Cytological studies of tobacco tissue cultures

Walter Julius Marten

The University of Montana

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CYTOLOGICAL STUDIES OF TOBACCO TISSUE CULTURES

by

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B.A. University of Montana, 1965

Presented in partial fulfillment of the requirements for the degree of

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1970

Approved by:

Chairman, Board of Examiners

Dean, Graduate School

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To my major professor, Dr. Richard A. Solberg, I would like to extend my deepest gratitude, for without his help, guidance, and perseverance, this research could not have been completed.

I would also like to thank my committee members, Dr. M. Chessin, Dr. C. C. Gordon, and Dr. T. J. Nimlos for their helpful comments and criticisms of this work.

Finally, I would like to dedicate this thesis to my wife, Sharon, whose patience, love, and understanding made it all possible.
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CHAPTER I. INTRODUCTION

The culturing of plant tissues has received ever increasing interest in recent years, due primarily to the ease of obtaining single cells, uniform tissues, both physiologically and morphologically, and regularly reproducible results. The primary aims of this study, in addition to basic training in cytological techniques, include:

(a) An analysis of the effect of cultural variables, media variations, temperature, and light differences, on cellular organelles.

(b) An analysis of the effect of cultural variables on cellular and tissue differentiation.

(c) Investigations of the effects of tobacco mosaic virus on (a) and (b) in cultures.

_Nicotiana tabacum, Nicotiana glutinosa_, a hybrid of these two species, and tobacco mosaic virus were chosen as experimental materials due to the wealth of previously published researches. The tobacco _culture H-196_, an A. C. Hildebrandt hybrid (Hildebrandt, 1958) was selected due to its rapid growth. Other cultures were experimented with but were eventually discarded due to their poor growth rates.

It was hoped that this study would contribute to the elucidation of the normal ontogeny of cellular organelles and cell differentiation and how virus multiplication is related to the structure and function of sub-cellular units.
LITERATURE REVIEW

The culturing of tissues in vitro has been used as a tool to study the cell and its organelles for the past 60 years. From these studies, workers have been able to obtain a clear picture of the cell, tissues, and plant formation. As early as 1900, through tissue culturing, it was purported that artificial embryos might eventually be grown from vegetative cells (Haberlandt, 1902). The use of tissue culturing as a tool became fully established in the 1930's with the work of White (1934) and others. From the 1930's to the present time the use of tissue culturing has entered into many facets of cytological study. Therefore, this review will include only literature pertaining to those areas of study which this thesis considers.

Media have been developed, modified, and remodeled (White, 1943; Hildebrandt, et al. 1946; Murashige and Skoog, 1962; Venketeswaran, 1965) as a result of studies on different tissues. Earlier media contained such added complex constituents as coconut milk, the composition of which is unknown. For this reason, the results were difficult to analyze with respect to specific growth factors. As a basic medium, White's medium, as modified by Murashige and Skoog (1962), was designed for use in bioassaying organic growth factors through the use of known organic and inorganic ingredients. This medium provides a four fold increase in fresh weight and a two fold increase in dry weight of tobacco pith and callus tissue in contrast to the medium of Hildebrandt, et al. (1946) and is thus the most satisfactory to date. Through the use of this medium in combination with several others, workers have been able to grow artificial embryos from vegetative cells (Steward, et al. 1963; Vasil and Hildebrandt, 1967), thus fulfilling Haberlandt's earlier claim.
Venketeswaran (1965) found that the production and accumulation of chlorophyll was highly influenced by the presence of indoleacetic acid and kinetin. By replacing the IAA or kinetin constituents with 2, 4-D he found no chlorophyll production in the callus. Another factor influencing chlorophyll production was light intensity. High intensities, 1350-2000 lux, tended to bleach the chloroplasts whereas in low, diffuse light, 325-540 lux, the chlorophyll synthesized in the cell was protected from bleaching (Venketeswaran, 1965). Bergmann and Berger (1966) observed that tissues of N. tabacum var. "Samsun" grown in the dark contained plastids consisting of only a few isolated thylakoids, some vesiculi, and tubuli, but no prolamellae bodies characteristic of plastids in dark grown leaves. These plastids were transformed to photosynthetically active chloroplasts when placed in the light. This transformation in white light is similar to the plant leaf's chloroplast formation in the blue and red regions of visible light. Little work has been done on the effects of various colored light in this area.

Chloroplasts in situ are surrounded by jackets of material which did not contain chlorophyll and which were pleomorphic (Wildman, et al. 1962). These jackets project long protuberances into the cytoplasm from which segments may break off, becoming indistinguishable from mitochondria. Mitochondria could, by uniting with the protuberances, completely lose their identity. Hongladaron, et al. (1966), Solberg and Bald (1962), Honda, et al. (1966), and Spencer and Wildman (1962) have found similar results.

With respect to some of the other organelles of the tobacco hair cell, Solberg and Bald (1962) described the nucleus as possessing furrows and invaginations in contrast to the smoothly spherical or ovoid
nuclei in cells in which cyclosis has ceased. They also described "cytoplasmic vesicles" in some of the regions of background parietal cytoplasm. These vesicles constantly changed shape but became statically ovoid or spherical when cyclosis ceased, due to curtailment of cyclotic forces.

Wu, et al. (1960) described the general cell morphology of H-239 (N. tabacum X N. glutinosa) in liquid shake cultures according to the age of the cultures. In the one - seven day old cultures the cells were predominantly meristematic, small, with large nuclei and small vacuolar spaces. Cellular activity was probably dominated by cell division. The tissue of the 7 - 28 day old callus possessed elongated and enlarged cells and a few tracheal elements. The 28 - 40 day old callus appeared in a senescent condition with cell contents gradually disintegrating. The plastids in these cells became small and finally disappeared. Some cells rejuvenated to produce one to many endogenous cells. The perforations in the cell walls, through which the plasmodesmata pass, appeared small and not well defined in tobacco tissue cultures in contrast to those of tobacco leaves which possessed large and quite distinct perforations (Kassanis, et al. 1958). Tracheid formation begins with the formation of the secondary wall, performed by the peripheral cytoplasm laying down a system of microtubules which form the characteristic thickenings of tracheal elements (Cronshaw, 1967).

It has been possible to regulate the degree of differentiation of the callus tissue with organic growth factors. Vasil and Hildebrandt (1966) were able to produce roots and shoots in the callus tissue of Cichorium (endive) by growing the callus on modified White's medium
containing 0.04 mg/l kinetin plus 1 mg/l IAA. No root or shoot formation was found using 0.2 mg/l kinetin plus 2 mg/l IAA in the medium. For continuous growth of healthy tobacco callus over a period of years, Murashige and Skoog (1962) found the concentration of 0.2 mg/l kinetin and 2.0 mg/l IAA in White's modified medium to work quite well. However, 0.04 mg/l kinetin and 1.0 mg/l IAA produced a much faster growing, loosely packed callus which produced roots and shoots within two to three weeks. In a later study, Vasil and Hildebrandt (1967) found that roots and shoots would differentiate with 1.5 mg/l IAA plus 5 mg/l kinetin in White's modified medium. They found negative results with respect to root and shoot differentiation in media with 1 mg/l IAA plus 0.04 mg/l kinetin, 1 mg/l IAA plus 1 mg/l kinetin, and 5 mg/l IAA plus 0.1 mg/l kinetin additives. With no IAA and 5 mg/l kinetin added to the medium, Vasil and Hildebrandt (1967) found profuse shooting and some rooting of the tobacco callus tissue. All of these results were produced with young callus of one or two subcultures. The older calli of several subcultures did not differentiate roots or shoots. Table I summarizes the above information.

Gibberellic acid was found to stimulate growth only in combination with IAA (Vasil and Hildebrandt, 1966). Growth stimulation was much greater with 2, 4-D than kinetin plus IAA but 2, 4-D tended to inhibit chlorophyll production (Venkesteswaran, 1965).

Other factors which influence growth rate are light and the initial size and age of the explant. The best growth of several different plant callus tissues was found in either total darkness or continuous white light of 200 foot candles (Vasil and Hildebrandt, 1966). It appeared that the photoperiod had little to do with root and shoot formation in
TABLE I.---Summary of root and shoot formation using various amounts of kinetin to IAA ratios. In mg/l.

<table>
<thead>
<tr>
<th>Investigators</th>
<th>Kinetin</th>
<th>IAA</th>
<th>Roots</th>
<th>Shoots</th>
</tr>
</thead>
<tbody>
<tr>
<td>Murashige and Skoog (1962)</td>
<td>0.64</td>
<td>2.0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>2.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.04</td>
<td>1.0</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

| Vasil and Hildebrandt (1967) | 5.0     | 1.5 | +     | +      |
|                              | 0.04    | 1.0 | -     | -      |
|                              | 1.0     | 1.0 | -     | -      |
|                              | 0.1     | 5.0 | -     | -      |
|                              | 5.0     | 0   | +     | +      |
tobacco callus. However, Pillai and Hildebrandt (1969) found that the light/dark ratio had a definite effect on differentiation in geranium callus tissue. They pointed out that in earlier work with various differentiating plant calli, tissues were first grown in liquid shake cultures and then transferred to solid media once differentiation had been initiated. Thus, once the differentiation had begun, there was no apparent requirement by the callus for the required light/dark ratio. The liquid shake simulated the appropriate photoperiod in initiating differentiation. Caplin (1961) found the relative increase in size of a callus tissue was inversely related to the initial weight of the explant. The subsequent callus growth decreased as the initial weight varied from 2.3 gm to 29.8 gm. The age of the callus tissue played an important role in the potential differentiation of the callus to roots and shoots (Murashige and Nakamo, 1964). The callus no longer produced roots and shoots after one and one-half to two years. From this work two types of calli were distinguished: slow-growing, compact calli, and more rapidly growing and differentiating calli. As mentioned previously, Vasil and Hildebrandt (1967) found similar results.

Tissue culturing as a tool has been of great use in the field of virology. One of the first problems researchers faced was determining the pathway for virus infection. For a virus to enter a cell, the cell first had to be wounded. Kassanis, et al. (1958) used a pricking method with a small needle to inoculate tobacco callus tissue with tobacco mosaic virus. More recently Murakishi (1966) inoculated four to six week old cultures by placing them in a vortex mixer with TMV for a few seconds. A similar method of inoculation was obtained by placing the tissues in liquid medium with the desired TMV concentration and then on
a tissue shaker (Wu, et al. 1960). The shaking provided enough wounding to permit the entrance of the virus.

Once in the cell, the virus sheds its protective protein coat and begins to duplicate itself. Young cultures of tobacco callus tissues, composed mainly of meristematic cells, were more resistant to TMV infection than five week old cultures which were found to contain the most virus (Wu, et al. 1960). This suggests that the TMV multiplication is competitive with cell division. Hansen and Hildebrandt (1966) concluded that some TMV infected calli remained healthy and that the concentration of the virus in callus cells was much less than that found in cells of systemically infected plants. TMV traveled at a rate of 1 mm/8 days in callus tissue (Kassanis, et al. 1958). This rate of movement is similar to the rate at which TMV travels in the tobacco leaf. The similarity exists even though the perforations in the cell wall for plasmodesmata of tobacco callus tissue are smaller and more indistinct than those of tobacco leaves.

Zaitlin and Boardman (1958) and Wu, et al. (1964) observed that the symptoms of TMV infection were not directly caused by the amount of virus synthesized. They further stated that the young leaves or callus show more symptom development than older leaves or callus that show few or no symptoms although they produce more virus. Wu, et al. (1960) theorized that the yellow-brownish coloration of the infected tissue may be the result of an accumulation of polyphenolic compounds originating from an increase in polyphenoloxidase activity.

No conclusive evidence has been set forth concerning the actual site of TMV multiplication. One school of thought theorizes the first site of TMV action is the chloroplast (Zaitlin and Boardman, 1958).
The nucleus is thought by others to be the first site of action of the virus (Bald and Solberg, 1961; Hirai and Hirai, 1964). Zaitlin and Boardman's (1958) contention was supported by their work with centrifugation of plastids associated with TMV. They found no TMV association with nuclear or mitochondrial fractions. Vesicular bodies containing ribosome-like particles have also been found within chloroplasts and mitochondria in virus infected tissues (Hrsgel and Break, 1964). However, there were no virus rods observed in the chloroplasts and mitochondria. Using immuno-fluorescence to determine synthesis and distribution of TMV antigen in tobacco, Nagaraj (1965) found a ring of antigen forming around the chloroplasts. No antigen was found around the nucleus or within the chloroplasts. Milne (1966) found similar results with Turkish tobacco inoculated with TMV. Electron microscopy revealed no association of the virus particles with the nucleus, but occasionally particles were found in invaginations or vacuoles in chloroplasts. Milne did not imply that the latter was an active association.

Bald and Solberg (1961), with their work on Turkish tobacco leaves infected with TMV U-1 and TMV U-5, using phase microscopy, observed that the nucleus was the first site of action. At the advent of TMV infection the chloroplasts began to adhere to the nucleus. Chloroplasts were seen to send out protrusions toward the nucleus prior to adhesion and away from the nucleus after adhesion. Those protrusions formed after adhesion were soon surrounded by mitochondria and spherosomes. Next, particularly with TMV U-5, bubbling was seen to occur around the nucleus. Soon after, the chloroplasts drifted away and "gray plates" began to be seen moving from the nucleus to the denser, more slowly moving cytoplasm. The nucleolus was noticed to have lost its optical density and appeared
smaller. With TMV U-1 a second site of activity, a vortex of cytoplasmic vesicles, appeared at a distance from the nucleus. This site was formed after the chloroplasts had aggregated around the nucleus. The virus crystals soon formed at this point and migrated toward the nucleus. The authors postulated from these observations that possibly the nucleolus was the source of virus nucleic acid and that the plastids were concerned with the synthesis of viral protein. In 1964, Hirai and Hirai, using a fluorescent antibody specific for TMV protein, obtained results in opposition to the later work of Nagaraj (1965). At six hours after inoculation a conglomeration of cytoplasmic network vesicles containing spherosomes and mitochondria was seen clustering around the nucleus. The fluorescent antibody was mainly confined to the nucleus at this time. As time proceeded, the fluorescence soon spread to the surrounding cytoplasm. No fluorescence was reported around the chloroplasts at the beginning as was later reported by Nagaraj (1965). TMV particles appeared after 30 hours. Later workers (Kolehmainen, et al. 1965) working with a N. tabacum hybrid (Samsun X White Burley) found a close association of TMV rods with the endoplasmic reticulum and ribosomes. From their work they hypothesized that the synthesis of the virus protein and/or the coating of the virus nucleic acid with protein is carried on in these areas.

Singh and Hildebrandt (1966) have described three major types of viral inclusion bodies which exist in most cells infected with TMV. They are the crystalline, characterized by fibrous needles and hexagonal crystals; the paracrystalline, characterized by gray plates; and the spherical bodies, characterized with or without enclosed crystals. In later work with virus infected tobacco callus, Singh and Hildebrandt
(1967) reported the formation of blebs in cytoplasmic strands and a large number of loosely held masses of cytoplasm which showed amoeboid movements. Also present was a development and accumulation of vesicles in the cytoplasm. The cell under the latter condition was usually senescent, but did divide in some cases.

Utilizing the literature as a basis for reference, research was conducted on the tobacco callus H-196. Basically this research has attempted the following:

(a) An analysis of growth differences of tissue grown on varying types of media.
(b) An analysis of growth differences of tissue grown in continuous light and tissue grown in continuous dark.
(c) An application of techniques for observation, initiation and growth of healthy and TMV infected callus tissue.
(d) An analysis of subcellular organelles in healthy callus tissue.
(e) An analysis as to the effects of TMV on subcellular organelles and tissues grown in light and dark conditions.
CHAPTER II. MATERIALS AND METHODS

GROWTH OF THE CALLUS

Five tissues were originally selected for the study. Four of these, H-196, M-222, G-252, and Wis-38 were obtained from the University of Oregon. The H-196 was a hybrid of N. glutinosa and N. tabacum, first isolated in Hildebrandt's laboratory. The M-222 was an isolate from N. tabacum. The G-252 was derived from N. glutinosa, and the Wis-38 is unknown. The fifth, a cultural strain of N. tabacum var. White Burley, designated as WM#1, was obtained by directly inoculating the basic medium with pieces of sterilized stem pith. White's medium, modified by Murashige and Skoog (1962), was elected as the basic medium. The calli were first grown in 125 ml. Ehrlenmyer flasks. Test tubes were later substituted for the Ehrlenmyer flasks to facilitate handling. To inspect callus differentiation, calli were grown on the basic medium with kinetin plus IAA variations. The liquid form of the basic medium was used on both a tissue rotator and a tissue shaker. All calli grown in the light were under continuous light conditions of 250-300 and 450-500 foot candles. The light source was Sylvania Gro-Lux white fluorescent and Sylvania fluorescent lamp banks. The containers for tissues to be grown in total darkness were wrapped in aluminum foil. In all experiments the temperature was maintained at 25 ± 4°C. Callus pieces approximately five millimeters in diameter and weighing about 200 milligrams, fresh weight, were transferred every six weeks. All transfers were made in a chamber which had been washed prior to usage with a 10 percent Amphyl solution. The chamber was also equipped with ultraviolet lamps which were turned on for a period of eight hours prior to transfers.
The tools used were also sterilized in the 10 percent Amphyl solution. Tobacco pith sections were sterilized on all exposed surfaces with 85 percent ethyl alcohol prior to culture. With these precautions, contamination was low, approximately 5 percent.

**INOCULA AND INOCULATIONS**

Tobacco mosaic virus U-1 strain was used as the inoculum for calli in those experiments dealing with viral effects. The source of the virus was *N. glutinosa* which had been infected with TMV U-1. Local lesions were cut from the inoculated plants and macerated with a pH 7.0 phosphate buffer in a Waring blender. After alternating low and high speeds of centrifugation, the viral solution was filtered through a 250 μm porosity Millipore filter to exclude any bacteria present. The filtrate was then tested on *N. glutinosa* to determine the relative amount of virus present. The four younger leaves of eight *N. glutinosa* plants were first sprayed with carborundum dust. The juice was then applied with the finger, stroking each leaf four times. Approximately 0.05 milliliters of juice was stroked on each half leaf. Three days later, the plants were examined for infection. Local lesions were counted per half leaf two days after initial appearance. An average was then obtained per half leaf. The inoculum was then diluted to a standard virus concentration and used to inoculate the calli. Inoculations on the calli were administered seven days after the young calli had been transferred. This seven day delay was to give the calli a chance to recover from any effects of the transferring process. A small glass needle was used as the inoculating tool, each callus being pricked 30 times. The controls were treated similarly, using distilled water as the inoculum.
CYTOLOGICAL METHODS

Those tissues to be permanently mounted were fixed with either Carnoy's or FAA fixing solution. They were then processed through the tertiary butyl alcohol series (Johansen, 1940), embedded in para plast, sectioned, and stained with safranin and fast green. Squash mounts of tissues for immediate observation were selectively stained with phloroglucinol and concentrated hydrochloric acid in order to detect the presence of tracheid formation more easily. Differentiating areas within the callus could be located with this technique. Most of the microscopic observations were made with live squash mounts, however, using a Zeiss phase microscope. Comparisons were made between control tissues and TMV U-1 infected tissues with regard to chloroplast number and development, "cytoplasmic vesicular" formation, cytoplasmic consistency and streaming, nuclear morphology, mitochondrial and spherosomal movement, TMV U-1 inclusion bodies, and tissue differentiation. Some Giemsa staining was attempted with negative results. Photomicrographs were taken to document observations.

BIOASSAY METHODS

To bioassay TMV U-1 in the host cell, the N. glutinosa local lesion half leaf technique was used. The calli were macerated in a tissue grinder using pH 7.0 phosphate buffer. The juice was then applied to previously carborundum-dusted N. glutinosa leaves by stroking with the finger four times. As mentioned before, each half leaf was covered with approximately 0.05 milliliters of inoculum. Three days later, local lesions were counted and the average per half leaf was obtained. Thirty-two half leaves were used in each bioassay of a callus. Bioassays were made
at zero, one, two, three, four, and five weeks after TMV U-1 was
inoculated into the seven day old calli. TMV multiplication was then
calculated by comparing the number of lesions produced by the standard
inoculum with the number of lesions produced by the macerated callus
juice.
CHAPTER III. RESULTS

GROWTH VS. TISSUE TYPE

At the beginning of the study five calli were chosen to work with. These five calli, M-222, G-252, Wis-38, WM#1, and H-196, were grown on White's medium as modified by Murashige and Skoog (1962). The callus designated H-196 grew faster and longer (two to four months) and was therefore selected as the basic callus to be used throughout the study.

TISSUE GROWTH VS. MEDIA

Calli were grown on the basic White's medium modified by Murashige and Skoog (1962). The medium was varied with respect to IAA and kinetin concentration, 2, 4-D additive, and to either solid or liquid consistency. The following three IAA and kinetin concentration combinations were chosen:

1) 0.2 mg/l kinetin, 2.0 mg/l IAA
2) 0.04 mg/l kinetin, 1.0 mg/l IAA
3) 5.0 mg/l kinetin, 25.0 mg/l IAA

Callus appearance

The calli growing on medium #1 were hard and dark green. The calli growing on medium #2 were loose and green, and the calli growing on medium #3 appeared very hard and brownish-green. Table II summarizes the appearances of the calli grown on the different media.

In one case, 2, 4-D was substituted for kinetin. The results of this substitution were similar to those observed by Venketeswaran (1965). The chloroplasts were absent and the callus appeared loosely aggregated and white-yellow.
TABLE II.---Effects of variations of kinetin and IAA content in the basic medium on the appearance of the calli.

<table>
<thead>
<tr>
<th>Treatment Number</th>
<th>Kinetin (mg/l)</th>
<th>IAA (mg/l)</th>
<th>Nature of Callus</th>
<th>Color of Callus</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>0.2</td>
<td>2.0</td>
<td>hard</td>
<td>dark green</td>
</tr>
<tr>
<td>H-196 grown in light (2)</td>
<td>0.04</td>
<td>1.0</td>
<td>loose</td>
<td>green</td>
</tr>
<tr>
<td>(3)</td>
<td>5.0</td>
<td>25.0</td>
<td>very hard</td>
<td>brownish green</td>
</tr>
</tbody>
</table>
Fresh Weight of Callus

The results of callus growth on the three media with respect to fresh weight are illustrated by Figure 1. All three tissues were comparable in fresh weight for the first three weeks. From the third week until the end of the sixth week, the callus growing on medium #1 increased in fresh weight at least 100 milligrams more than those calli growing on the other two media. Accordingly, medium #1 was chosen as the basic medium for the remainder of the study.

Tissue Differentiation

Table III summarizes the effects of different IAA and kinetin contents on the growth of roots and shoots. There was no formation of roots or shoots with any of the three media. The highest level of differentiation was the formation of vascular tracheal elements.

Tissue Growth vs. Method of Culture

Once the type of callus and the basic medium had been chosen, four methods of culture were tested: the tissue shaker and the tissue rotator with liquid media cultures; 125 ml. flasks and 15 ml. test tubes with solid media cultures. Following are the results obtained with each of these.

The tissue shaker with liquid media cultures failed to produce any positive results with respect to growth of calli. Pectinase was used to break down intercellular connections but produced negative results, thus the shake method was abandoned.

The rotator was used to obtain tissue growth of single cells and small clones in liquid culture. On rare occasions growth was obtained, but when the tissues were transferred to nutrient agar medium they
Figure 1.—Fresh weight of H-196 grown on White's modified medium with three concentration combinations of kinetin and IAA.

- ○ - 0.2 mg/l kinetin, 2.0 mg/l IAA
- △ - 0.04 mg/l kinetin, 1.0 mg/l IAA
- □ - 5.0 mg/l kinetin, 25.0 mg/l IAA
Fresh weight of callus in milligrams

Age of callus in weeks
TABLE III—Effects of variations of kinetin and IAA content in the basic medium on differentiation.

<table>
<thead>
<tr>
<th>Treatment Number</th>
<th>Kinetin mg/l</th>
<th>IAA mg/l</th>
<th>Tracheal Elements</th>
<th>Roots</th>
<th>Shoots</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>0.2</td>
<td>2.0</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H-196 grown in light</td>
<td>0.04</td>
<td>1.0</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(3)</td>
<td>5.0</td>
<td>25.0</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
soon died. Since so few positive results were obtained, it was decided
to use nutrient agar medium instead of the nutrient liquid medium.

One hundred and twenty-five ml. Ehrlenmyer flasks containing solid
media were next employed. This method produced much callus growth and
was therefore used almost exclusively throughout the first stages of
the research. Fifteen ml. test tubes were substituted later to facili­
tate handling.

**TISSUE GROWTH VS. PHOTOPERIOD**

The basic callus, H-196, was grown on White's modified medium
(Murashige and Skoog, 1962) with 0.2 mg/l kinetin and 2.0 mg/l IAA, in
either continuous dark or continuous light. Figure 2 illustrates the
results obtained. Those tissues grown in complete darkness showed a
much greater growth rate in terms of fresh weight than did those grown
in light. After growing in continuous light for six or seven weeks,
some of the tissues began to die. These tissues, if placed in total
darkness, were soon rejuvenated and began to grow rapidly once again.

In neither the light-grown nor the dark-grown tissues were shoots
and roots produced. Again the highest level of tissue differentiation
was the formation of vascular elements.

Cultures grown in the dark were white in color and quite loose in
construction as compared with the hard, dark-green callus grown in the
light.
Figure 2.---Fresh weights of H-196 grown in continuous dark and continuous light. Kinetin-IAA concentration was 0.2 mg/1, 2.0 mg/1 respectively.

▲ - continuous dark

○ - continuous light
CYTOLOGICAL INVESTIGATION OF HEALTHY TISSUES

COMPARED WITH TMV INFECTED TISSUES

Chloroplast number and development

The chloroplasts were found scattered throughout the cytoplasm, moving with the cytoplasmic flow. There were an average of 20 chloroplasts per cell in the light-grown calli. Those tissues grown in the dark possessed no distinguishable chloroplasts. However, when the dark-grown tissues were placed in the light, chloroplasts soon developed. In all tissues observed no chloroplast protuberances were ascertained as described by Solberg and Bald (1962). The resolution of the phase microscope at the higher magnifications made such observation difficult in that the average size of the chloroplasts was 4 μ. There were no distinguishable differences between the chloroplasts of the healthy and the TMV infected tissues.

Nuclear Morphology

The nuclei were usually found to be near or against the cell wall and were the center of cytoplasmic activity. They appeared in a constant state of activity, possessing invaginations and furrows with which cytoplasmic strands were associated. Their appearance was ovate, continually changing in shape, but never becoming entirely spherical. The nucleus became spherical only when the cell ceased living and cyclosis stopped. These observations correspond closely with those of Solberg and Bald (1962). As with the chloroplasts, there appeared no apparent differences between nuclei of infected and healthy tissues. Figure 3 illustrates a nucleus in a living cell.

Cytoplasmic Streaming, Consistency, and Vesicular Formation

In healthy tissues there could be seen much cytoplasmic activity.
Figure 3.---The living cell of a callus showing the nucleus and adjacent cytoplasmic strands. 600X
Cytoplasmic streaming was found both around the periphery of the cell and through the central vacuole. The cytoplasm was observed to have protuberances extending through the central vacuole linking and producing new cytoplasmic strands at will. Vesicles, as described by Solberg and Bald (1962), could be seen in the background parietal cytoplasm. They appeared mobile and constantly changed shape due to cyclotic forces. When the cell died, the cyclotic forces ceased, and consequently the vesicles soon became spherical and discontinued movement. At this time they grouped together forming spherical patterns of various sizes. Figure 4 illustrates the network after death of the cell. Figure 5 shows their active state in the living cell. When the cell began to cease activity as the result of injury, caused either by tapping the cover slip or saturating the cell with a solution of a pH significantly different from that of the cell, small spheres of cytoplasm formed as blebs from the cytoplasmic strands. These grew in size to form large spherical networks, similar to the dead cytoplasmic vesicle network. At the same time, the cell contents congregated on this cytoplasmic network. Closer observation of this phenomenon revealed a smaller network of irregularly shaped vesicles similar to that of the living cytoplasmic vesicles of Solberg and Bald (1962). Photomicrographs of these were attempted, but due to poor resolution at 2000X, the photomicrographs showed nothing discernible. In all these purposefully injured cells it was difficult to distinguish between the cytoplasmic vesicles of Solberg and Bald (1962) and the cytoplasmic network formed by blebs.

In contrast, the TMV infected tissue showed some striking differences with respect to cytoplasmic streaming, consistency, and
Figure 4.---A dead cell from a callus showing the "cytoplasmic vesicle" network. 400X
Figure 5.---A living cell from a callus showing the active "cytoplasmic vesicle" network. The cell wall is in the upper right hand corner. 1600X
vesicular formation. Those results obtained by purposely injuring a cell closely resembled those of the TMV infected cells. Small spheres of cytoplasm were seen to form blebs from the cytoplasmic strands. These blebs of cytoplasm appeared denser than the background cytoplasm or the cytoplasm of the strands. The blebs either enlarged, forming large spherical networks, or returned to the cytoplasmic strand. It also appeared that this denser cytoplasm would form ridges within the cell. Again, these ridges appeared and disappeared as the cyclotic forces of the cytoplasm moved them from one place to another. The spherical networks formed by the blebs were very similar to those cytoplasmic vesicles of dead cells as described by Solberg and Bald (1962). The only difference was that this network was in the TMV infected living cell. Portions of cytoplasm were occasionally observed floating freely within the spherical networks. Closer observation of these free floating cytoplasmic masses indicated cytoplasmic vesicle networks similar to the vesicles of Solberg and Bald (1962). At no time were viral inclusion bodies, such as crystals or gray plates, observed in the cytoplasm. The only evidence of any infection were the aforementioned results. These results correspond very closely with those of Singh and Hildebrandt (1967).

Mitochondrial and Spherosomal Movement and Observations

In both the TMV infected tissues and healthy tissues mitochondria and spherosomes were observed with great difficulty due to the similarities between their density and that of the cytoplasm. Consequently, it was difficult to observe their movements and associations of mitochondria and spherosomes with one another and other cellular bodies. Therefore, little will be said of them other than that they were present in both
the infected and healthy tissues.

Tissue Differentiation

Although the calli did not develop roots and shoots, a simple vascular system was observed in both the healthy and TMV infected tissues. These areas were located throughout the calli, at the center and on the periphery. None of these areas was seen to be connected to adjacent vascular areas. Several types of tracheids were observed, the most common being the short reticulate-scalariform tracheal elements. Helical and pitted tracheal elements were also observed, but less frequently. Figures 6, 7, and 8 illustrate the different types of tracheal elements observed. The longer tracheal elements could be construed to be vessel members except no perforation plates were observed.

Phloem in these vascular areas was not definitely ascertainable. Figures 9 and 10 could show phloem elements or immature tracheal elements. Several cells of this type were observed, and in a few instances, deteriorating cytoplasm with a nucleus was found. In Figure 9 an element can be seen on the periphery of the callus with no tracheal or phloem elements adjacent to it. In Figure 10 an immature vascular element is adjacent to or connected with a tracheal element. In both instances the tissue was stained with phloroglucinol and concentrated HCl. The element in question took up very little of the stain which is evident by the very light red color. This stain is specific for lignin which comprises the secondary walls of tracheal elements. In some instances the element in question took up none of the stain. Other than these unusually structured cells, no evidence of phloem elements was found. This does not mean they were not present, but they were not
Figure 6.—Reticulate-scalariform tracheal element in a callus cell stained with phloroglucinol and concentrated HCl. 400X
Figure 7.—Border pitted tracheal element with adjacent reticulate-scalariform tracheal element (arrow) in callus cell stained with phloroglucinol and concentrated HCl. 400X
Figure 8.---Helical tracheal element in callus cell stained with safranin and fast green. 400X
Figure 9.---Immature tracheal element (?) in callus tissue stained with phloroglucinol and concentrated HCl.  400X
Figure 10.—Immature tracheal element (?) with adjacent reticulate-scalariform tracheal element (arrow) in callus cell stained with phloroglucinol and concentrated HCl. 400X
easily observed, which coincides with the results of other workers (Karsten, 1965). Phloem cells possess very little lignin in their walls, making them difficult to observe using the above stain. Again, these results were observed in both healthy and infected tissues.

**TMV Multiplication in the Callus**

To measure the rate of multiplication of TMV in the callus, a standardized reference inoculum was used. This purified inoculum was found to produce an average of 20 lesions per half leaf. When the calli were inoculated with the known viral concentration, rate and amount of TMV multiplication was found to be proportional to the size and the age of the callus. As the callus increased in fresh weight, so did the number of viral particles up to a limit which, in this case, was dependent upon the age of the calli. Figure 11 illustrates the fact that growth of the callus was somewhat deterred due to TMV infection. Comparing the responses of light-grown and dark-grown tissues to virus infection and multiplication, those tissues which were grown in the dark had greater virus multiplication than those tissues grown in the light. Figure 12 illustrates this observation. The dark-grown tissue's response to TMV is similar to whole plants which, if placed in the dark for a period prior to inoculation, become more susceptible to TMV infection. But in the whole plants, after the initial infection, plants grown under continuous light conditions produce more TMV than plants grown under dark conditions. The latter did not seem the case with the calli as Figure 11 clearly points out.

A note of clarification should be made here concerning the results depicted in Figure 11. The one week old callus showed no TMV
Figure 11.---Fresh weights of TMV-infected calli and healthy calli grown in dark and light conditions.

▲ - healthy uninoculated callus grown in dark
■ - TMV inoculated callus grown in dark
○ - healthy uninoculated callus grown in light
□ - TMV inoculated callus grown in light
Figure 12.---TMV titer in light-grown calli vs. dark-grown calli.

▲ - dark-grown calli
○ - light-grown calli
lesions when bioassayed due to the small amount (0.005 ml. ± 0.002 ml.) of inoculum used. This callus was inoculated and then bioassayed immediately in order to check for "background" TMV. There was none.
CHAPTER IV. DISCUSSION

ANALYSIS OF GROWTH OF TISSUES

Of the three media selected at the beginning of the research, the one which fulfilled the greatest number of preconditions and was subsequently chosen was White's medium as modified by Murashige and Skoog (1962), with 0.04 mg/l kinetin and 1.0 mg/l IAA. Calli grown on this medium appeared green in color and had a loose texture. The medium produced a very fast rate of growth although no root or shoot formation occurred. This may not have been attributable to the medium, but perhaps to the age of the callus. It had been continually subcultured since it was received from Hildebrandt's laboratory in 1958. Workers (Murashige and Nakamo, 1964; Hildebrandt, 1969) have suggested that after several subcultures, a callus tends to lose its ability to differentiate roots and shoots. It is recommended that more varied media and also younger callus tissue be used in further work.

The greatest degree of differentiation observed was the formation of vascular tissue, specifically, tracheal elements. The tracheal elements were grouped and scattered throughout most of the callus tissue. There were no phloem elements observed, but according to Karsten (1965), they are not easily detected.

Minimal success using the tissue rotator and the tissue shaker can perhaps be attributed, again, to the age of the callus. There is a definite need for utilizing such a mechanism since it can provide the researcher with single cell clones, a simple method of TMV inoculation, and direct observation of single cell differentiation. Pillai and Hildebrandt (1969) inferred that the tissue shaker may be directly con-
nected with callus differentiation of roots and shoots; the shake method may be a differentiation initiator as is photoperiod.

The best explant size was found to be 200 mg. A slow growing callus resulted if an explant larger than 200 mg. was used. These results coincide with those of Caplin (1961) who found that the relative increase in size of a callus was inversely related to the initial weight of the explant.

Light intensity was another factor in influencing the growth of the calli. The greatest rate of callus growth was obtained in total darkness. Continuous light seemed to inhibit callus growth somewhat. Vasil and Hildebrandt (1966) found that the best growth rates were obtained in either total darkness or continuous light of 200 footcandles. In further studies it is suggested that varying photoperiods be experimented with in order to find optimal growing conditions. The photoperiod in this research was found to have little to do with differentiation. Tissues grown both in continuous light and total darkness differentiated vascular tissue at the same rates. However, Pillai and Hildebrandt (1969) state that there is a direct connection between photoperiod and tissue differentiation.

ANALYSIS OF CELLULAR ORGANELLES OF
HEALTHY AND TMV INFECTED CALLUS TISSUES

There appeared to be no differences between the cellular organelles of healthy and infected tissues. Nuclei possessed invaginations and furrows as Solberg and Bald (1962) had stated. Cells grown in the light had an average of 20 chloroplasts. Mitochondria and spherosomes were equally hard to distinguish in both the infected and healthy tissues. However,
there were some marked differences in the cytoplasm of the two tissue types. In the infected cells, the cytoplasmic strands were observed to produce blebs of cytoplasm. These blebs formed cytoplasmic spherical networks or disappeared into the cytoplasmic strands. The cytoplasmic spherical networks were similar to the cytoplasmic vesicles of inactive healthy tobacco hair cells of Solberg and Bald (1962). Singh and Hildebrandt (1967) state that these networks and blebs are the result of high concentrations of TMV, even though no viral inclusion bodies are present. The results in this research also show no viral inclusion bodies, but the fact that virus was present in the tissue was confirmed by positive bioassays. Also present in the TMV infected cells were mobile ridges of cytoplasm caused by slower moving, more dense cytoplasm. These results are again similar to those of Singh and Hildebrandt (1967). In summary, the most striking difference between the infected and the healthy cells appeared in the cytoplasm.

**ANALYSIS OF THE EFFECTS OF TMV ON THE CALLUS TISSUES**

The average fresh weight of the calli with TMV was 150 mg. less than the fresh weight of the healthy calli after four weeks of growth. Until that time there was from 20 to 50 mg. difference between the two. It could be postulated that TMV multiplication was competing with cell division which produced fewer cells and, consequently, less fresh weight. Wu, et al. (1960) stated that the multiplication of TMV may compete with cell division and is greatest in five week old cultures.

These conclusions pertain to tissues grown in the light. In contrast, infected tissues grown in the dark were noticeably different. The greatest TMV concentration occurred in two week old tissues and then
steadily declined. Even considering the differences in growth between healthy light-grown and healthy dark-grown tissues, TMV multiplication as measured by bioassays was not proportional. No explanation for this phenomenon could be found in the literature, except that when whole tobacco plants are placed in the dark for a period of time prior to TMV infection, the plants are more susceptible to infection. This problem remains to be adequately resolved.
CHAPTER V. CONCLUSIONS

The following are some concluding remarks concerning the major observations obtained in this research along with some suggestions for further work.

1. The callus used, H-196, produced no differentiation beyond vascular tissue. In future studies, several things should be checked in conjunction with this lack of differentiation:
   a) A younger tissue should be used.
   b) A greater range of media variations should be tested.
   c) A variety of photoperiods should be tested.
   d) The tissue shake method should be refined and employed.

By using these modifications, perhaps tissue differentiation would proceed beyond vascular tissue formation, possibly producing whole tobacco plants.

2. The calli grown in the dark grew faster than those grown in the light. Again, in future investigations, a series of different light regimes should be used to better understand such results.

3. The infected tissue showed no viral inclusion bodies typical of TMV infection; however, three cytoplasmic alterations were observed: a) mobile cytoplasmic ridges; b) cytoplasmic blebs; c) spherical cytoplasmic networks. These were presumably caused by widely different densities of the cytoplasm which are uncommon in healthy tissues. In future work it would be interesting to investigate why there were only cytoplasmic alterations present, to the exclusion of typical viral inclusion bodies.

4. TMV infected tissues growing in the dark produced proportionate-
ly more TMV than those growing in the light.
BIBLIOGRAPHY


