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Tissue culture of Dioscoreophyllum cumminsii

Jeffrey Lee Koons

The University of Montana

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TISSUE CULTURE OF DIOSCOREOPHYLLUM CUMMINSII

By

Jeffrey L. Koons

B.A., University of Montana, 1974

Presented in partial fulfillment of the requirements for
the degree of
Master of Arts
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Approved by:

David E. Bildenbach
Chairman, Board of Examiners

Dean, Graduate School

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Tissue Culture of Dioscoreophyllum cumminsii

Director: Dr. David E. Bilderback

Tissue cultures from the seeds of Dioscoreophyllum cumminsii were initiated and maintained on a modified medium of White with coconut milk (CM). Growth could not be maintained on this medium without CM, but good growth was obtained on the medium of Murashige and Skoog, using one-half the macronutrients. Cultures grown on this defined medium displayed distinct nodules and darker, senescent tissue. This medium was used to study the response to 2,4-dichlorophenoxyacetic acid (0, 10^{-7}, 10^{-6} and 5 \times 10^{-6} \text{M}), zeatin (0, 10^{-6} and 10^{-5} \text{M}), and gibberellic acid (0, 5 \times 10^{-8} and 5 \times 10^{-6} \text{M}).

When 2,4-D was either deleted or supplied at 10^{-7} \text{M}, roots were produced and the culture became senescent. Good growth was maintained at higher auxin concentrations. Tissue was examined using both light and electron microscopy, which showed that the nodules were organized into four zones. The outer two zones were meristematic, containing large starch accumulations and vacuolar protein deposits, while the inner zones were highly vacuolate with the cells becoming progressively degenerate towards the core of the nodule.

Growth was stimulated by zeatin at low auxin concentrations, but did not affect growth at higher auxin concentrations. Growth could be maintained by zeatin in the absence of auxin. Tissue produced in response to zeatin appeared unorganized, but did contain localized growth centers. Starch accumulations and vacuolar protein deposits were found within these centers.

Tissue grown on medium containing 10^{-6} \text{M} 2,4-D and 10^{-5} \text{M} zeatin was supplied with GA_3, and growth was doubled during two passages. New growth, in response to GA_3, was typically callus-like, with the cells containing large nuclei and nucleoli. The nucleoli contained one to several large vacuoles which were not found in tissues on other media.
ACKNOWLEDGMENTS

I would like to express my sincerest thanks to Dr. David E. Bilderback, chairman of my committee, for all of the help, time, and encouragement which he gave me, both as a graduate and as an undergraduate student.

I would also like to thank Dr. R. S. Medora for his help during this study, as well as the other members of my committee, Dr. C.N. Miller and Dr. G.P. Mell.

Thanks also to the Research Advisory Council for grants (849-9/ZA and 841-4/C) which provided some supplies and summer salary for this work.
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CHAPTER 1

INTRODUCTION

Non-Carbohydrate Sweeteners

In 1969, the Food and Drug Administration banned the use of cyclamates leaving saccharin as the only sweetener permissible in food supplies of the United States (Inglett, 1974). Many dietetic and medicinal needs, as well as the rising cost of sugar, have spurred an intensified search for a safe, alternative sweetener (Morris, 1976). Diverse types of compounds elicit a sweet taste (Table 1). Many of these compounds, however, have not been proven safe for human consumption. Research on the physiological basis of taste has progressed in recent years, but the diversity of sweeteners has hindered the development of a general model explaining the mechanism of taste.

Some possible alternative sweetening agents have been found in naturally occurring foods. In 1852, W.F. Daniell described a berry which, when chewed, caused normally sour or bitter tasting foods to taste sweet (Daniell, 1852 as cited in Kurihara and Beidler, 1968). This berry is produced by Synsepalum dulcificum, and the
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1 after Inglett 1974
taste-modifying substance has been shown to be a glycoprotein (Kurihara and Beidler, 1968).

Although some D-amino acids (Solms et al., 1965 as cited in Morris et al., 1972) and a dipeptide ester (Mazur et al., 1969) are known to taste sweet, no true protein possessing this quality had been found until recently. In 1972, two independent research groups reported the isolation and characterization of a sweet tasting protein (Monellin) from the berry of Dioscoreophyllum cumminsii (Morris et al., 1972; Van Der Wel, 1972; Morris and Cagan, 1972; Van Der Wel and Loeve, 1973). The molecular weight of this protein is approximately 10,500. Taste threshold tests indicated the protein was about 3,000 times sweeter than sucrose by weight. On a molar basis, the relative sweetness of the protein would be at least an order of magnitude greater than this.

Two other sweet tasting proteins were isolated from the fruit of Thaumatalcoccus danielli. These proteins, Thaumatin 1 and 2 have molecular weights of approximately 21,000 and 20,000, respectively. Taste threshold tests showed them to be about 1600 times sweeter than sucrose by weight and approximately $10^5$ times sweeter on a molar basis (Van Der Wel and Loeve, 1972).

These three proteins are the sweetest compounds known (Van Der Wel, 1972). Proteins as sweeteners would
be of obvious importance both dietetically and medicinally for those people requiring sugar substitutes such as diabetics. Since the plants which produce these proteins are not under cultivation at the present time, desirable quantities cannot be obtained for widespread use.

Tissue Culture of Dioscoreophyllum Communisii

The possible use of cell cultures of higher plants as a source of desirable chemical products has been recognized since the 1950s (Routien et al., 1956). Useful compounds such as alkaloids, steroids, proteins, and antimicrobial agents have been isolated from plant tissue cultures (Puhan and Martin, 1971; Staba, 1969; Steck and Constabel, 1974) and it is reasonable that sweet proteins could also be produced using this technique.

Dr. R.S. Medora became interested in producing sweet proteins from tissue cultures of Synsepalum dulcificum, Thaumatococcus danielli, and Dioscoreophyllum cumminsii. Cultures of Dioscoreophyllum cumminsii were successfully initiated from seeds, but cultures of Thaumatococcus danielli and Synsepalum dulcificum have not been obtained.

There are three phases to this research project; the successful initiation and maintenance of the desired cultures, the manipulation of growth and differentiation
by chemical stimulation, and finally, the determination of the presence of sweet protein in the tissue cultures.

This thesis describes the growth and differentiation of *Dioscoreophyllum cumminsii* tissues cultured on different media containing various types of growth regulators.
Tissue cultures of *Dioscoreophyllum cumminsii* Diels, were obtained from Dr. R.S. Medora. These cultures were initiated from seeds purchased from Seed Supply Agency, Akim-Oda, Ghana. Each seed was flame sterilized, and the seed coat removed. The endosperm, containing the embryo, was cut longitudinally, and each half was placed in a 125 ml Erlenmeyer flask containing 20 ml of a modified White's medium with 0.75% agar (Appendix A). This medium was supplemented with coconut milk (CM: 15% v/v) and 2,4-D \( (9 \times 10^{-6} \text{ or } 1.8 \times 10^{-5} \text{ M}) \). After 4 weeks, these subcultures were transferred to a standard medium containing 1.8 \( \times 10^{-6} \text{ M} \) 2,4-D. All media used were adjusted to pH 5.7-5.8 with 0.1 N NaOH after dissolving the agar, and all cultures were grown in constant darkness at 27°C ± 2 and 64% relative humidity.

Further studies were conducted using the medium of Murashige and Skoog (1962), henceforth abbreviated M and S (Appendix B). In addition, the macronutrient components
were halved (1/2 M and S) and various concentrations of 2,4-D (0, 10^{-7}, 10^{-6} and 5 \times 10^{-6} M), zeatin (0, 10^{-6} and 10^{-5} M), and gibberellic acid (0, 5 \times 10^{-8} and 5 \times 10^{-6} M) were added singly or in combination.

An attempt was made to initiate shoots using the 1/2 M and S macro- and micro-nutrients with the vitamins recommended by Linsmeier and Skoog (1965), 10^{-6} M 2,4-D and 10^{-5} M zeatin. This medium was further supplemented with 160 mg/L adenine sulfate, 340 mg/L NaH_{2}PO_{4} \cdot H_{2}O and 100 mg/L L-Tyrosine (Thorpe and Murashige, 1970).

All M and S media and modifications contained 1% agar.

Growth was measured as the net increase in fresh weight for each 30 day growth period. On passage to fresh medium, approximately 250 mg of tissue was transferred after preferentially removing the darker, senescent tissue.

**Light Microscopy**

Tissue was fixed in buffered 3% gluteraldehyde (0.05 M potassium phosphate, pH 6.8), dehydrated in a graded ethanol series and embedded in methacrylate plastic (Feder and O'Brian, 1968). The tissue was sectioned at approximately 1 and 3 \mu m, stained with a periodic acid-Schiff procedure (Feder and O'Brian, 1968) and counter stained with aniline blue-black (Fisher, 1968).
**Electron Microscopy**

Tissue was fixed in 3% buffered gluteraldehyde (0.05 M potassium phosphate, pH 6.8) and postfixed in 2% Osmium (buffered, pH 6.8). The tissue was rinsed twice in buffer, dehydrated in a graded ethanol series and embedded in either a modified Mollenhauer, Epon-Araldite 506 mixture (1964; Appendix C) or Spurr's medium (1969). Silver and silver-gold sections were cut with a glass knife on a Sorvall MT-2 ultramicrotome and picked up on either Formvar or Collodion covered copper grids. The sections were stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963). Pictures were taken with a Zeiss EM 9 S-2 electron microscope.
CHAPTER 3

RESULTS

Standard Maintenance Medium

Tissues obtained from Dr. R.S. Medora had been maintained on the standard medium for approximately nine months. A random sample of each culture had been transferred to fresh medium at 30 day intervals. These tissues were extremely hard, compact, and heterogenous with each callus consisting of both light yellow and dark brown tissue. Growth was slow and did not appear to be localized in any region of the callus, and roots were infrequently produced.

During this study, the 2,4-D concentration in the standard medium was increased to 5 x 10^{-6} M, which completely inhibited root production. The majority of the darker tissue was preferentially removed at each passage to fresh medium. After 14 transfers, growth had increased considerably when compared with the initial growth rates on the standard medium with increased auxin (Figure 2). New growth was confined to that portion of the tissue directly in contact with the medium. This zone of growth was light yellow while the distal portion of the culture was dark brown. The consistency of these cultures remained unchanged.
Figure 1. Average growth of 18 cultures after one passage, and after 14 passages on the standard medium.
GROWTH—MG FRESH WEIGHT

STANDARD MEDIUM

T1

T14
Chemically Defined Medium

Coconut milk (CM) was deleted from the standard medium to eliminate unknown growth factors. After two passages, tissue transferred to this medium without coconut milk grew much slower (Figure 2) and became increasingly dark as growth decreased. The modified medium of White would not support growth in the absence of coconut milk.

Subcultures from the standard medium also were transferred to M and S and 1/2 M and S medium containing the same concentration of 2,4-D as the standard medium. Tissue grown for three passages on the 1/2 M and S medium grew rapidly while growth on the M and S medium declined (Figure 3). The decrease in growth on the M and S medium was also accompanied by a progressive darkening of the tissue.

Dramatic morphological changes occurred during the first passage on the 1/2 M and S medium. Distinct, light yellow nodules appeared together with a very dark, brown-black tissue (Figures 4, 5). New growth was derived from these nodules and the amount of growth varied depending upon the relative amounts of nodular and dark tissue. Friability increased greatly in conjunction with the change in morphology. The tissue became extremely soft and appeared more moist.
Figure 2. Average growth during two consecutive passages on the standard medium with and without coconut milk (CM). 12 cultures per treatment.
GROWTH - MG FRESH WEIGHT

STANDARD MEDIUM

+CM  -CM
Figure 3. Average growth during three consecutive passages on M and S and 1/2 M and S medium. Ten cultures per treatment.
Figure 4. Typical nodular morphology of cultures grown on 1/2 M and S(defined) medium. Approx. 3X

Figure 5. Nodular culture cut in half showing distinct nodules and dark tissue. Approx. 3X
Auxin

Growth. Tissue was subcultured from the standard medium and transferred to 1/2 M and S medium containing $5 \times 10^{-6}$ M 2,4-D for two passages. Similar morphological changes occurred as described above, which were characteristic of growth on this defined medium with auxin. These cultures were then transferred to fresh medium containing either $10^{-7}$, $10^{-6}$ or $5 \times 10^{-6}$ M 2,4-D and grown for three passages. Several cultures were transferred to medium without auxin; however, growth measurements were not taken.

Growth rates appeared comparable (Figure 6), although the cultures grown on medium supplied with either $10^{-6}$ or $5 \times 10^{-6}$ M 2,4-D seemed healthier. New growth on medium containing the lowest auxin concentration was limited to root production. In cross section, these roots had triarch xylem and the cortical cells contained numerous starch grains, most prominently in those cells surrounding the stele (Figure 7).

Morphology. Those cultures grown on medium lacking auxin rapidly produced roots, both with and without root hairs (Figure 8). Following root production, the cultures became very dark and necrotic. Growth could not be maintained without auxin.
Figure 6. Average growth during three passages on the defined medium containing different 2,4-D concentrations. 13 cultures per treatment.
Figure 7. Root cross section. Arrows depict xylem differentiation. Phase-contrast. Approx. 660X

Figure 8. Roots produced after two passages on the defined medium without auxin. Approx. 3X
All cultures grown on the low auxin supplement also produced roots and eventually stopped growing although the length of time involved in this sequence was increased. Roots arose from nodular tissue; however, these nodules did not appear to form root primordia exclusively as root number did not correspond to the number of nodules present.

One-half of the cultures supplied with $10^{-6}$ M 2,4-D exhibited roots but not as extensively as those cultures described above. Decreased growth and senescence did not occur following root production. No roots were formed by tissues in the highest auxin treatment.

**Histology.** Nodules were excised from tissue grown on the defined medium containing $10^{-6}$ M 2,4-D, and fixed for both light and electron microscopy.

The nodules were organized into four general regions. 1) A thin fluffy layer of loosely attached cells, 2) a meristematic mantle of cells, 3) an interior zone of large, highly vacuolate cells, and 4) a central core of randomly differentiated tracheal elements and degenerating cells (Figure 9). These regions were not sharply defined, as the cytology of the cells changed progressively from one region to the next.

Cells of the outer zone and meristematic mantle contained combinations of small and large vacuoles (Figure
Figure 9. Longitudinal section of nodule. Numbered arrows depict zones described in text. Approx. 260X

Figure 10. Cells of nodular zones 1 and 2. S=starch grain, P=protein inclusions. Approx. 2600X
Within the vacuoles were large granular dots.
Within the vacuoles were large protein deposits, both free or aligned along the membrane. Numerous starch grains occupied the cytoplasm adjacent to the nucleus.

With the electron microscope, the large protein bodies were found both closely appressed to the membrane and free within the vacuoles (Figures 11, 12, 13). Fibrillar material was also present. Those bodies appressed to the membrane were apparently in the process of being formed. Small vesicles, containing cytoplasmic like material, were commonly found associated with these bodies (Figure 14). Rough endoplasmic reticulum and polysomes indicated active protein synthesis. Numerous vesicles were seen fused with the plasma membrane presumably depositing cell wall material (Figure 13). A small lacuna was commonly observed within the nucleolus, and the nuclei were very fibrillar in appearance. Numerous multi-lobed starch grains were also present within the plastid.

Toward the interior of the nodules, cells became larger and more vacuolate than those of the outer zones, with both protein and starch accumulation diminishing (Figures 15, 16). The nucleus and cytoplasm were much more peripheral although cytoplasmic strands commonly traversed the vacuole.

Cells of the core region appeared to be degenerating, and starch and large protein deposits were not observed (Figure 17). The cytoplasm and mitochondria appeared very
Figure 11. Cells of zone 2. S=starch grain, V=vacuole, N=nucleus. Approx. 6200X
Figure 12. Montage of zone 2 cell. NC=nucleolus, N=nucleus, P=protein, S=starch, VS=vesicle. Approx. 7200X
Figure 13. Cell from zone 2. Arrow depicts region illustrated in Figure 14. Approx. 7200X

Figure 14. Detail of Figure 13. VS=vesicles, PR=polysome, RER=rough endoplasmic reticulum, P=protein. Approx. 64,000X
Figure 15. Cells of the outer region of zone 3. Approx. 2600X
Figure 16. Cell detail from outer region of zone 3. S=starch, P=protein. Approx. 18,000X

Figure 17. Degenerating cell of zone 4. CW=cell wall, M=mitochondrion. Approx. 28,500X
dense. Some tracheal elements differentiated and intercellular gaps formed due to cell breakdown (Figure 9).

Dark tissue from the above cultures was observed with the electron microscope and was found to be necrotic, containing only disorganized membrane profiles (Figure 18).

**Auxin/Cytokinin**

**Growth.** Tissue from cultures maintained on the standard medium were transferred to 1/2 M and S medium containing either $10^{-7}$, $10^{-6}$ or $5 \times 10^{-6}$ M 2,4-D for three passages. The number of cultures on each treatment was increased during each transfer by subdividing previous cultures. Each auxin treatment was then randomly divided into three groups and transferred to fresh medium containing $10^{-6}$ or $10^{-5}$ M zeatin. Two cultures from each auxin treatment were transferred to medium containing $10^{-5}$ M zeatin but lacking auxin.

During three passages on medium containing zeatin, growth varied both between auxin treatments as well as through time (Figure 19). Tissue from the low auxin treatment without zeatin steadily decreased in growth while those supplied with either concentration of zeatin grew increasingly well with each passage. All of these cultures had produced roots during the three passages on medium with $10^{-7}$ M 2,4-D and had become progressively
Figure 18. Detail of cell from the dark, senescent tissue. Mb=membrane, CW=cell wall. Approx. 93,000X
Figure 19. Average growth during 3 consecutive passages on the defined medium with variable 2,4-D and zeatin concentrations. 6 or more cultures per treatment.
darker and necrotic. The zeatin fortified medium reversed this sequence. New roots did not appear and the tissue did not become darker.

The healthiest and most consistent growth was obtained from cultures grown on medium containing $10^{-6}$ M 2,4-D and $10^{-5}$ M zeatin. At this auxin level, $10^{-5}$ M zeatin stimulated growth as compared to the controls.

Growth on the high auxin treatment was not stimulated by addition of zeatin although the growth during the third passage with $10^{-5}$ M zeatin might indicate some stimulation after prolonged exposure.

Tissue grew well on the medium lacking auxin but containing zeatin. Growth rates were not recorded but after three passages the cultures seemed healthy despite the production of some roots. Growth could be maintained without auxin in the presence of zeatin.

**Morphology and Histology.** The characteristic nodular morphology produced by tissue on 1/2 M and 5 with auxin persisted with the addition of zeatin, but some morphologically distinct tissue also appeared along the sides of the parent culture in contact with the medium. This new tissue was light yellow-white in color and seemingly not nodular. All tissue treated with zeatin became much more rigid than that prior to treatment.

This distinctive new growth was excised from cultures
grown on $10^{-6}$ M 2,4-D and $10^{-5}$ M zeatin and fixed for light microscopic observation. Although this tissue seemed superficially less differentiated than the parent tissue, distinct growth centers were still found. These appeared as distinct masses of small, densely cytoplasmic cells surrounded by larger, highly vacuolate cells (Figure 20). In cross section, cells around the periphery of these centers contained starch grains, and the cells in the interior were somewhat more vacuolate than those surrounding them (Figure 21). Mitotic cells were found in both regions as were proteinaceous inclusions within the vacuoles.

Viewed longitudinally these centers appeared to be organized in a similar manner as nodules grown on other media (Figure 22). Highly vacuolate cells formed a thin layer covering the densely cytoplasmic meristematic mantle. Cells to the interior were somewhat more vacuolate and random tracheids were found in the central core region (Figure 23). The interior cells differed from those in the corresponding region of nodules grown on media containing auxin alone. These cells were more cytoplasmic and did not degenerate in the core region. No starch accumulation was found, although protein deposits were found throughout the growth center.
Figure 20. Growth centers in tissue produced in response to $10^{-6}$ M 2,4-D and $10^{-5}$ M zeatin. Approx. 260X

Figure 21. Detail of Figure 21. Arrows depict starch grains, P=protein, Mi=mitotic cell. Approx. 600X
Figure 22. Longitudinal section from tissue produced in response to 10^-6 M 2,4-D and 10^-5 M zeatin. Approx. 260X

Figure 23. Core region of Figure 23. T=tracheid, P=protein. Approx. 600X
Auxin/Cytokinin/Gibberellic Acid

**Growth.** Tissue was grown on 1/2 M and S medium containing $10^{-6}$ M 2,4-D and $10^{-5}$ M zeatin for two passages. As stated previously, this medium produced the most consistent growth from this tissue. The cultures were then transferred to fresh medium containing either $5 \times 10^{-8}$ or $5 \times 10^{-6}$ M GA$_3$ and growth recorded during two passages (Figure 24).

Growth was dramatically increased by both GA treatments after two passages; however, growth stimulation occurred more rapidly in response to the lower GA concentration.

**Morphology and Histology.** Cultures grown on a medium containing GA$_3$ were morphologically similar to those grown on zeatin. The relative amount of the light, non-nodular tissue was, however, greatly increased. Most of the increased growth was due to proliferation of this tissue rather than the residual nodular part of the culture.

No distinct growth centers were observed in this tissue after treatment with GA$_3$. The cells were approximately 2.5 times larger than cells in the meristematic mantle of nodules (Figure 25). These cells had very lightly staining cytoplasm which generally appeared to
Figure 24. Average growth of 11 cultures grown on the defined medium with $10^{-6}$ M 2,4-D, $10^{-5}$ M zeatin, and variable $GA_3$ concentrations for two consecutive passages.
GROWTH - MG FRESH WEIGHT

GA\textsubscript{3-}M 0 5 \times 10^{-8} 5 \times 10^{-6}

10^{-6} M 2, 4-D
10^{-5} M ZEATIN
Figure 25. Cell from tissue produced in response to $10^{-6}$ M 2,4-D, $10^{-5}$ M zeatin, and $5 \times 10^{-8}$ M GA$_3$. Phase-contrast. S=starch grains, N=nucleus. Approx. 2600X

Figure 26. Nucleolus of cell from tissue produced as in Figure 25. V=vacuole, NC=nucleolus. Approx. 10,000X
be peripheral. The nuclei and nucleoli were both large, the latter containing large vacuoles or several lacunae (Figure 26). Very small starch grains were distributed randomly through the tissue and protein deposits were rare. Cell walls were very thin and convoluted.
CHAPTER 4

DISCUSSION

Standard Medium

After growing more than a year in culture, *Dioscoreophyllum* tissues maintained on the standard medium continued to increase in growth and change morphologically. Preferential selection of tissue for subculture has been found to increase growth and decrease organization of tissues (Butenko, 1964), and the removal of the dark, senescent tissue is apparently responsible for this increase in growth through time.

Coconut milk, or one of a number of complex natural plant extracts, is frequently added to nutrient medium to encourage initiation of callus or to maintain growth. Coconut milk is rich in substances which may enhance or affect tissue growth (Shantz and Steward, 1955; Tulecke et al., 1961; Pollard, et al., 1961; Letham, 1974; Van Staden and Drews, 1974, 1975), but its use precludes quantification of the medium composition which is desirable for studies of growth, morphogenetic capacity, or biosynthetic processes (Hildebrandt, 1970).

The modified medium of white could not support
growth without the addition of coconut milk. The defined 1/2 M and S medium produced good growth and was used to study the effects of 2,4-D, zeatin, and gibberellic acid on growth and histology of *Dioscoreophyllum* tissues.

**Defined Medium**

**Auxin**

Most tissues grown in culture require exogenously supplied auxin to initiate the callus and maintain growth. The auxin, 2,4-D, is considered to be the most potent auxin in stimulating callus growth of isolated tissue, while concurrently inhibiting organized development (Murashige, 1974; Venverloo, 1973). *Dioscoreophyllum* tissue, however, when grown on the defined medium containing relatively high concentrations of 2,4-D is highly organized into nodular growth centers. New growth of the tissue is confined to a broad zone of cell division at the periphery of the nodules.

The anatomy and cytology of the nodules indicate that they are regions of potential organ formation. Large accumulations of starch and protein, as well as the formation of zones of cell division, have been found to form in tobacco callus tissues prior to shoot formation (Thorpe and Murashige, 1968, 1970; Ross, et al., 1973). However, nodules of *Dioscoreophyllum* tissue did not arise
from completely unorganized tissues, and shoots were not produced when the medium was supplemented with substances used to induce shoot formation in tobacco cultures (Thorpe and Murashige, 1970).

The cellular changes, from the periphery to the core of the nodules, appears to be both temporal as well as spatial. As new cells are formed at the periphery, cells to the interior progressively degenerate. Further organization of the nodules was not observed, and it appears that differentiation of these tissues is arrested at the nodular stage in the presence of auxin.

**Dioscoreophyllum** tissue responds rapidly to decreased auxin supplements by producing roots and becoming necrotic. This rapid response indicates a relatively low endogenous auxin content in the tissue. Good growth could be maintained only with relatively high auxin concentration in the medium. No exogenous cytokinins were required for maintenance of growth. Witham (1968) has suggested that very effective auxins like 2,4-D can lower the requirement of exogenous cytokinins and can stimulate growth without it.

When exogenous auxin concentrations are decreased, roots differentiated in conjunction with the nodular tissue and appeared more rapidly and profusely when dark tissue was present in the culture. The nodules are not
simply root precursors as several roots may arise from one common area of a nodule while other nodules may be devoid of roots. The role of the dark tissue in root formation is unclear. However, it possibly may play a role in establishing a physiological or nutritional gradient within the cell mass which, in conjunction with a decrease in exogenous auxin, enhances root production. Studies by Ross and Thorpe (1973) have indicated that the establishment of a physiological gradient within a culture may be in part responsible for shoot initiation.

**Auxin/Cytokinin**

The role of auxin-cytokinin interactions in regulating the pattern of organogenesis in cultured tissue was first suggested by Skoog and Miller (1957) and has been reviewed periodically (Butenko, 1964; Murashige, 1974). High auxin/low cytokinin ratios favors root formation, and the reverse favors the formation of shoots.

This study, as well as other investigations, appears to contradict this general organogenic pattern. Nishi et al. (1975) found that rice cultures produced roots only when auxin was deleted from the medium, and Kaul and Sabharwal (1972) found root production was inhibited at high auxin concentrations. In intact plants, auxin tends to enhance root initiation while inhibiting further root development, which may explain the behavior of these
tissues.

Zeatin has been isolated from coconut milk (Van Staden and Drewes, 1974) and for this reason was chosen for studying the effect of exogenously supplied cytokinins. Tissue which had been grown on the defined medium without cytokinin became soft and more moist. The first observable response to cytokinin was a hardening of the tissue. A similar response to higher cytokinin levels was noted in tobacco cultures (Lance et al., 1976).

The auxin requirement for growth of Dioscoreophyllum tissue can be replaced by the addition of zeatin as tissue was successfully subcultured on medium without auxin but supplemented with $10^{-5}$ M zeatin. Similar responses were obtained with certain strains of tobacco cultures (Syōno and Furuya, 1972). These authors indicated that cytokinin (kinetin) may be influencing endogenous auxin concentrations.

Growth responses to zeatin varied with the concentration of 2,4-D present. Tissue grown on $10^{-7}$ M 2,4-D, which normally would become necrotic during several passages, steadily increased in growth. Zeatin consistently stimulated growth of tissue grown on $10^{-6}$ M auxin although it did not appear to enhance growth at higher auxin levels. Cytokinins may act synergistically with auxin at certain levels to promote cell division and growth (Letham, 1969)
although the cytokinin (kinetin) was found to inhibit growth at high auxin concentrations (Witham, 1968). According to the scheme of Skoog and Miller (1957), a balance of auxin and cytokinin favors rapid growth of unorganized tissue. The best growth obtained from Dioscoreophyllum tissue was when a moderate auxin concentration was supplemented with cytokinin. However, it must be kept in mind that the tissues on this medium never become completely disorganized.

Distinct growth centers were found embedded in an unorganized matrix of tissue consisting of highly vacuolate parenchymatous cells. These growth centers were more compact and localized than the larger, broad nodules observed on medium containing auxin alone. Starch and protein deposits were observed although their distribution within the growth center was different from that found in the nodules. Addition of zeatin did not induce organ formation although possible organ forming centers were present. It is unclear whether these centers were derived directly from the nodules or differentiated from unorganized tissue produced by the nodules in response to zeatin.

Auxin/Cytokinrin/Gibberellic Acid

Gibberellic acid, GA₃, has been shown to promote cell division (Digby, et al., 1964), induce fresh weight increases (Murashige, 1964) and to inhibit starch accumu-

Growth of Dioscoreophyllum tissue was dramatically stimulated in response to GA_3, although this growth seemed largely due to cell enlargement. The tissue was completely disorganized and no localized growth centers were found in tissue produced in response to GA_3. This tissue was typically callus-like, with large thin-walled cells, little starch accumulation, peripheral cytoplasm and an enlarged nucleus and nucleolus. The nucleolus was very distinctive in this tissue, both in size and in containing one to several large vacuoles. Large nucleoli and nucleolar vacuoles have been observed in a wide variety of cells. Several studies have shown that nucleoli do become enlarged and vacuolate in response to GA_3 (Gifford and Nitsch, 1969; Chapman and Jordan, 1971). Other work has attributed this response to 2,4-D (Vasil, 1973).

The role of the nucleolar vacuoles is unclear. Johnson and Jones (1967) found contractile nucleolar vacuoles in cultured tobacco cells and suggested that the vacuoles may be involved in the release of some soluble product associated with RNA metabolism. Johnson (1969) correlated the degree of vacuolation of the nucleolus with RNA synthesis although Barlow (1970) found that the
capacity to synthesize RNA was not related to nucleolar vacuolation. Furthermore, Rose, et al. (1972) found that nucleolar vacuoles formed when RNA metabolism is slowed down. It is difficult to say, at this time, what is responsible for this distinctive nucleolar morphology, although it does appear to reflect a change in the physiological state of these cells.
CHAPTER 5
CONCLUSIONS

The growth of tissue in static cultures may vary, not only between species, but also between tissues from different sources from the same plant (Butenko, 1964). Preferential selection of tissues, as well as the length of time in cultures may also cause variation in response to identical conditions (Murashige and Nakamo, 1965; Butenko, 1964). Growth rates of Dioscoreophyllum cultures grown for short periods of time under identical conditions varied greatly. The organization of the cultures precluded the subcultures of qualitatively identical tissues of similar weights so that statistical analysis of growth rates could not be employed. However, distinct trends, both in growth and histology could be identified.

Coconut milk, which is rich in cytokinins (Letham, 1974), was required for growth on the standard medium. However, tissue grew well on 1/2 M and S without cytokinins indicating that cytokinins may not be the component of coconut milk needed to maintain growth. However, the differences in the organic and mineral constituents of the two media may differentially affect the cytokinin requirement of the tissue. Furthermore, Linsmeier and
Skoog (1965) found that some of the vitamins supplied in the modified White's medium are unnecessary and in fact, may inhibit growth of tobacco cultures. These vitamins perhaps may be inhibiting growth in the absence of coconut milk.

Large, vacuolar deposits of protein were found in the organized tissue grown on the defined medium both with and without zeatin. Cultures are currently being grown for a preliminary assay for sweet protein. Maximum growth of Dioscoreophyllum tissue on the defined medium was in response to gibberellic acid. The cells produced, however, do not contain proteinaceous deposits and generally stain very lightly for cytoplasmic protein. This tissue, although faster growing, may not be a desirable source for the sweet protein.

The sweet protein is water soluble and comprises more than 50% of the soluble protein in the berry. The function of this protein in the berry tissue is unknown although it would seem to play a role in seed dispersal. Histological and developmental investigation of the berry could provide useful information for inducing cultures of Dioscoreophyllum to produce this significant product. If the sweet protein is accumulated in the vacuoles of the cells of the fruit, the protein deposits observed with the light and electron microscopes in the cultured cells may be sweet
protein also.

The work described in this thesis is the first investigation of the in vitro growth of Discocoryphillum in response to different known growth regulators. New species in culture require extensive investigation as different auxins and cytokinins, as well as other factors, may prove to be more effective in increasing growth and altering tissue organization. This work represents only the beginning of the groundwork necessary for manipulation of this species in culture to produce sweet proteins.
Tissue cultures from the seeds of *Dioscoreophyllum cumminsiii* were obtained from Dr. R.S. Medora, which had been initiated and maintained on a modified medium of White with coconut milk. The cultures were comprised of both light yellow and dark brown tissue and were extremely hard. During transfer, the dark tissue was preferentially removed, and after 14 passages, growth increased. Growth could not be maintained on this medium without coconut milk, but good growth was obtained when the tissue was transferred to the medium of Murashige and Skoog (1962) using one-half the macronutrients (1/2 M and S).

The morphology of the cultures changed when grown on the 1/2 M and S medium, with distinct nodules and dark senescent tissue appearing. Electron and light microscopy showed that the nodules were organized into four zones, the outer two being comprised of meristematic cells containing starch accumulations and vacuolar protein deposits. Within these outer zones was a region of highly vacuolate cells and the core of the nodule contained random tracheal elements and degenerating cells. The dark tissue was non-living. The cultures grown on this medium became
much softer. This medium was used for studying the response of this tissue to plant growth regulators.

When 2,4-D was either deleted or supplied at $10^{-7}$ M, roots were produced and the cultures became senescent. Good growth was maintained with auxin concentrations of either $10^{-6}$ or $5 \times 10^{-6}$ M.

Cultures were then grown on these auxin concentrations for three passages before the addition of zeatin which was supplied at either 0, $10^{-6}$ or $10^{-5}$ M. Growth was then recorded for three passages. Growth was stimulated by zeatin in the high auxin treatment, but growth was maintained without auxin if zeatin was present. New growth in response to zeatin appeared unorganized, but distinct growth centers were found within a matrix of callus-like tissue.

Tissue was then grown on $1/2$ M and S containing $10^{-6}$ M 2,4-D and $10^{-5}$ M zeatin, which produced the best and most consistent growth for three passages. Gibberellic acid was then supplied at concentrations of 0, $5 \times 10^{-8}$ or $5 \times 10^{-6}$ M. Growth doubled during two passages in response to both concentrations of $GA_3$ and new growth was typically callus-like. The cells contained large nuclei and nucleoli and little starch or protein accumulation. Large nucleolar vacuoles were present which may indicate a significant change in the physiological state of this tissue.
BIBLIOGRAPHY


APPENDIX A

MEDIUM USED BY MEDORA
FOR CALLUS CULTURES

**Macronutrients**--White, 1963

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**Micronutrients**--White, 1963


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Amino Acids & others--Staba and John, 1962

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Adjuncts

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APPENDIX B

MURASHIGE & SKOOGS MEDIUM*

Mineral Salts

### Major elements

- NH₄NO₃: 1650 mg/L
- KN₀₃: 1900 mg/L
- CaCl₂.2H₂O: 440 mg/L
- MgSO₄.7H₂O: 370 mg/L
- KH₂PO₄: 170 mg/L
- Na₂-EDTA: 37.3 mg/L
- FeSO₄.7H₂O: 27.8 mg/L

### Minor elements

- H₃BO₃: 6.2 mg/L
- MnSO₄.4H₂O: 22.3 mg/L
- ZnSO₄.4H₂O: 8.6 mg/L
- KI: 0.83 mg/L
- Na₂MoO₄.2H₂O: 0.25 mg/L
- CuSO₄.5H₂O: 0.025 mg/L
- CoCl₂.6H₂O: 0.025 mg/L

\[1.5 \text{ ml/L of a stock solution containing } 5.57 \text{ g FeSO₄.7H₂O and } 7.45 \text{ g Na₂-EDTA per liter of H₂O.}\]

### Organic constituents

- Sucrose: 30 g/L
- Glycine: 2.0 mg/L
- Agar: 10 g/L
- myo-Inositol: 100 mg/L
- Nicotinic acid: 0.5 mg/L
- Pyridoxin: HCl: 0.5 mg/L
- Thiamin: HCl: 0.1 mg/L

APPENDIX C

Modified Mollenhauer (1964) Epon-araldite 506

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