS OF SEVERAL EXPERIMENTS UNDER SIMILAR CONDITIONS OF ROOM TEMPERATURE IN LIGHT.

PLATE 1. REDWOOD BURL OF *SEQUOIA SEMPERVIRENS* (LAMB.) ENDL. IN SPROUTING CONDITION. X 1/2.

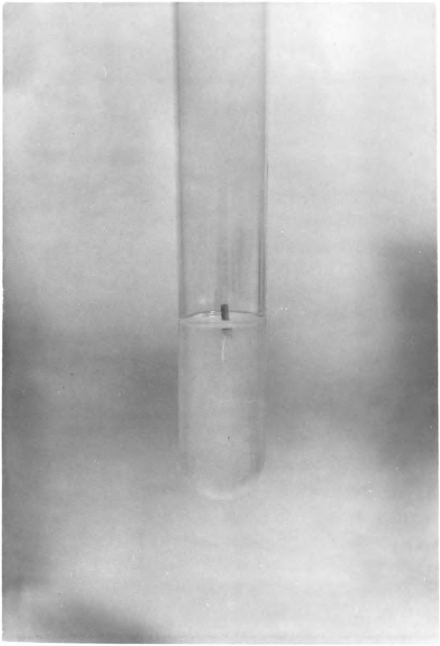


PLATE 2. FRESHLY CUT SEGMENT FROM SHOOT OF REDWOOD BURL. X 1.

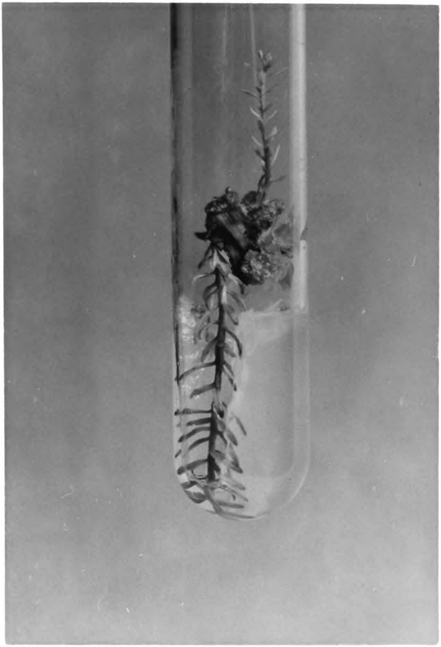


PLATE 3. TYPICAL GROWTH OF SHOOTS AND CALLUS FROM A SEGMENT AFTER EIGHTY DAYS. X 1 1/2.

PLATE 4. SHOOT TIPS OF SEQUOIA BURL SHOOTS GROWING IN CULTURE. X 1 1/2.

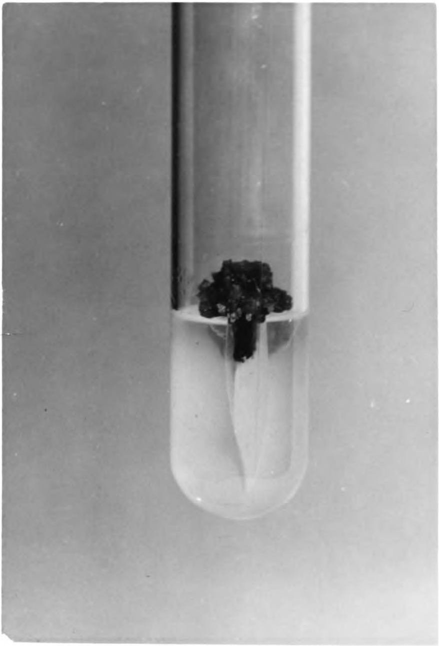


PLATE 5. TYPICAL GROWTH OF CALLUS FROM AN EXCISED SEGMENT OF SEQUOIA BURL SHOOT. X 1 1/2.

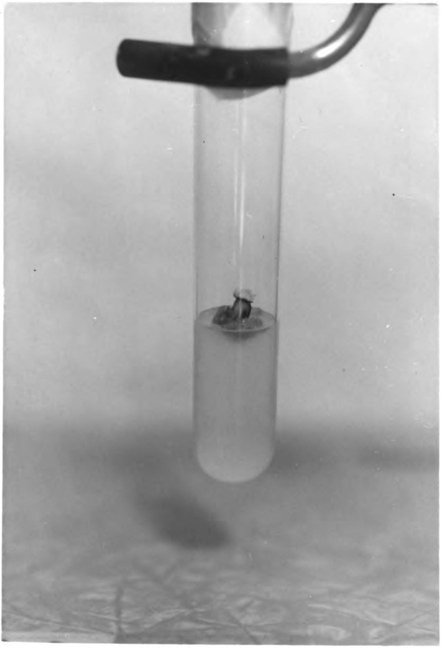


PLATE 6. DEVELOPMENT OF CALLUS AT BOTH ENDS OF BURL SHOOT SEGMENT GROWN IN DARKNESS AT 24° C. X 1.

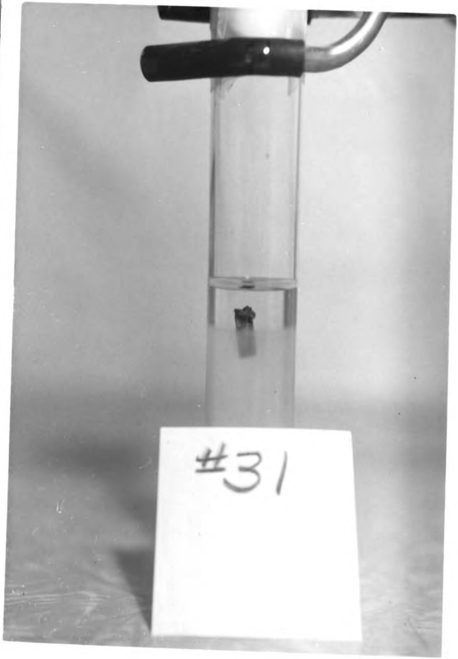


PLATE 7. METHOD OF GROWING SUBMERGED CULTURES. X 1.

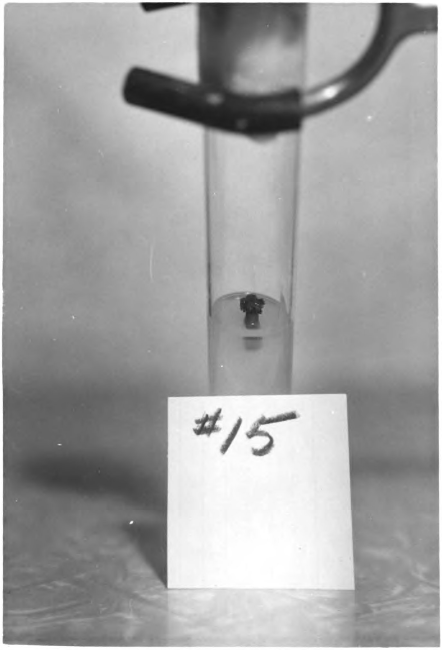


PLATE 8. TYPE OF CALLUS PRODUCED BY SUBMERGED CULTURES (LIQUID REMOVED). X 1.

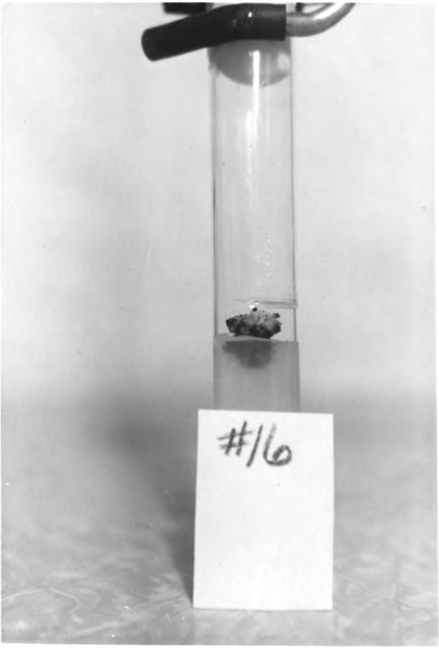


PLATE 9. PRODUCTION OF CALLUS BY NON-SUBMERGED CULTURE. X 1.

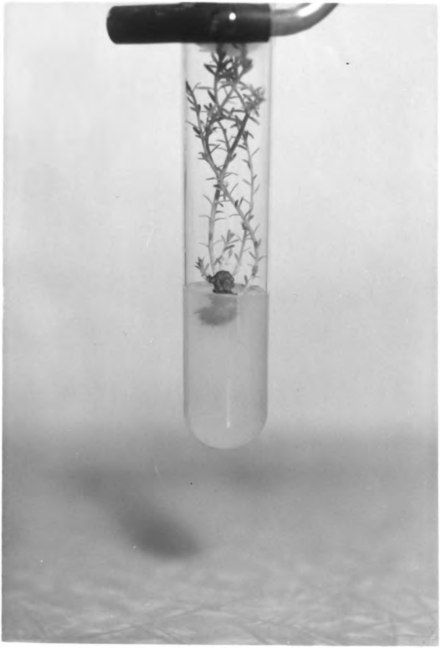


PLATE 10. PRODUCTION OF SHOOTS BY NON-SUBMERGED CULTURE. X 1.