THE FORMATION OF NICOTINE AND A STUDY OF THE MORPHOLOGY IN CALLUS DERIVED FROM MARYLAND-872 TOBACCO

By

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THE FORMATION OF NICOTINE AND A STUDY OF THE MORPHOLOGY IN CALLUS DERIVED FROM MARYLAND-872 TOBACCO

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PREFACE

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CHAPTER I

INTRODUCTION

Nicotine is the major secondary metabolite of various Nicotiana species. In the intact plant, nicotine is produced mainly in the root and is subsequently transported to the shoot (1). Nicotine has also been found in tobacco root organ cultures (2).

Several authors (3-9) have investigated nicotine production in tobacco callus tissue cultures in vitro. Shiio and Ohta (9) determined optimal levels of various regulators for nicotine production in tobacco callus. As levels of growth regulators were raised above the low optimal concentrations, nicotine production was inhibited. Therefore, low concentration of growth regulators are required for nicotine production.

Low concentrations of growth regulators in the medium could result in formation of a partially differentiated callus culture (10). Lignification, vascularization, and organogenesis may occur. A correlation between nicotine production and differentiation in tobacco callus tissues was suggested by Tabata et al. (7) in 1971 since callus with vegetative buds had about twice the nicotine content as callus without buds. Thus, differentiation resulting from low concentrations of growth regulators may be precursory to nicotine production.

The following experiments were designed to help clarify the relationship between differentiation and nicotine production in tobacco callus tissue. The objective of this research was to establish a callus

tissue culture of Nicotiana tabacum variety Maryland-872 which would produce nicotine and to study its morphology.

CHAPTER II

LITERATURE REVIEW

A. Nicotine Production in Tobacco Tissue Culture

It is well-known that nicotine is synthesized mainly in the roots of Nicotiana species (1). Several studies on the effect of 2,4-dichloropheoxyacetic acid (2,4-D) on nicotine production in the intact plant have been performed by Yasumatsu and co-workers (11-13). Experiments on ^{14}C-2,4-D translocation in hydroponically grown plants showed that less than two percent of the radioactivity was transported from the leaf to the root while 79 percent of the ^{14}C-2,4-D was translocated from the root to the leaf after five days (11). Foliar application of 2,4-D to hydroponically grown N. tabacum reduced incorporation of $^{14}CO_2$ into nicotine by 86 percent. This would indicate that 2,4-D reduced the nicotine content of the tobacco plant by decreasing nicotine biosynthesis (12). Simultaneous foliar application of 2,4-D and gibberellic acid (GA) appeared to reduce nicotine content additively (13).

Several authors have studied nicotine production in tobacco callus tissue culture:

1964	Speake et al. (3)	N. tabacum var. "Virginia"
1966	Benveniste et al. (4)	N. tabacum var. "P-19" habituated
1967	Furuya et al. (5)	N. tabacum var. "Bright Yellow"
1968	Tabata et al. (6)	var. "Wisconsin-38"

1971	Tabata et al. (7)	N. tabacum var. "Bright Yellow-12"
1971	Furuya et al. (8)	N. tabacum var. "Bright Yellow"
1973	Shiio and Ohta (9)	N. tabacum var. "Bright Yellow"

In these studies several conclusions, which are discussed below, were reached concerning tissue origin, the state of callus organization, and the effect of growth regulators on nicotine production.

Speake et al. (3) isolated and identified nicotine from several lines of N. tabacum tissue in culture. Nicotine production was observed through twenty-three transfers of cell cultures derived from root, stem, and leaf. All possessed the ability to produce nicotine. This suggested that all cells of the plant have similar genetic potential and that the predominance of the root as the site of nicotine biosynthesis is due mainly to organization which is partially retained in root organ cultures (1, 2). This may account for the high nicotine content in root organ cultures (29 µg/mg dry weight) when compared with cell cultures (7 µg/mg dry weight). Shiio and Ohta (9) had similar results with tobacco seedlings. Callus derived from seedling root showed higher nicotine content than callus derived from the hypocotyl. This was considered to result from the initially induced callus retaining some of its differentiated character. The differences in nicotine content observed in callus from explants of the hypocotyl and root slowly disappeared during successive transfers. The level of nicotine was greatly dependent upon the types and concentrations of regulators present in the medium. Nicotine production was inhibited as the concentration of regulators was increased over optimal levels.

Furuya et al. (5) reported the presence of nicotine and anatabine

in callus grown on medium containing 1.0 ppm indole-3 acetic acid (IAA). Later, Furuya et al. (8) studied the differences in composition of callus maintained on 1.0 ppm IAA-medium and 1.0 ppm 2,4-D medium for five years. The IAA callus contained nicotine, scopoletin, scopolin, and small amounts of anatabine and anabasine. The 2,4-D callus had no tobacco alkaloids present and the scopoletin and scopolin occurred in smaller amounts than in IAA callus. Callus transferred from 2,4-D medium to IAA-medium recovered the ability to produce nicotine. When callus was transferred from IAA-medium to 2,4-D medium, the nicotine in callus maintained on 2,4-D medium might be attributed to an inhibitory effect of 2,4-D on nicotine biosynthesis. This is similar to the results of Yasumatsu et al. (12) observed in the intact plant. Tabata et al. (7) reported that 2,4-D inhibited bud and nicotine formation while in the absence of 2,4-D many buds and much nicotine were produced. The pool sizes of both glutamic and aspartic acids, probably the initial intermediates leading to the formation to the pyrrolidine and pyridine rings of nicotine, respectively, were reduced in callus maintained on 2,4-D medium. Shiio and Ohta (9) observed that 2,4-D was inhibitory to nicotine synthesis at all concentrations and that 1.0 ppm 2,4-D almost completely inhibited nicotine production.

Shiio and Ohta (9) found that kinetin in the presence of 0.1 ppm α-naphthaleneacetic acid (α-NAA) is inhibitory to growth and nicotine production in callus. They observed no organogenesis. However, Tabata et al. (7) had reported that kinetin promoted both the growth and nicotine production in callus derived from "Bright Yellow" pith. As kinetin concentration was increased from 0 to 2.0 ppm, the fresh weight, nicotine content, and number of buds per culture increased. Not all the

culture developed vegetative buds. It was found that callus with buds had about twice the nicotine content as callus without buds. No roots were formed, but the callus was not checked microscopically for root primordia. Tabata suggested that a relationship may exist between nicotine synthesis and differentiation in the cultures.

The inhibitory effects of α-NAA and IAA on nicotine production were weaker than that of 2,4-D (9). One-hundred ppm IAA inhibited nicotine synthesis while ten ppm was optimal for nicotine synthesis. The optimal concentration of α-NAA was 0.1 ppm and 10.0 ppm was required to completely inhibit nicotine production. Tissue grown without the addition of α-NAA was observed to have higher dicarboxylic amino acid content than callus grown on medium with α-NAA present (14, 15). Changes in activities of nitrate reductase, malate dehydrogenase, glutamate dehydrogenase, and aspartate aminotransferase were found to affect the amino acid levels (15). However, the amino acid levels at 0.1 ppm α-NAA may not have been investigated and the reduced aspartic and glutamic acid levels at higher concentration may correspond to the inhibition of nicotine production. This may still be consistent with the effect of 2,4-D on levels of these amino acids and nicotine production observed by Tabata et al. (7).

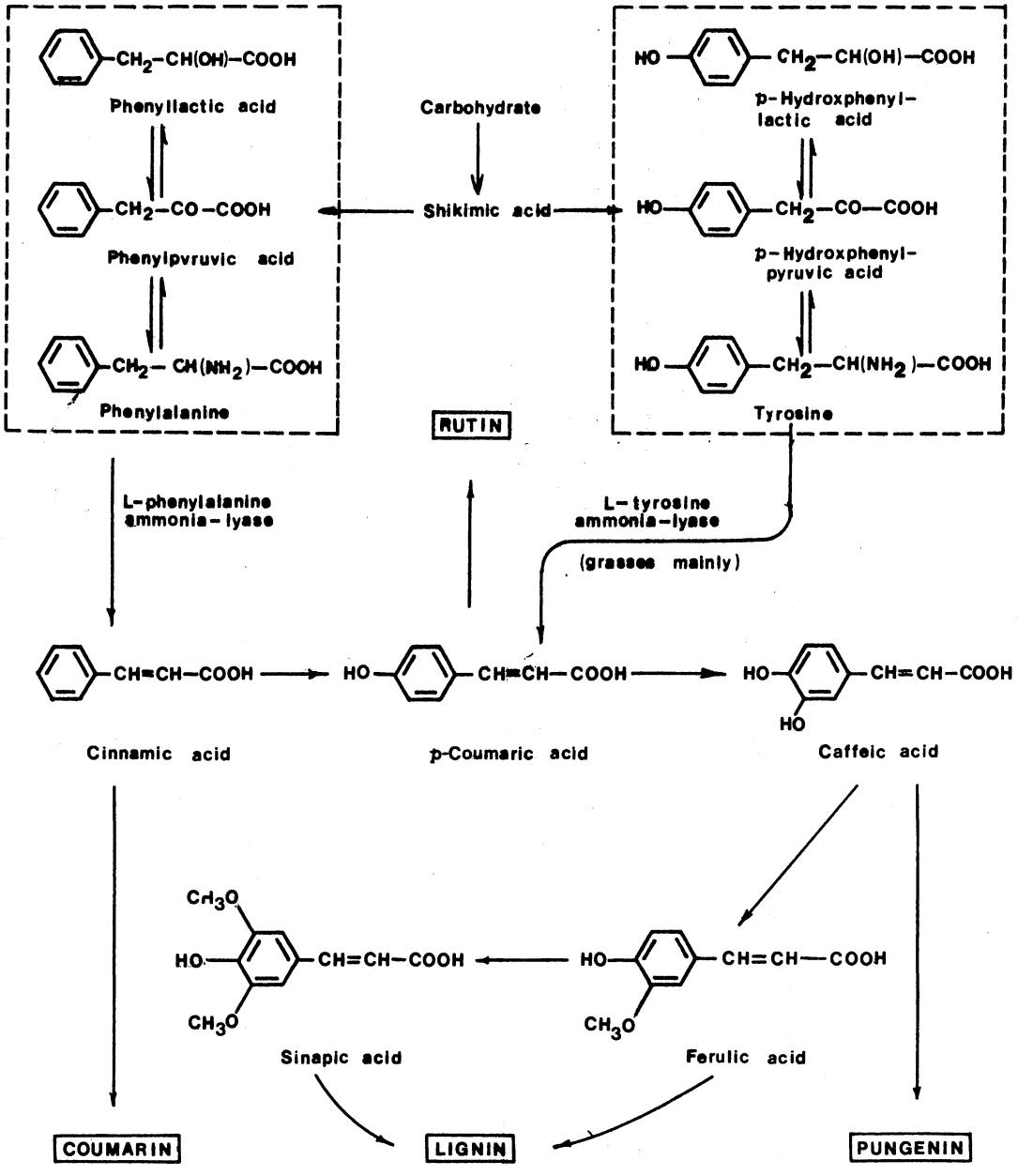
B. Histogenesis in Callus Cultures

Since low concentrations of growth regulators allowed nicotine production and high concentrations inhibited it, the callus may have been in some partially differentiated state. When cultures are actively growing, the callus usually contains mostly parenchymous cells with a few localized groups of meristematic cells. Very few, if any, vascular

tissues are found. Vascular tissues, varying from tracheid-like cells to organized vascular bundles or nodules containing xylem and phloem, may be found in slow-growing cultures. The slow growth may be a result of growth inhibition by auxins or result from a long period without subculture. These observations were made in a series of microscopic studies (10).

Much basic knowledge of the lignification process has been elucidated (16). By studying different sections of bamboo plants, they could study metabolic changes which occur in the plant during various stages of lignification. The apex showed little lignification while lower sections had lignified vessels and sieve tubes. The activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were found to increase just below the apex and then remain constant in older tissues. Oxygen uptake and the C_6/C_1-ratio decreased towards the base of the plant; this paralleled an increase in the rate of lignification. These results indicate that the pentose phosphate pathway may predominate in the respiratory processes of lignifying tissues. Maximum activities of 5-dehydroquinate hydrolase and 5-dehydroshikimic acid reductase was found immediately below the apex. These high activities result in the high shikimic acid content of tissue below the apex. Young tissues probably synthesize large amounts of shikimic acid for conversion to phenylalanine and tyrosine which are required for protein synthesis. In older tissues, the emphasis is shifted to lignification for which a smaller shikimic acid pool is adequate. Phenylalanine deaminase and tyrase activities are at a maximum just below the apex, corresponding to the increase in concentrations of trans-cinnamic and trans-p-coumaric acids (Figure 1). Siegel (35) has demonstrated

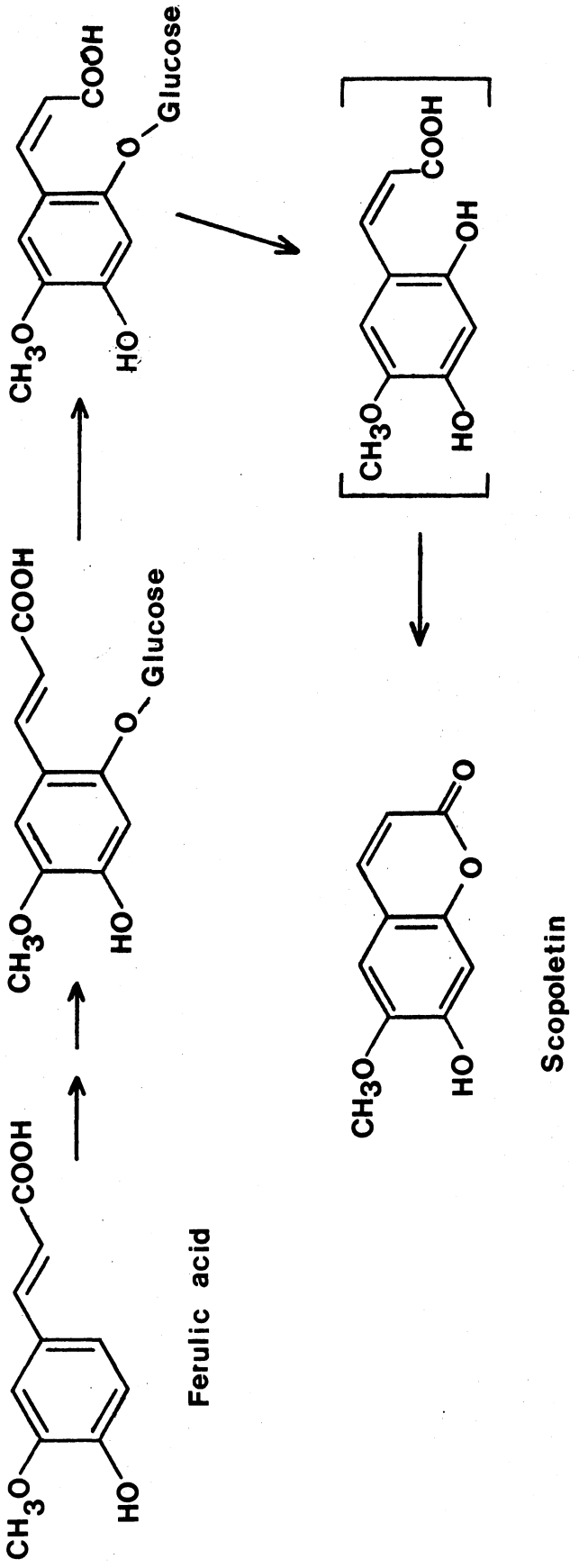
Figure 1. Biosynthesis of Cinnamic Acid Derivatives in Vascular Plants (17)


Phenyllactic acid
Phenylpyruvic acid
Phenylalanine
Carbohydrate
Shikimic acid
p-Hydroxphenyl-lactic acid
p-Hydroxphenyl-pyruvic acid
Tyrosine
RUTIN
L-phenylalanine ammonia-lyase
L-tyrosine ammonia-lyase
(grasses mainly)
Cinnamic acid
p-Coumaric acid
Caffeic acid
Sinapic acid
Ferulic acid
COUMARIN
LIGNIN
PUNGENIN

correlations between peroxidase activity and lignification in a series of studies. Thus, lignin is definitely a product of phenylpropane metabolism.

The levels of scopoletin and scopolin in tobacco tissue cultures are greatly affected by kinetin. Skoog and co-workers (19, 20) found that formation of both scopoletin and scopolin is a function of kinetin added to the medium. Bergmann (21) found a similar correlation between the amount of kinetin administered and the lignin content of N. tabacum variety Samsun tissues, determined as Klason lignin. The lignin produced was mainly deposited in the cell walls of tracheids but was also found in the middle lamella and on the outside of the walls. The tracheids stained with phloroglucinol-HCl and gave a weak Maule reaction, indicating mainly coniferaldehyde groups with a few syringylpropane sidechains. Lignin on cell walls gave only a positive phloroglucinol reaction indicating only coniferaldehyde groups. Bergmann (21) found that increasing the kinetin concentration in cell suspensions of N. tabacum inhibited uptake of oxygen. This effect could be reversed by addition of pyruvate, succinate, or α-ketoglutarate. Experiments with malonate and arsenite, specific inhibitors of the citric acid cycle, indicated that in cells with kinetin in the medium a larger amount of glucose is oxidized via the pentose phosphate pathway than in normal cells in which the Embden-Meyerhof pathway is the major route of glucose oxidation. Bergmann suggested that both lignin and scopoletin (Figure 2) are products of phenylpropane metabolism. Kinetin could activate the phenylpropane metabolism by inhibiting the Embden-Meyerhof pathway and thus shunting glucose-6-phosphate into the pentose phosphate pathway. This would raise the levels of intermediates necessary for shikimic acid synthesis

Figure 2. Biosynthesis of Scopoletin in Higher Plants (17)



Ferulic acid

Scopoletin

and phenylpropane metabolism (21). Thus, there is some correlation between Bergmann's work on lignification in tobacco callus tissue and Hamaguchi's studies on bamboo (i.e., lignin is a product of phenylpropane metabolism in tissue in which glucose is mainly metabolized via the pentose phosphate shunt).

Studies by Schafer and Wender (22) indicate that the scopolin/scopoletin ratio may be of more importance than either alone in regard to lignification. Calcium ion concentration in the medium was found to affect the degree of lignification in Wisconsin-38 callus. This effect of calcium was first observed in sunflower crown gall tissue (23). Lipetz (18) later observed that as the calcium ion concentration was increased more peroxidase leaked into the medium and less lignification occurred. He proposed that calcium ion concentration may control the concentration of peroxidase on the cell wall.

The ability of auxin and kinetin to induce development of meristems implicates these substances in organogenesis. Skoog and Miller (24) showed that Wisconsin-38 callus tissue grows actively and remains parenchymous in medium containing an appropriate IAA to kinetin ratio (e.g., 2.0 ppm IAA:0.2 ppm kinetin). If the auxin to kinetin ratio is decreased, leafy shoots are initiated. With a medium promoting bud initiation, addition of tyrosine (210 ppm) enhances the process. Bud formation is suppressed and roots are initiated if the auxin to kinetin ratio is increased. Gibberellin prevents formation of shoots and roots (25). Although gibberellin inhibited neither vascularization nor cell division, it did inhibit differentiation of nodular structures with intense meristematic activity which are apparently forerunners of shoot primordia. Cambium-like activity continued in regions scattered

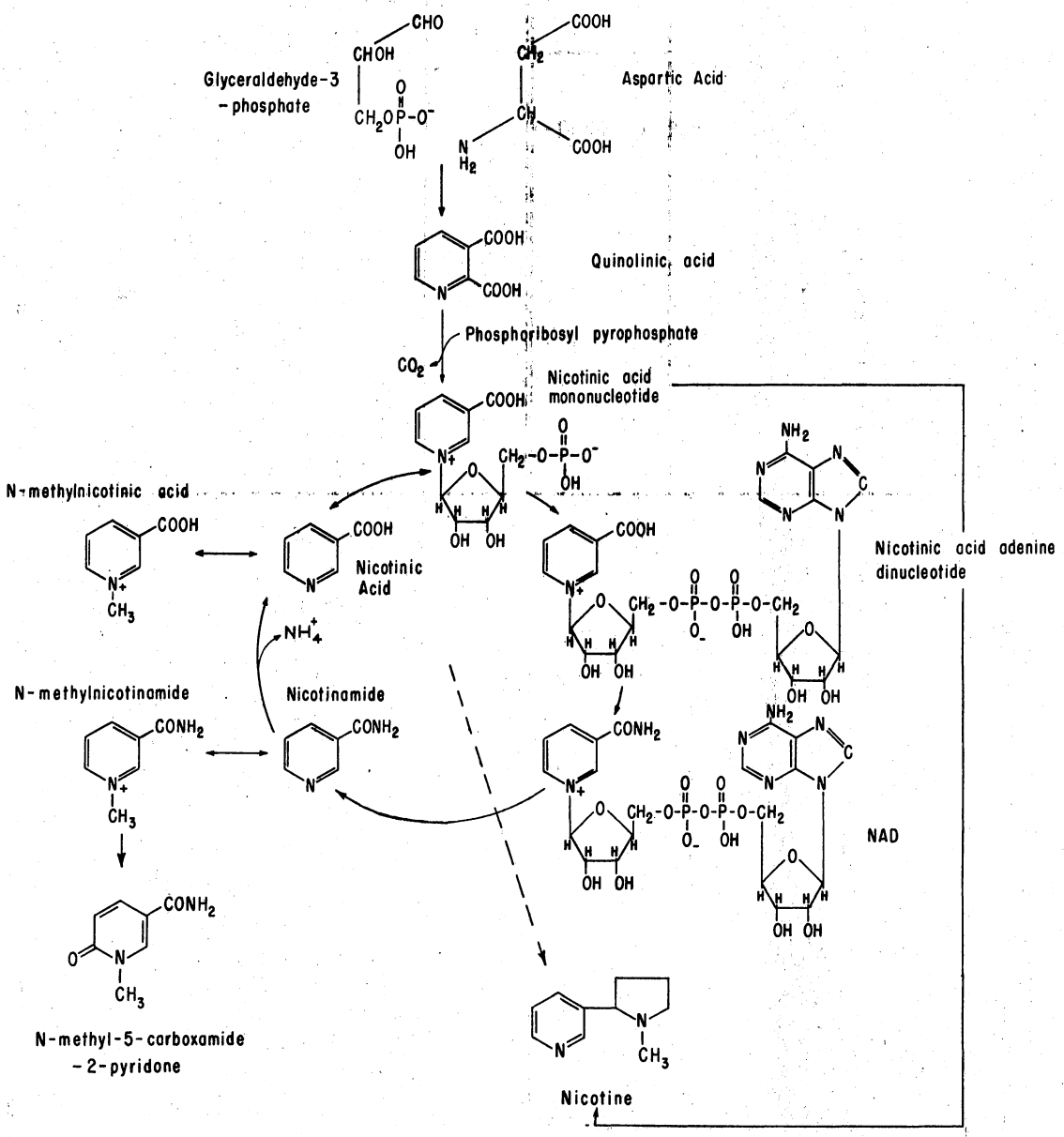
throughout the tissues with and without gibberellin. Therefore, vascularization and organogenesis may be two distinct processes.

Many experiments have involved explant material in which callus is still attached to the organ fragment from which it was derived (10). Often callus tissues when continually subcultured lose their ability to initiate organs. Old established cultures which retain this ability are the exceptions.

C. Relationship of the Pyridine Nucleotide Cycle to Nicotine Biosynthesis

Labeling patterns have been used to investigate the biosynthesis of pyridine alkaloids in higher plants. Studies indicate that the pyridine moieties of nicotinic acid mononucleotide, nicotinic acid adenine dinucleotide, and nicotinamide adenine dinucleotide--as well as quinolinic acid, nicotinic acid, and nicotinamide—are incorporated into ricinine in Ricinus communis L. (26, 27). Thus, quinolinic acid and intermediates in the pyridine nucleotide cycle (Figure 3), which was postulated by Waller et al. (26), serve as precursors for ricinine. Other studies showed that the pyridine nucleotide cycle is operational in tobacco plant and that it can act as a source of intermediates in nicotine biosynthesis (28).

Figure 3. The Pyridine Nucleotide Cycle (26)


Glyceraldehyde-3 - phosphate
Aspartic Acid
Quinolinic acid
Phosphoribosyl pyrophosphate
CO_2
Nicotinic acid mononucleotide
N-methylnicotinic acid
Nicotinic Acid
Nicotinic acid adenine dinucleotide
NH_4^+
N-methylnicotinamide
Nicotinamide
NAD
N-methyl-5-carboxamide - 2-pyridone
Nicotine

CHAPTER III

MATERIALS AND METHODS

A. Materials and Chemicals

1. Plants

Small tobacco plants, Nicotiana tabacum variety Maryland-872, were obtained and transplanted into Bethany silt loam soil at the agronomy farm of Oklahoma State University in Stillwater on May 27, 1974. Two non-flowering plants approximately one-and-one-half meters tall were taken for tissue culture work on October 25, 1974. (See Figure 4.)

2. Reagents

Media. L-Glutamine, L-asparagine, L-aspartic acid, 2,4-D, kinetin as the potassium salt, and agar (Type IV) were purchased from Sigma Chemical Company, St. Louis, Missouri. Pyridoxine-hydrochloride, thiamin-hydrochloride, nicotinic acid, IAA, and GA were purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio. Inositol was purchased from Fisher Scientific Company, Fair Lawn, New Jersey and α-NAA was obtained from Eastman Kodak Company, Rochester, New York. Sucrose was purchased from J. T. Baker Chemical Company, Phillipsburg, New Jersey.

Chemical Reagents. Nicotinic Acid-6-^{14}C (NA-6-^{14}C), 26.2 mc/mM, was obtained from Nuclear-Chicago. Azaserine, azaleucine, and

Figure 4. Tobacco Variety Maryland-872 in an Oklahoma Field

6-azauracil were obtained from Calbiochem, Los Angeles, California. Solvents and chemical reagents were of analytical reagent grade unless otherwise noted.

Tobacco Alkaloids. The nicotine standard used was from Nutritional Biochemicals Corporation and showed very little contamination with anabasine or nornicotine upon gas liquid chromatographic analysis. L-Nornicotine was purchased from A & A Laboratories, Incorporated, Plainview, New York. Other alkaloid standards had been collected by Dr. G. R. Waller over a number of years from friends and associates.

3. Apparatus

Precoated thin-layer chromatographic plates, Silica Gel GF, 250-microns and 500-microns thick, were purchased from Analtech, Incorporated, Newark, Delaware. The Swinny adapter and Teflon filters were purchased from Millipore Corporation, Bedford, Massachusetts.

B. Methods

1. Tissue Culture

Media. Four totally synthetic growth media, which were modifications of media developed by Brown and Lawrence (29), were used. They all contained the major and minor elements of Murashige and Skoog (30), nicotinic acid (0.5 ppm), pyridoxine-hydrochloride (0.1 ppm), thiamin-hydrochloride (0.1 ppm), inositol (100 ppm), glutamine (100 ppm), asparagine (100 ppm), aspartic acid (100 ppm), sucrose (20 g/l), and agar (8 g/l). Only the growth regulators were varied in the different media as seen in Table I.

TABLE I

GROWTH REGULATOR COMPONENTS OF THE MEDIA

Media	Growth Regulator Components
BL1	α-NAA, 0.1 ppm; Kinetin, 1.0 ppm
BL2	2,4-D, 1.0 ppm; Kinetin, 0.5 ppm
SM	2,4-D, 1.0 ppm; Kinetin, 0.5 ppm; GA, 1.0 ppm
SUM	2,4-D, 1.0 ppm; GA, 1.0 ppm; IAA, 0.5 ppm; Benzyladenine, 1.0 ppm

The media was adjusted to pH 5.7-5.8 with 1N sodium hydroxide before adding the agar. Media was heated on a hot plate until the agar was completely dissolved and then poured into 25 mm x 150 mm tubes at 20 ml per tube, 18 mm x 150 mm tubes at 10 ml per tube, and 125 ml flasks at 40 ml per flask. Tubes were capped with Kap-put closures stuffed with non-absorbent, long-fiber cotton and flasks were stuffed with this cotton. After autoclaving at 15 psi for 15 minutes, they were stored in a cold room at 4° C until needed. Small tubes were slanted to give more surface area.

Explants and Subculture. Stem sections of tobacco variety Maryland-872 were surface sterilized in 0.1 percent Tween-20 in 70 percent ethanol for three minutes and then rinsed three times with glass distilled water. Pith tissue was removed with a cork borer, sectioned, and placed on the various media. These cultures were then placed in the

growth chamber. Callus induced on one medium was subsequently subcultured on the same medium at eight-week intervals. Subsequently, all callus will be referred to by the medium on which it was maintained, e.g., BL1 callus. Subcultures will be designated S1, S2, and S3; e.g., S1-BL1 is the first subculture on BL1 medium.

All transfers were made in a transfer room in Room 441E of the Physical Sciences Building or in a laminar flow Edge GARD hood from the Baker Company, Incorporated, Sanford, Maine.

Growth Conditions. All cultures were kept in a growth chamber, Model AL80-4W, which had been modified to give a 16-hour light cycle of fluorescent lighting. A fan was also installed so that the temperature could be maintained at about 25° C.

2. Isolation of Metabolites

Three 10 g samples from callus grown on each medium were taken for extraction. Each sample was extracted in a mortar and pestle with 30 ml methanol. The tissue was then transferred to a flask and extracted two more times with methanol while sonicating. The extracts were pooled and taken to dryness on a rotary evaporator made by Buchler Instruments, Fort Lee, New Jersey, at temperatures less than 40° C. The residue was then extracted with 10 ml 0.5 N HCl. The acid extracts were centrifuged at 3500 x g for ten minutes at room temperature. Centrifugation removed most components that gave emulsions upon subsequent ether extraction. The acidic extract was extracted once with an equal volume of ether. The aqueous phase was then taken to pH 9.5 with 2N NaOH and extracted three times with ether. Anhydrous sodium sulfate was used to dry the pooled, basic ether phases. The ether was filtered and 10 ml 0.5 N HCl

was added to convert nicotine to a salt form. The solution was then taken to dryness on the rotary evaporator. The residue which was predominantly nicotine-hydrochloride was extracted with two 1 ml aliquots of methanol:ammonium hydroxide (9:1 v/v) and brought to 2 ml. Extracts were then transferred to two-dram vials which were sealed with Teflon disc-fitted caps and stored in the refrigerator until subsequent analysis. Nicotine standards were run through the extraction procedure and a nicotine recovery curve was constructed (Figure 5).

3. Chromatography and Instrumental Analysis

Gas Liquid Chromatography (GLC). Gas liquid chromatographic analyses were performed on a modified Barber-Coleman Model 5000 gas chromatograph equipped with a flame ionization detector (31). Liquid injection was used to introduce the sample onto the 10-feet x 1/4-inch glass column packed with 15 percent SE-30 on Anakrom ABS, 60-80 mesh. Quantitation was done by constructing a nicotine standard curve (Figure 6). The gas chromatograph response was linear with respect to nicotine injected.

Thin-Layer Chromatography (TLC) and Ultraviolet Spectroscopy. The callus extracts were also submitted to thin-layer chromatography Silica Gel GF plates, both analytical (250 microns) and preparative (500 microns). The developing system was chloroform:methanol:ammonium hydroxide (60:10:1 v/v/v). Nicotine bands were Drangendorff positive and appeared as absorbing spots in a Model C-3 Chromata-Vue ultraviolet light box, manufactured by Ultra-Violet Products, Inc., San Gabriel, California. UV-absorbing bands corresponding to nicotine standards were scraped from the plates with a Swinny Adapter-Aspirator assembly (32).

Figure 5. Nicotine Recovery Curve for the Extraction Procedure Used

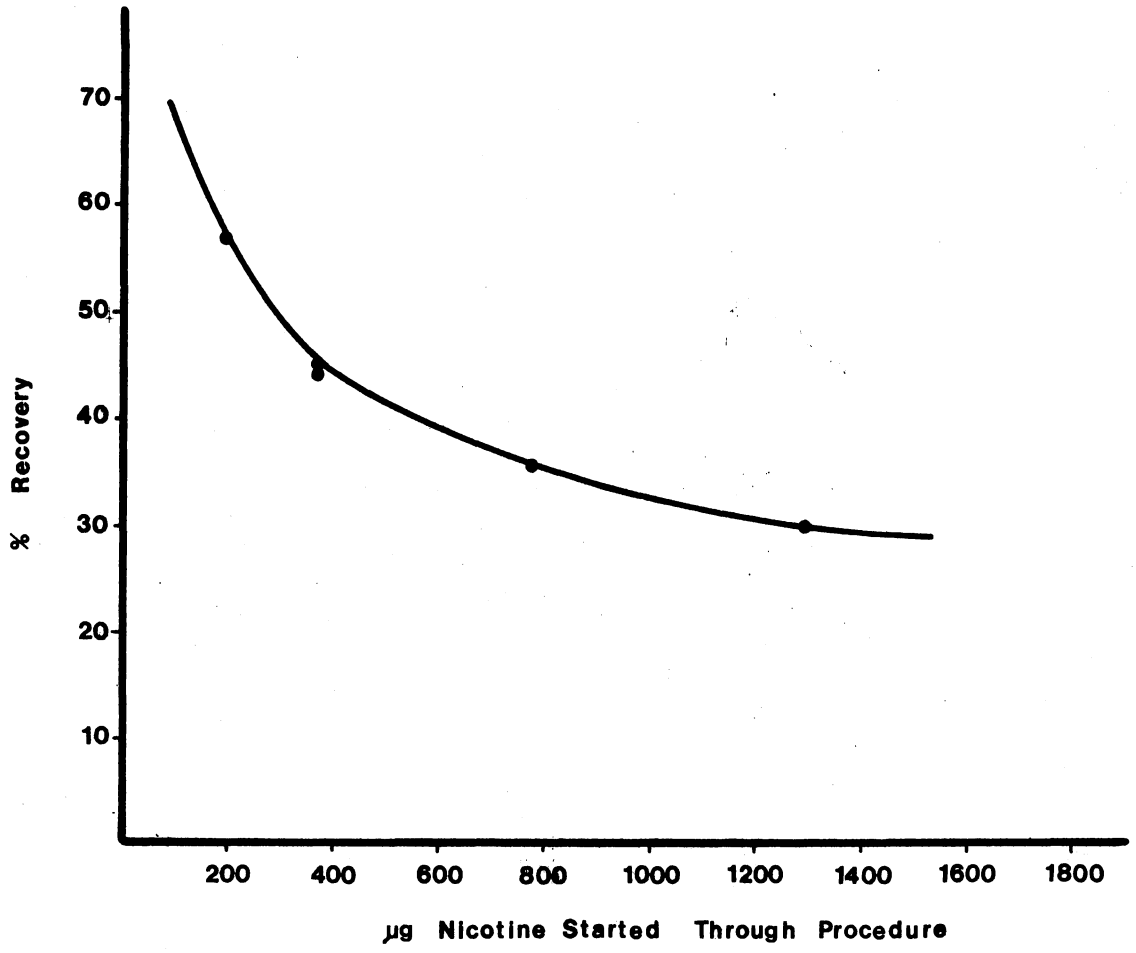
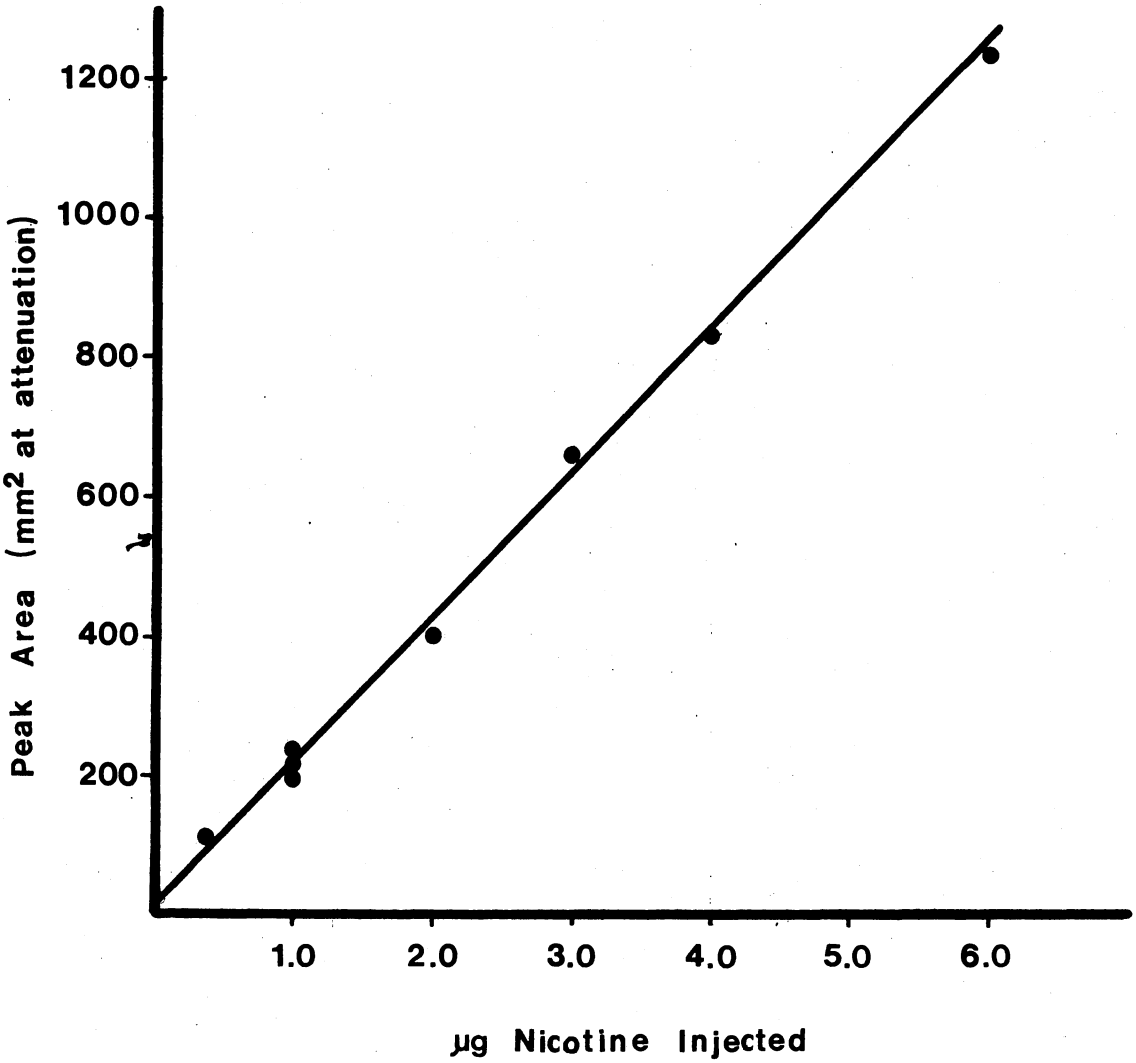

70
60
50
40
30
20
10
% Recovery
200
400
600
800
1000
1200
1400
1600
1800
µg Nicotine Started Through Procedure

Figure 6. Nicotine Standard Curve for Gas Liquid Chromatography


1200
1000
800
600
400
200
Peak Area (mm2 at attenuation)
1.0
2.0
3.0
4.0
5.0
6.0
µg Nicotine Injected

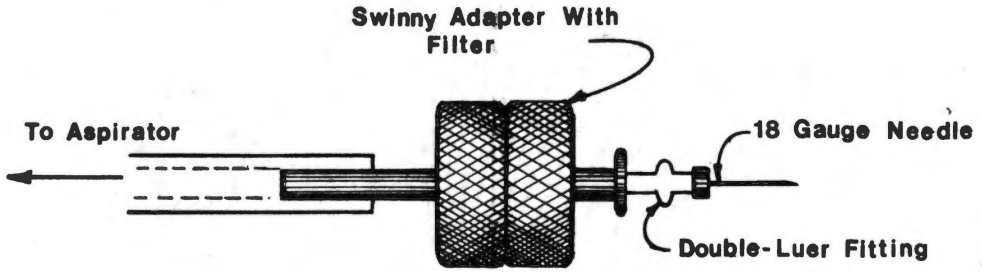
The adapter with a 13.0 mm Mitex (Teflon) filter, 10.0 microns pore size, was connected to an aspirator at one end and an 18-Gauge needle via a double Luer Teflon fitting at the other end. The needle and tubing to the aspirator were then removed and a 5.0 ml Luer-Lok syringe full of methanol was connected (Figure 7). The silica gel was then eluted into a centrifuge tube calibrated "to contain" until 2.5 ml was reached. The eluate was transferred to a 3.0 ml quartz cuvette and scanned from 300 nanometers to 220 nanometers in a Hitachi-Perkin Elmer 124 Double Beam Spectrophotometer. Quantitative determination was achieved by constructing a nicotine standard curve of absorbance at 262 nanometers versus nicotine concentration (Figure 8).

4. Histochemical Methods

Callus tissue with and without shoot formation was removed from culture tubes and immediately fixed in FPA (formalin, 10 ml; propionic acid, 5 ml; 95 percent ethanol, 50 ml; water, 35 ml) for at least 24 hours. The callus was then dehydrated through Zirkle's modified t-butanol series (33) and embedded in paraffin. Cross-sections and transverse sections were cut at 10 microns and attached to slides with Haupt's adhesive. Paraffin was removed and the slides were hydrated before staining.

Safranin-Fast Green (34). Fast green stains non-lignified material. Safranin is less specific for lignin than phloroglucinol or the Maule reaction since it reacts with phenolic hydroxyl groups in general (16). Safranin-Fast Green was used generally in studying the tissue morphology.

Figure 7. Assembly for Scraping and Eluting Bands From Thin-Layer Chromatography Plates


Swinny Adapter With
Filter
To Aspirator
18 Gauge Needle
Double-Luer Fitting

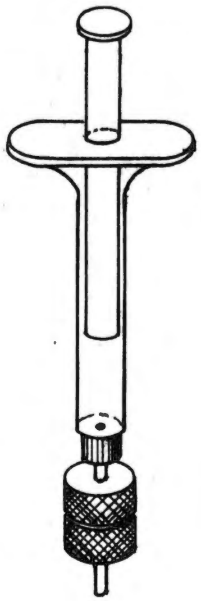
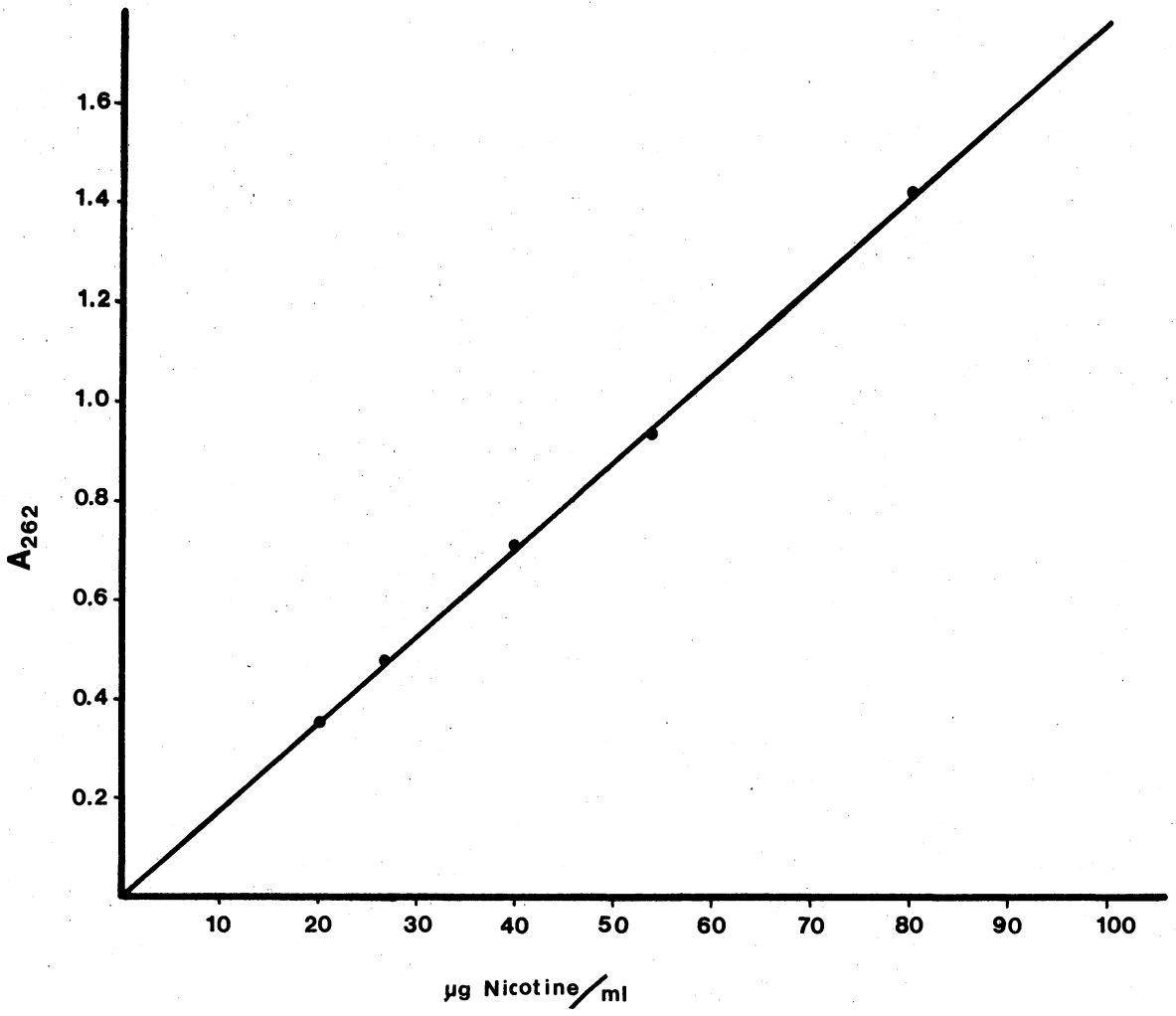


Figure 8. Nicotine Standard Curve for the Hitachi-Perkin Elmer Double Beam Spectrophotometer


1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
A_{262}
10
20
30
40
50
60
70
80
90
100
µg Nicotine/ml

Phloroglucinol-HCl. The sections were de-waxed and hydrated. One drop of 20 percent HCl saturated with phloroglucinol was placed on the section and covered with a coverslip (35). This stain reacts with coniferaldehyde groups in lignin to give a purple color (16). These are not permanent slides since the color fades in 30-45 minutes (35).

Maule Color Reaction. This reaction is positive only for lignins with significant amounts of syringylpropane units although the mechanism of the reaction is unknown (16). The sections were stained by treating successively in one percent aqueous permanganate, two percent HCl, and making a wet mount in a few drops of ammonium hydroxide (33).

Fluorescence Technique. Paraffin was removed and the sections were hydrated. Then wet mounts were made in a few drops of 0.005 percent water soluble aniline blue in a 0.15 M phosphate buffer at pH 8.65. Callose could be observed after about ten minutes by fluorescence microscopy. Callose fluoresced bright yellow-green and lignin fluoresced a less intense blue-white when observed with a mercury arc HBO 200-watt light source with an ultraviolet exciter filter Schott BG12 and a Schott OGI barrier filter (36).

CHAPTER IV

RESULTS AND DISCUSSION

A. Tissue Growth

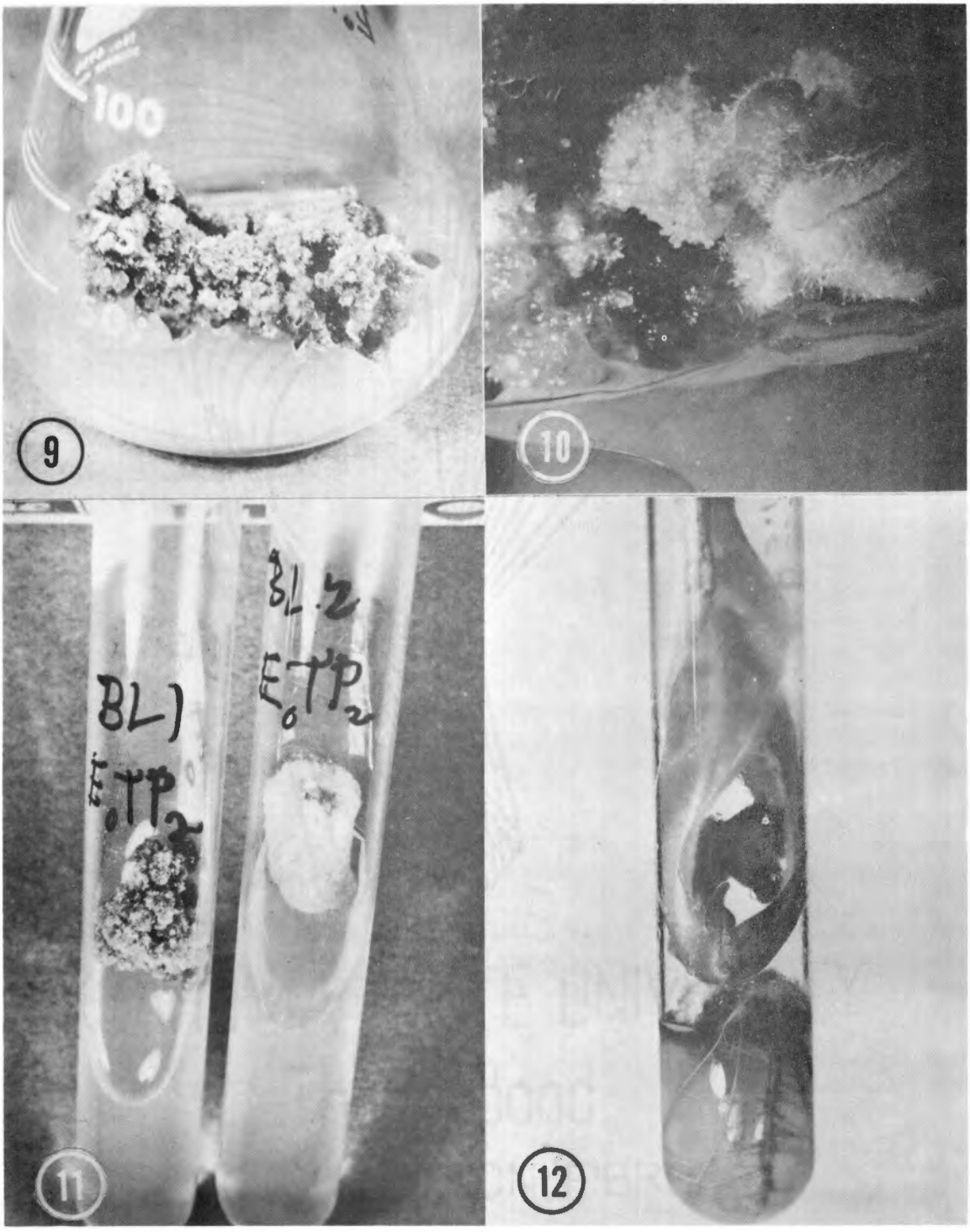
Callus tissue derived from pith tissue of N. tabacum variety Maryland-872 was induced on four different media and maintained on the same media through three subcultures. SUM callus was compact and tan. Growth was poor and no nicotine was detected. When grinding this callus in the mortar and pestle, a fish odor similar to trimethylamine was noted. SM callus was compact and light green. Growth was poor and no nicotine was detected. BL2 callus was compact and tan. This callus grew slowly with a hard central core and with the more friable tissue toward the periphery being in contact with the medium. No nicotine was detected. Thus, 1.0 ppm 2,4-D was sufficient to inhibit nicotine production as observed by Shiio and Ohta (9) in "Bright Yellow" callus. BL1 callus grew fairly well. BL1 callus, which developed shoots and vegetative buds in some cases, was green and more friable than the other callus tissues (Figures 9, 10, and 11). The buds continued to elongate without root formation. This morphogenic response is similar to that observed by Tabata et al. (7) in "Bright Yellow-12" callus and Skoog and Miller (24) in stem callus of "Wisconsin-38." One culture in the third BL1 subculture did have root formation (Figure 12). The root appeared to have its origin at the base of the shoot. As the callus was subsequently subcultured, fewer of the cultures showed shoot formation. This

Figure 9. Callus Derived From Maryland-872: S2-BL1 Callus in a Flask Culture

Figure 10. Callus Derived From Maryland-872: Bud Formation on S2-BL1 Callus

Figure 11. Callus Derived From Maryland-872: S2-BL1 and S2-BL2 Callus on Slant Cultures

Figure 12. Callus Derived From Maryland-872: S3-BL1 Culture With Root Formation


100
9
10
BL1
E.TP2
BL2
E.TP2
11
12

is typical of callus which is repeatedly subcultured (10). Nicotine was detected in BL1 callus. Observations of tobacco callus are summarized in Table II. Comparing the ultraviolet scan of nicotine standard with that of the spot eluted from the TLC plate, verifies that nicotine was produced in the callus (Figure 13). A growth curve of the third subculture was constructed over a seven-week period (Figure 14).

TABLE II

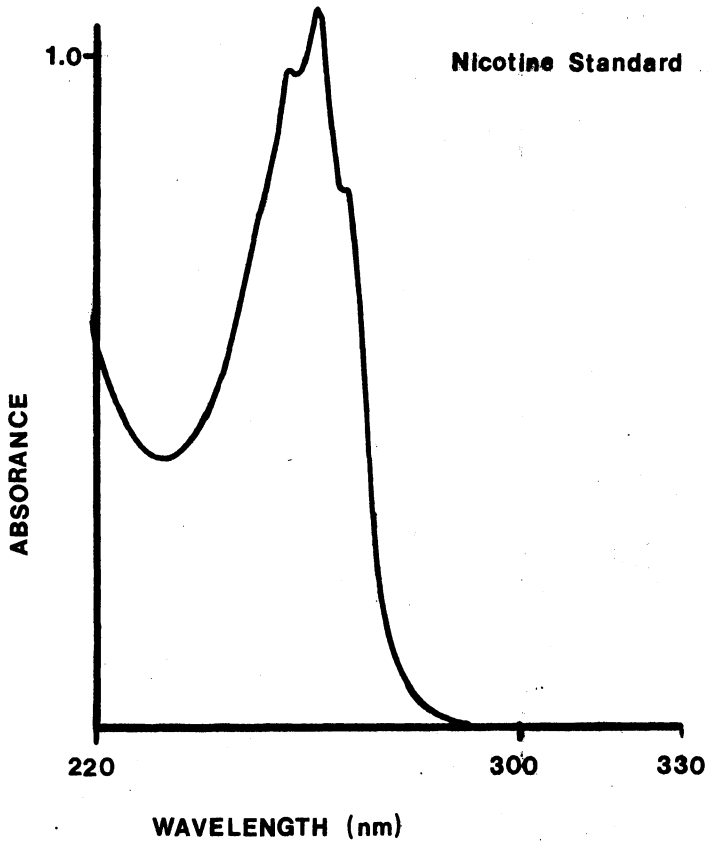
OBSERVATIONS OF TOBACCO CALLUS ON VARIOUS MEDIA

Media	Observations	Alkaloid
SUM	compact, tan, fishy odor	none
SM	compact, light green	none
BL1	compact, green, shoot formation	nicotine
BL2	compact, tan	none

B. Nicotine Production

Original tissue of Maryland-872 was analyzed for nicotine content (Table III) using TLC and the spectrophotometer. With 500 microns Silica Gel GF plates, the R_f value for nicotine was 0.71 in chloroform: methanol:ammonium hydroxide (60:10:1). Root tissue was not analyzed since it was not used in explanting.

Figure 13. Spectrophotometric Scans of Nicotine Standard and TLC Spot Corresponding to Nicotine Isolated From BL1 Callus


1.0
Nicotine Standard
ABSORANCE
220
300
330
WAVELENGTH (nm)

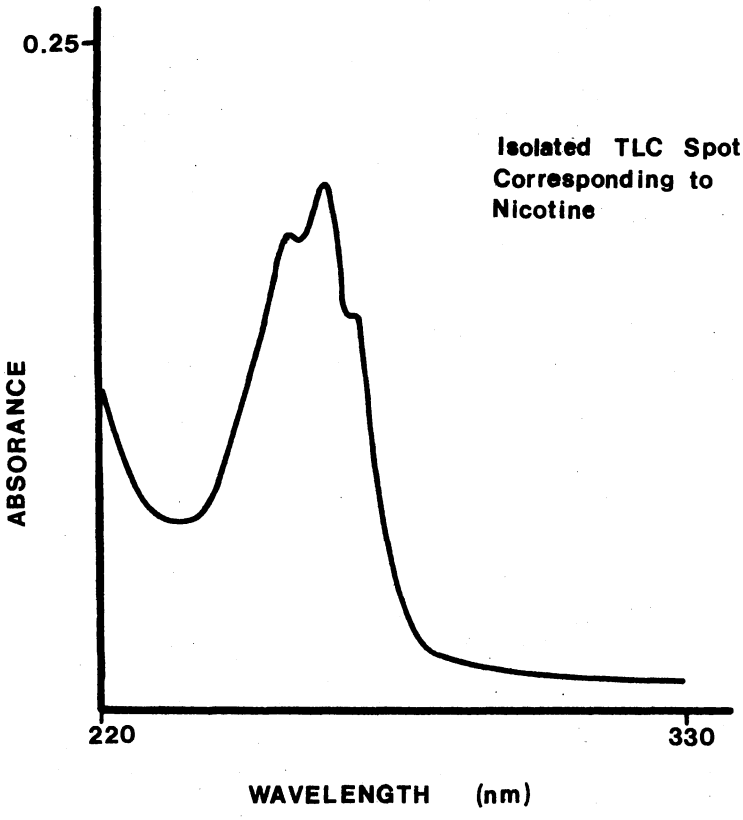

0.25
Isolated TLC Spot
Corresponding to
Nicotine
ABSORANCE
220
330
WAVELENGTH (nm)

Figure 14. Growth Curve of S3-BL1 Callus. Indicated feature is the Number of callus cultures sampled (n).

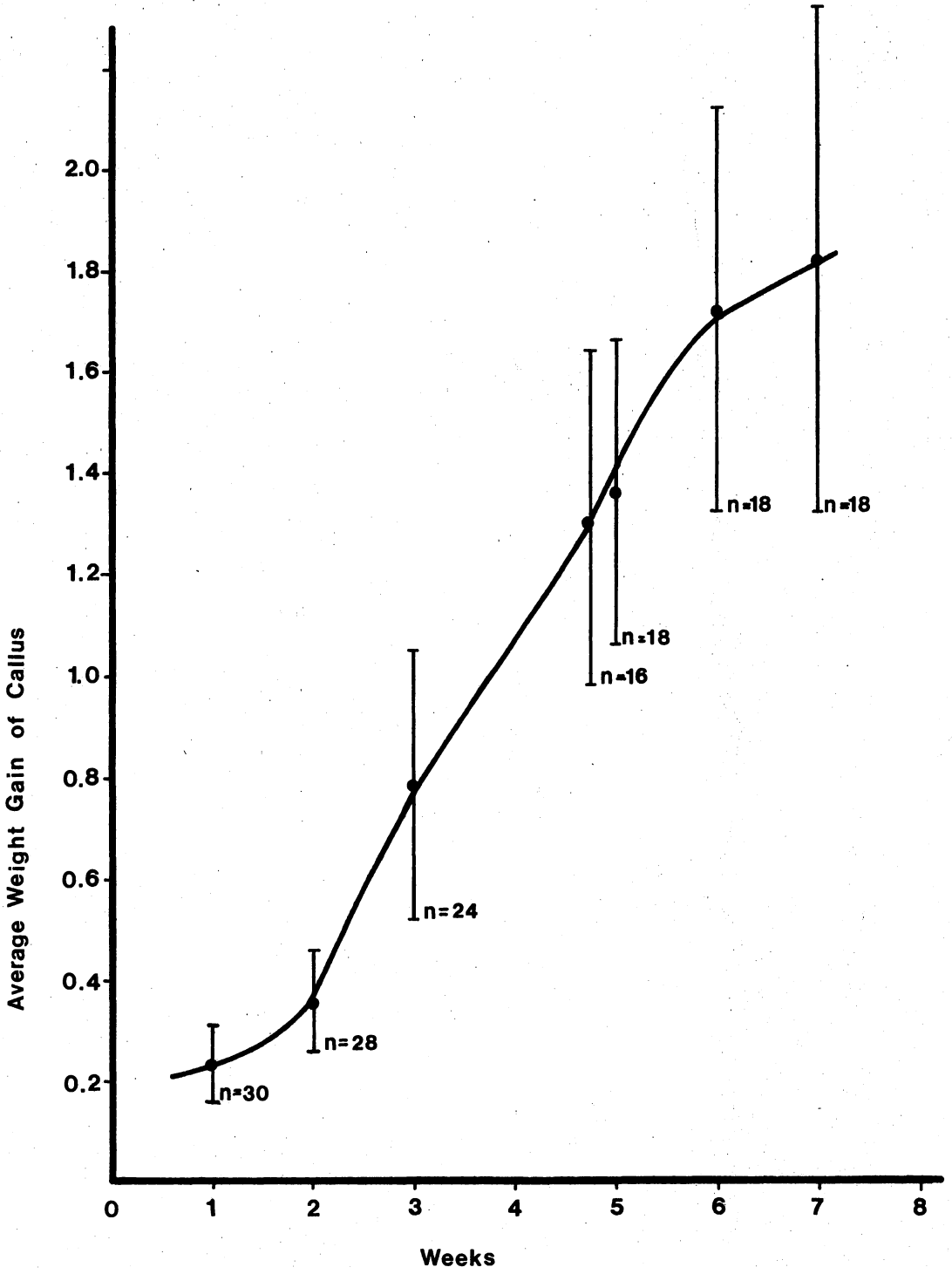

Average Weight Gain of Callus
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
7
8
Weeks
n=30
n=28
n=24
n=16
n=18
n=18
n=18

TABLE III

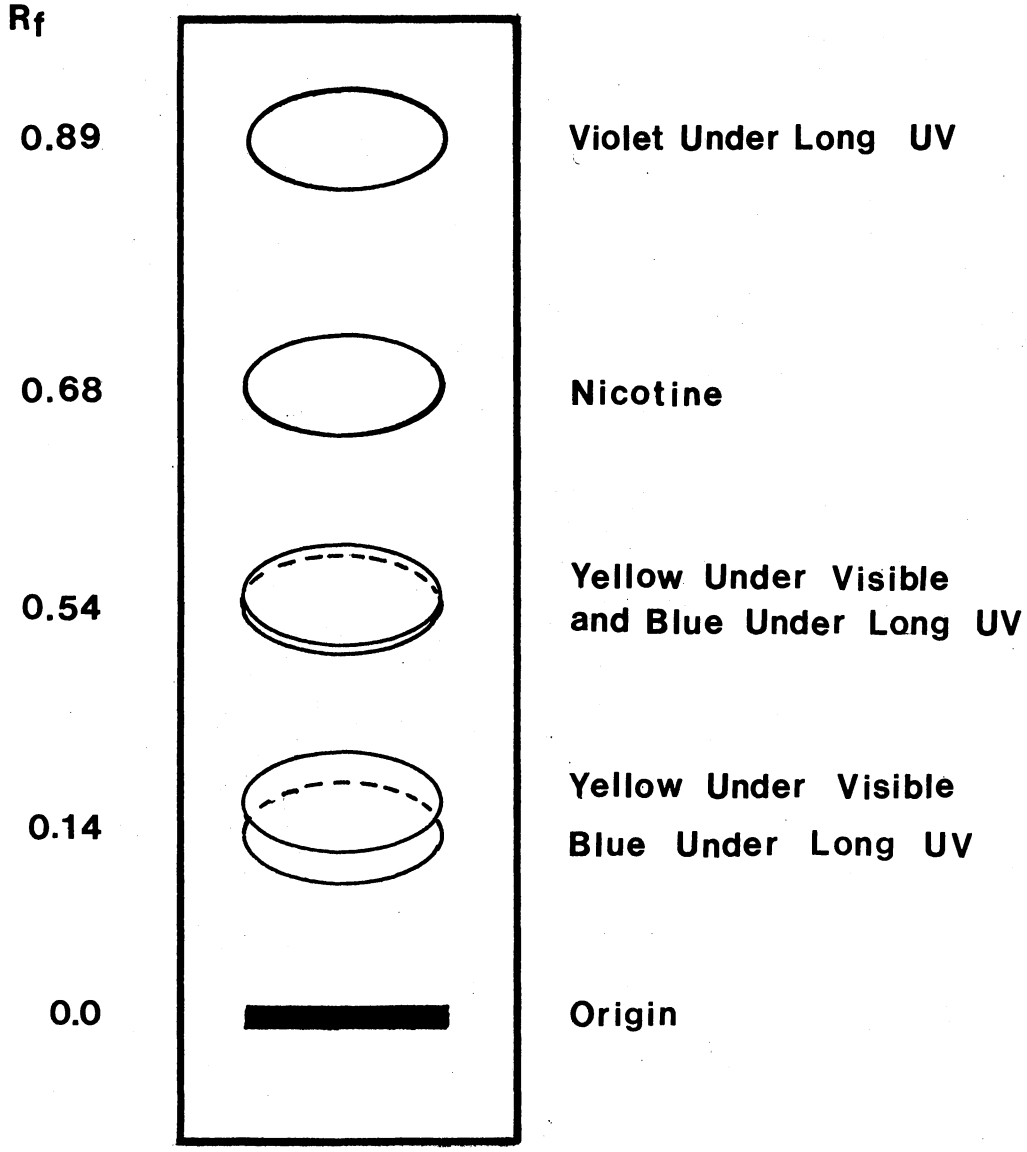
NICOTINE ANALYSIS* OF MARYLAND-872
EXPLANT MATERIAL

Tobacco Tissues	Nicotine Content (ug/g fresh weight)
stem epidermis	268 ± 30
leaf	1070 ± 51
pith	406 ± 43

*Average of three samples in which nicotine was determined spectrophotometrically after TLC purification.

BL1 callus from the first subculture was analyzed by TLC and the spectrophotometer. Spots other than nicotine were observed on TLC plates after development as seen in Figure 15. However, they were not identified and did not give a positive Dragendorff reaction. The results of the nicotine analysis (Table IV) showed that callus with vegetative buds and shoots had about five times the nicotine content of callus without buds or shoots. This corresponds with the results of Tabata et al. (7), who first observed that Bright Yellow-12 callus with vegetative bud formation had higher nicotine content than callus without buds. Callus with shoots removed had higher nicotine content than callus without shoot formation. This suggests that the shoot may induce the callus to produce more nicotine. The high nicotine content observed in the leaf suggests that nicotine may be synthesized in the callus and transported to shoot tissue as it is translocated to the leaf in the

Figure 15. Thin-Layer Chromatogram of Extract From S3-BL1 Callus


R_f
0.89
Violet Under Long UV
0.68
Nicotine
0.54
Yellow Under Visible
and Blue Under Long UV
0.14
Yellow Under Visible
Blue Under Long UV
0.0
Origin

intact plant.

TABLE IV

NICOTINE ANALYSIS* OF S1-BL1 CALLUS

Callus Tissues	Nicotine Content (ug/g fresh weight)
callus without buds or shoots	14.5 ± 2.4
callus with buds and shoots removed	71.3 ± 10.1
shoots excised from callus	335.0 ± 20.1

*Average of three samples in which nicotine was determined spectrophotometrically after TLC purification.

During the growth of the third subculture on BL1, nicotine content was analyzed by GLC on 15 percent SE-30. Tobacco alkaloids were sufficiently resolved (Table V). The nicotine content decreased greatly during the first two weeks and did not reach high levels again until after the fifth week (Figure 16). Nicotine is probably used as a nitrogen source until the callus assimilates nitrogen sources from the medium. Nicotine was the only tobacco alkaloid detected in Maryland-872 callus; this is reasonable since 96 percent of the total tobacco alkaloid in the intact plant is nicotine (37).

Figure 16. Nicotine Content of S3-BL1 Callus Over a Seven-Week Growth Period

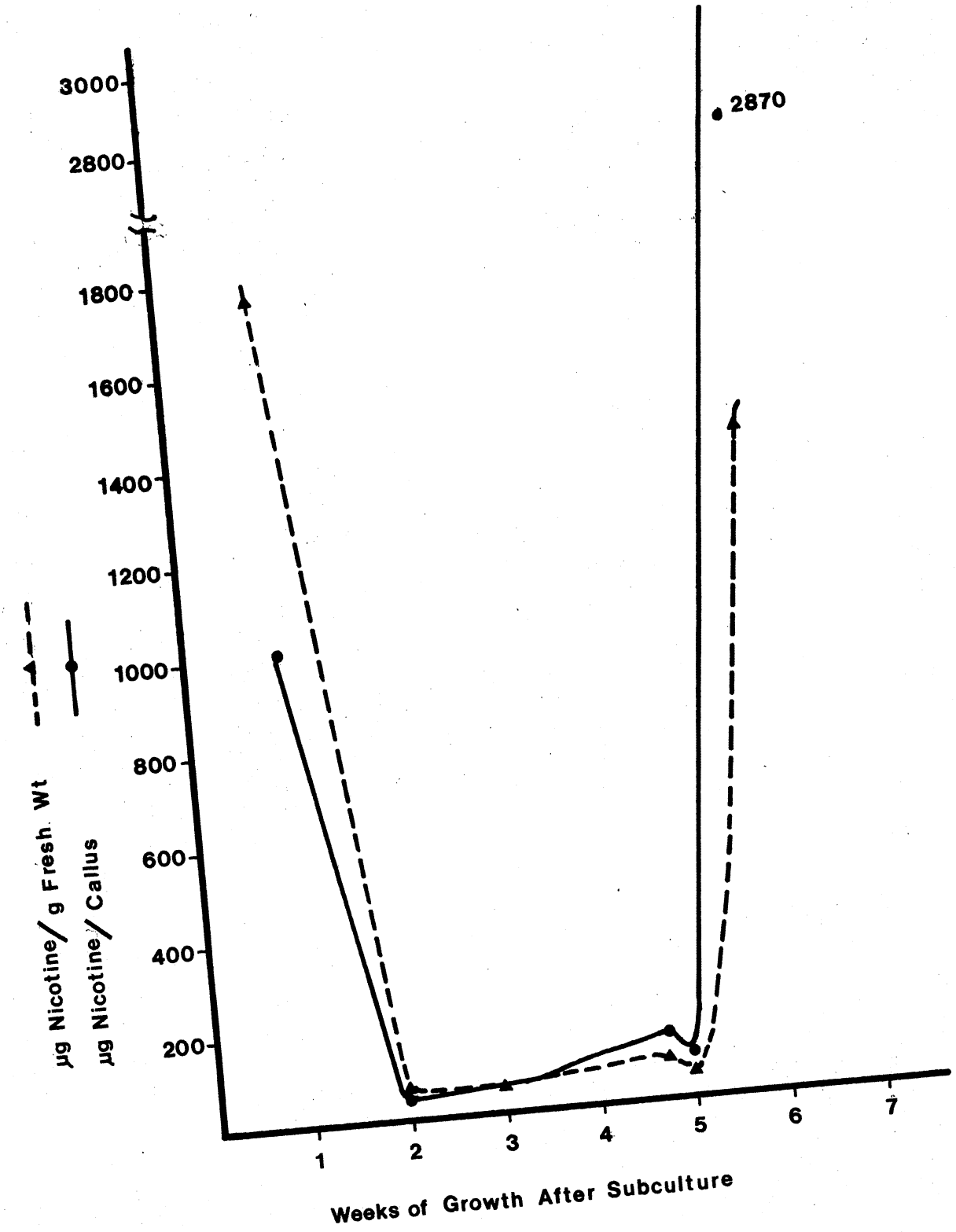

3000
2800
1800
1600
1400
1200
1000
800
600
400
200
2870
µg Nicotine/g Fresh Wt
µg Nicotine/Callus
1
2
3
4
5
6
7
Weeks of Growth After Subculture

TABLE V

RETENTION TIMES OF TOBACCO ALKALOIDS
(15% SE-30: 178° COLUMN TEMP.
200° FLASH HEATER
240° DETECTOR
45 ml/MIN.)

Alkaloid	Rt (Min)
nicotine	5.8
anabasine	9.2
nornicotine	7.2
actinidine	5.3
myosmine	7.3

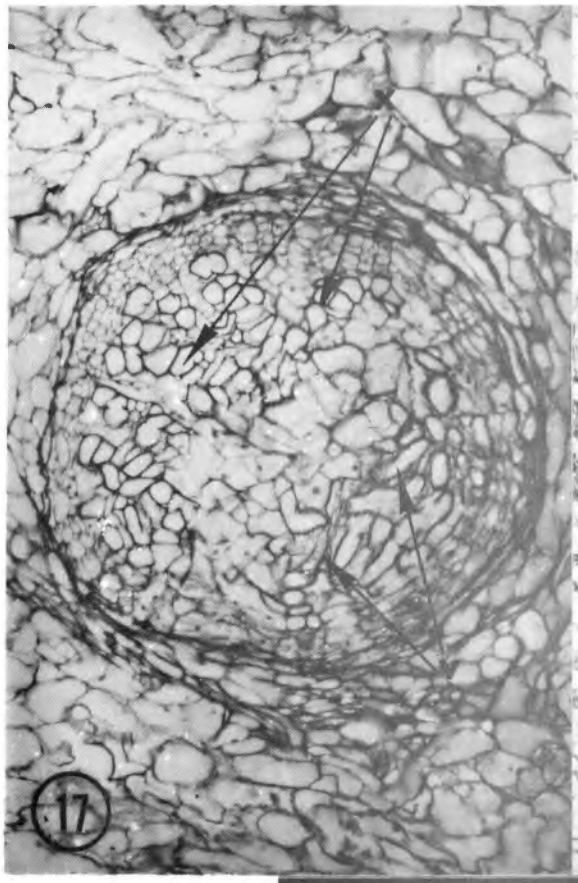
C. Histological Studies

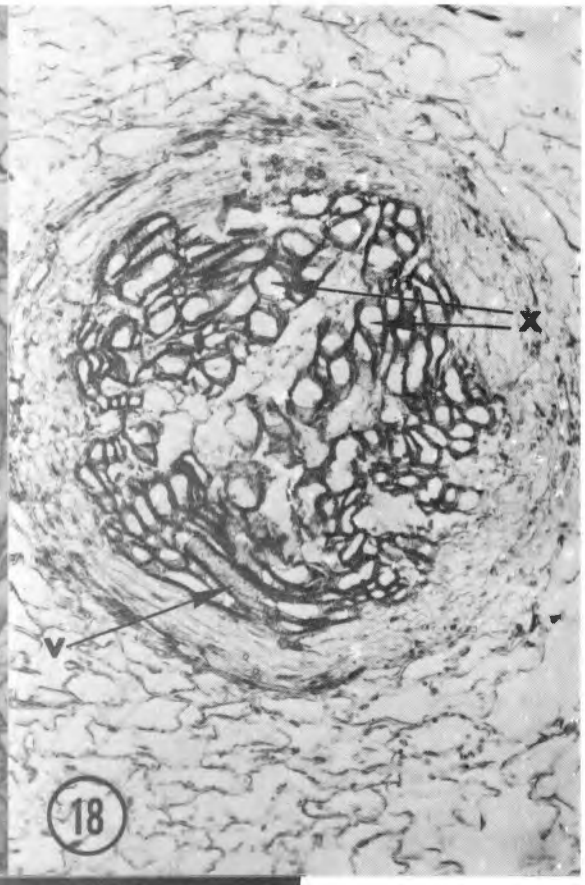
BL1 Callus without shoot formation was sectioned sagittally and in cross-section. Nodules containing xylem and phloem, similar to those observed by other researchers (10), were found scattered randomly throughout the callus. The appearance of these nodule-like structures may be dependent upon (1) origin of the callus, (2) the extent to which the callus has been subcultured, (3) the composition of the culture medium, (4) the extent of immersion in the culture medium, and (5) growth activity of the callus (10). Lignified xylem stained red with safranin (Figure 17) gave a positive phloroglucinol (Figure 18) reaction, and gave a negative Maule reaction. Sections mounted in 0.005 percent aniline blue solution were viewed with a fluorescence microscope. Lignin in the xylem fluoresced blue-white while callose plugs in

Figure 17. Photomicrograph of S3-BL1 Callus Without Shoot Formation: Cross-Section of a Nodule Stained With Safranin-Fast Green. Indicated features are the Pitted Vessels (v) and the Xylem (x). Magnification: 110X.

Figure 18. Photomicrograph of S3-BL1 Callus Without Shoot Formation: Cross-Section of a Nodule Stained With Phloroglucinol-HCl. Features indicated are the Xylem (x) and Pitted Vessels (v). Magnification: 220X.

Figure 19. Photomicrograph of S3-BL1 Callus Without Shoot Formation: Cross-Section of a Nodule Stained With Aniline Blue and Viewed With a Fluorescence Microscope. Features indicated are the Xylem (x) and Phloem (p). Magnification: 220X.


17


x
v
18


p
x
19

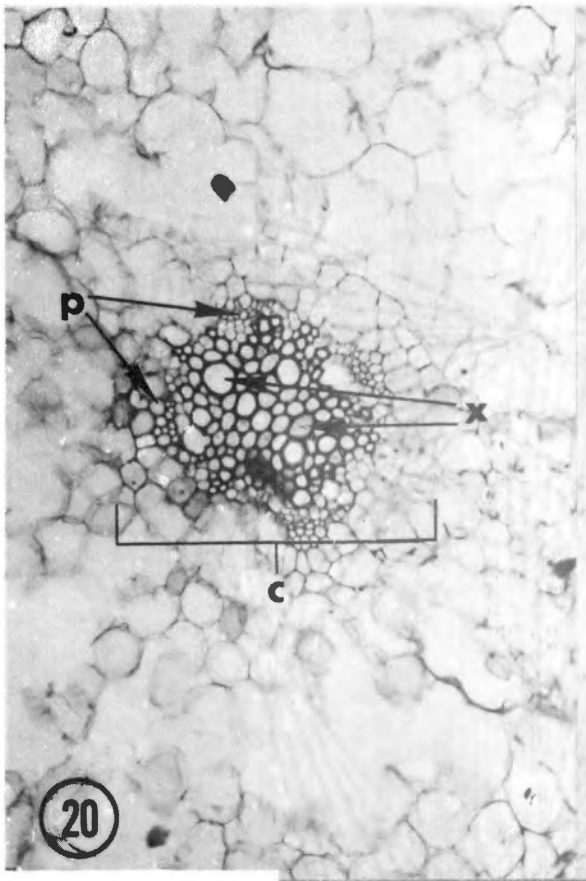
phloem fluoresced bright yellow-green (Figure 19). All these nodules appeared spherical with xylem at the center and phloem at the periphery. In section the nodules appear analogous to the vascular pattern in primary roots. The primary root, which appeared in one of the third subcultures, was sectioned with a freezing microtome. Sections showed that the vascular pattern in the root (Figure 20) was similar to a node in cross-section; i.e., an exarch system. In root, it is typical for phloem strands to occur at the periphery of the vascular cylinder with alternating xylem strands and with xylem or pith present at the center of the cylinder (38). The xylem in root sections stained more intensely with phloroglucinol (Figure 21) than did the xylem in nodules of the callus, but this is typical of frozen and paraffin sections. Sections of root were also observed under the fluorescence microscope (Figure 22).

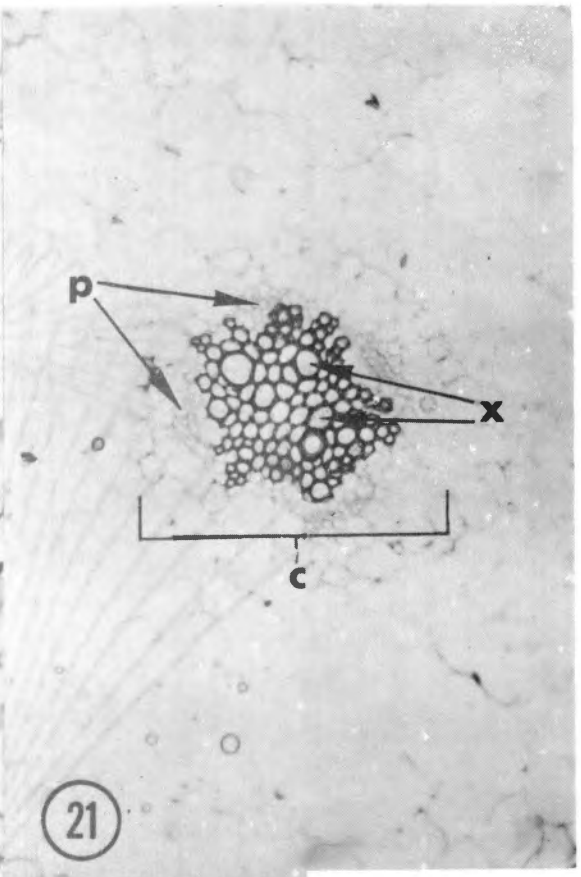
By taking sagittal sections of BL1 callus with shoot formation, it was possible to trace the shoot vascular tissue into the callus tissue. The vascular tissue of the shoot was contiguous with vascular tissue of a nodule (Figure 23). This nodule had xylem near its base adjacent to parenchymatous tissue (Figure 24). Thus, interaction between the shoot and callus seems plausible. Murashige (25) observed that shoot formation was contingent upon formation of nodule-like structures with high meristematic activity, such as those found in our BL1 callus without shoots. Xylem in the shoot did not stain with safranin as intensely as xylem in the nodule; this indicates that xylem in the shoot is less lignified than xylem of the nodule. Some nodules in the sections were similar to those in callus without shoots. Other nodules were much larger and more diversified (Figure 25).

Figure 20. Photomicrograph of Section of the Root Formed in the Third Subculture of BL1 Callus: Cross-Section Stained With Safranin-Fast Green. Features indicated are the Vascular Cylinder (c), the Xylem (x), and the Phloem (p). Magnification: 220X.

Figure 21. Photomicrograph of Section of the Root Formed in the Third Subculture of BL1 Callus: Cross-Section Stained With Phloroglucinol-HCl. Features indicated are the Xylem (x), Phloem (p), and Vascular Cylinder (c). Magnification: 220X.

Figure 22. Photomicrograph of Section of the Root Formed in the Third Subculture of BL1 Callus: Cross-Section Stained With Aniline Blue and Viewed With a Fluorescence Microscope. Features indicated are Xylem (x) and Phloem (p). Magnification: 220X.


p
x
c
20


p
x
c
21

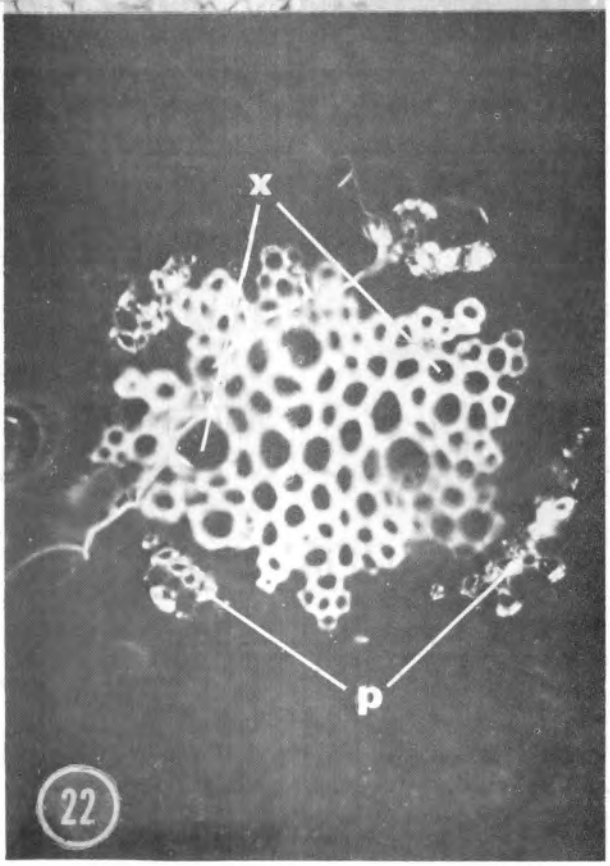
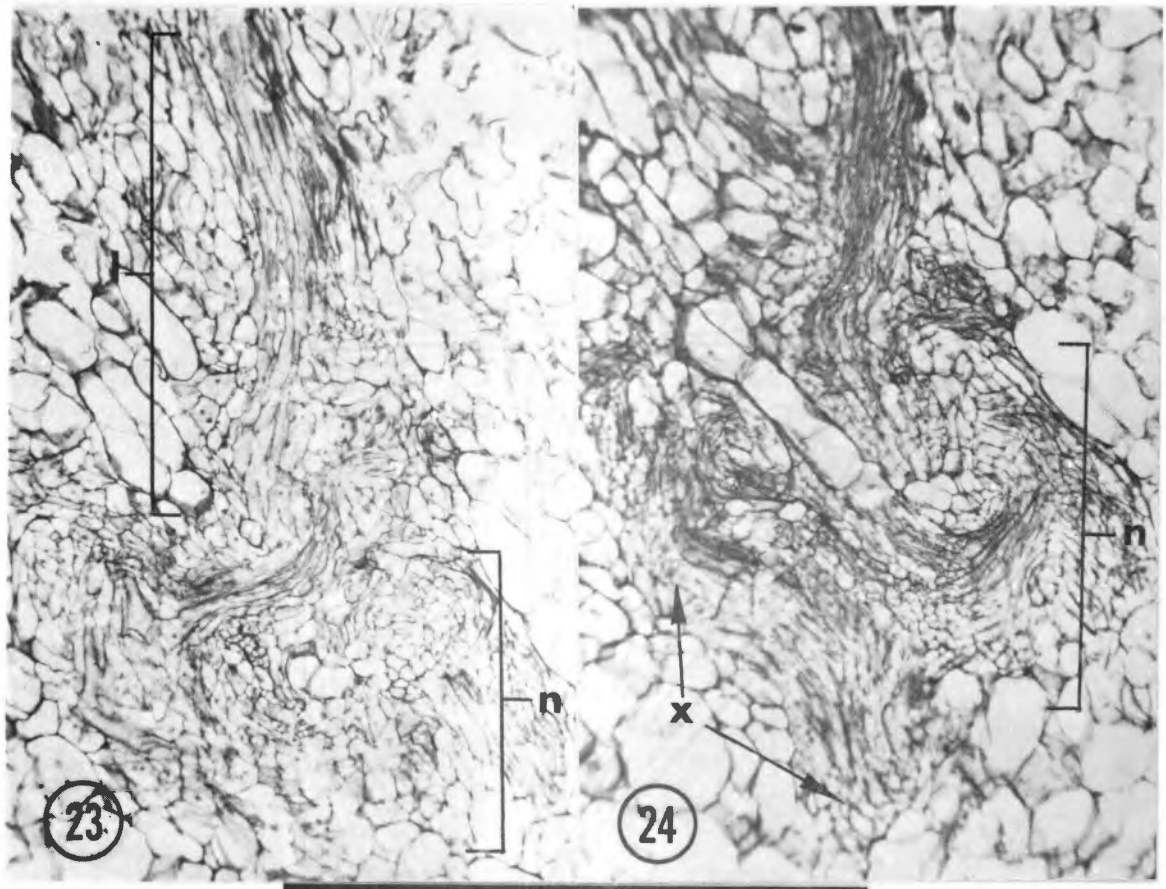
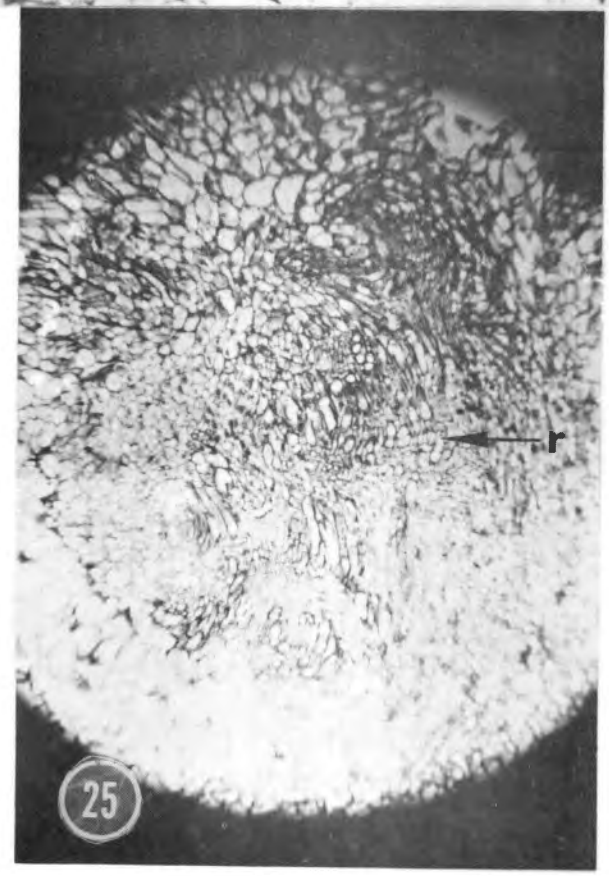

x
p
22

Figure 23. Photomicrograph of Sagittal Section of S3-BL1 Callus With Shoot Formation: Sagittal Section Through the Leaf-Callus Junction Stained With Safranin-Fast Green. Features indicated are: Vascular Bundle from the shoot in longitudinal section (l), and the Nodule within the callus (n). Magnification: 110X.

Figure 24. Photomicrograph of Sagittal Section of S3-BL1 Callus With Shoot Formation: Closer View of Xylem at the Base of the Nodule. Features indicated are: the Nodule (n) and Xylem at the base of the nodule (x). Magnification: 220X.

Figure 25. Photomicrograph of Sagittal Section of S3-BL1 Callus With Shoot Formation: Large Nodule in Sagittal Section Stained With Safranin-Fast Green. Feature indicated is the Xylem Ray (r). Magnification: 30X.


l
n
23
n
x
24


r
25

D. Incorporation Studies of NA-6-^{14}C Into Nicotine

Eleven mature tobacco plants were injected into the pith with various amounts of nicotine precursors and inhibitors of the pyridine nucleotide cycle. Results of the incorporation studies are shown in Table VI.

TABLE VI

RESULTS OF INCORPORATION STUDIES IN INTACT PLANTS IN THE FIELD

Plant	Treatment	Nicotine Content (mg/g fresh weight)	Nicotine Radioactivity
74-1-0	None	---	----
74-2-0	None	304	None
74-3-0	15 μC NA-6-^{14}C	161	None
74-4-0	15 μC NA-6-^{14}C	389	Trace
74-5-1	15 μC NA-6-^{14}C + 0.3 g azaserine	332	None
74-6-2	15 μC NA-6-^{14}C + 0.3 g azaleucine	378	None
74-7-3	15 μC NA-6-^{14}C + 0.1 g azauracil	273	Trace
74-8-0	None	610	None
74-9-0	None	220	None
74-10-0	15 μC NA-6-^{14}C	---	----
74-11-0	15 μC NA-6-^{14}C	246	Trace

The incorporation was allowed to occur for 24 hours in the first seven plants and for 48 hours in the last four. The plants were harvested and frozen. The plants were cut into small sections and macerated in a Waring blender. The slurry was then steam distilled and the condensate was acidified with HCl and treated as in the Methods section, Isolation of Metabolites. Nicotine was purified by TLC and determined spectrophotometrically. One ml of the basic extract was diluted in 19 ml Bray's solution and the specific activity was determined by liquid scintillation counting.

Incorporation was slight; this indicates that nicotinic acid may not have been transported from the pith to the root where nicotine biosynthesis occurs.

CHAPTER V

SUMMARY

The purpose of the research described was to establish a callus tissue culture of tobacco variety Maryland-872 which would produce nicotine and to study the morphology of the callus. It was found that vascular differentiation occurred in BL1 callus tissue in which nicotine was produced. Nodule-like meristematic structures containing xylem and phloem which were similar to primary root were scattered throughout the tissue. BL1 callus with shoot formation had higher nicotine content than BL1 callus without shoot formation. Vascular tissue in the shoot was contiguous with vascular tissue of a nodule present in the callus. Therefore, interaction between the shoot and callus may have resulted in higher nicotine production in BL1 callus with shoot. Since shoot tissue had a much higher nicotine concentration than the callus with shoots removed it is suggested that nicotine produced in the callus tissue may be transported to the shoot. This would be similar to the intact plant in which nicotine produced in the root is transported to the shoot (1).

Studies of incorporation of radioactive precursors into nicotine were not attempted with BL1 callus. The incorporation studies in the intact plants gave negative results, indicating that NA-6-^{14}C was not transported from the pith tissue to the root. A cell suspension culture that synthesizes nicotine should be developed and incorporation into nicotine should be examined. Cell suspension cultures in which nicotine

is produced would also be ideal for attempts to isolate enzymes catalyzing the final steps in nicotine biosynthesis, i.e., some pyridine nucleotide cycle intermediate into nicotine. Cell suspension cultures would also be suitable for studies on the effects of various inhibitors of the pyridine nucleotide cycle upon incorporation of radioactive precursors into nicotine.

Since it has been observed that low concentrations of growth regulators (0.1 ppm α-NAA and 0.1 ppm kinetin in BL1 callus) are required for nicotine production in tobacco callus tissue (9), it is suggested that in future studies on alkaloid formation in tissue culture that the organization of the tissue (e.g., vascularization, lignification, organogenesis) should be observed since organized growth may even occur in suspension cultures (10). Various alkaloidal secondary metabolites have been isolated from tissue cultures, e.g., coniceine and N-methylconiine in callus cultures of Conium maculatum, tropane alkaloids in callus and suspension cultures of various Datura species, indole alkaloids in callus culture of Vinca roseus (39), and diterpenoid alkaloids in callus cultures of Delphinium ajacis (40). The scopolin/scopoletin ratio may be an easy method to follow the lignification process since scopolin, scopoletin, and most alkaloids may be isolated simultaneously via extraction (8).

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VITA[2]

Thomas Michael Heinze

Candidate for the Degree of

Master of Science

Thesis: THE FORMATION OF NICOTINE AND A STUDY OF THE MORPHOLOGY IN CALLUS DERIVED FROM MARYLAND-872 TOBACCO

Major Field: Biochemistry

Biographical:

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