GROWTH AND ALKALOID CONTENT OF *ERYTHROXYLON COCA* CALLUS CULTURES

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INTRODUCTION

Tissue Culture

The first attempts at culturing isolated plant cells on artificial media were begun by Haberlandt in 1902. His work was unsuccessful, and this failure was probably due to the choice of mature plant cells and to the lack of knowledge as to what nutrients were needed to support cell growth (7). Success was later achieved with the prolonged culture of excised roots of tomato (31). The first truly successful cultures of plant tissues were the cultures of carrot and tobacco cambial tissues (7, 44).

Tissue culture involves the establishment and maintenance of various plant parts such as cells, tissues or organs on artificial media under aseptic conditions (28). Culture of material other than plant tissues is referred to by the plant part used, i.e., anther, meristem, pollen, cell, and organ cultures, although the term tissue culture is often used to collectively describe these techniques (11). Once established, cultures may be induced to produce roots and shoots or callus tissue, depending on the desired goals. Numerous references are available which describe the preparation of explants and media and the establishment and maintenance of cultures (11, 28).

The uses of plant tissue culture are varied: (1) rapid clonal propagation of selected varieties; (2) production of pharmaceuticals and other plant products; (3) *in vitro* breeding and genetic improvement

of crops; and (4) recovery and preservation of disease-free material and germplasm (28). In addition, the uses of tissue culture can be extended into the fields of biochemistry, pathology and physiology (11).

Media

The main requirements of cells in culture can be broken down into three categories: inorganic salts, organic substances, and natural complexes. The inorganic salts found in most tissue culture media are based upon the major and minor essential elements needed for growth of whole plants. Major essential elements are nitrogen, phosphorus, calcium, potassium, iron, sulfur and magnesium. In addition, carbon, hydrogen, and oxygen are supplied by sugar, water and the air. Minor essential elements vary among media formulations, but generally included are: boron, zinc, manganese, iodine, and copper (29).

Organic components of nutrient media include: (1) a carbohydrate source, which is generally sucrose; (2) vitamins, i.e., thiamin, inositol, nicotinic acid, and pyridoxin; (3) amino acids and amides; and, (4) growth regulators, auxins, cytokinins, and gibberellins (29).

Natural complexes are used when a chemically defined medium fails to initiate and support growth *in vitro*. One disadvantage of natural complexes is their variability. A wide variety of materials have been used as natural complexes: liquid coconut and corn endosperm; banana, orange, and tomato juice; malt and yeast extracts; and, casein and lactalbumin hydrolysates (28).

Totipotency and Cytodifferentiation

Haberlandt's attempts to culture and to control the growth and development of single plant cells recognized the totipotent nature of

plant cells (7). According to the concept of cell totipotency, each plant cell contains the genetic information necessary for its development into a complete plant with the biochemistry characteristic of that plant (25). However, cells which have differentiated to the point where they are unable to "de-differentiate" or return to a less differentiated state no longer are considered to be totipotent (30).

While plant cells contain identical genetic information, plant growth and development results from the selective expression of this genetic material (25). The process whereby cells become biochemically and morphologically different from one another as a result of selective gene expression is termed cytodifferentiation. Cytodifferentiation can be defined as the ordered sequence of changes in the structure and function of cells by which cells arising in the plant meristem become the specialized non-dividing cells of the plant body (36).

The factors which control gene expression and cytodifferentiation are not well understood, but basically cells differentiate because of the short and long range influences exerted upon them by other cells. For example, as cell division continues, the inner cells are subject to changing physical forces. Concentration gradients of plant hormones and metabolites also arise in the cell mass. These physical and chemical influences elicit changes in the patterns of gene activity of the affected cells (24, 36).

Production of Secondary Metabolites in Culture

In light of the totipotent nature of plant cells, the question arises as to whether potentially totipotent cells could be induced to behave biochemically as they would in a complete plant without having to

go through the plant's entire process of growth and development (25). The ability to synthesize plant metabolites in culture would be of value because of the various secondary metabolites produced by plants. Secondary metabolites are compounds which are not considered to be essential for plant growth but which are in many cases of medicinal or food value to man. These secondary metabolites include alkaloids, flavoring compounds, oils, enzymes, and terpenoids (12).

In some cases, laboratory synthesis of secondary compounds may be too difficult or expensive to replace extraction from plant material. Tissue culture could provide a means of increasing plant material under controlled conditions for extraction, for biotransformation of precursors of desired compounds, or for the release of metabolites into the surrounding medium (12). Furthermore, the use of the tissue culture for secondary metabolite production presents the following advantages: (1) production independent of environmental factors such as climate, plant pests, and seasonal changes; (2) more stringently controlled drug production; (3) decreased need for land; and, (4) more uniform raw materials (45).

However, although cell cultures can be grown on a large scale, the production of secondary metabolites in culture is not yet commercially feasible. The reason for this is that the factors controlling metabolite production are not well understood and levels in vitro are often lower than in the plant (23, 40). However, metabolite levels in cultures can be increased in various ways. The addition of precursors (9, 39), the selection of high yielding and stable cell lines (38, 46), and modifications of the media and cultural conditions (46) have all increased the level of plant metabolites in culture. Thus, it may be possible to

develop an economical means of producing valuable plant products *in vitro*. In view of the possible advantages of secondary metabolite production in culture and because no work has been done in this area with the coca plant, the growth and alkaloid content of *Erythroxylon coca* Lam. callus cultures was studied.

LITERATURE REVIEW

Alkaloids

The term alkaloid or "alkali-like" was first proposed by W. Meissner in 1819. Alkaloids are basic compounds mainly of plant origin which contain nitrogen in a heterocyclic ring (30). Many of these compounds have significant pharmacological activity as in the antispasmodics, hyoscyamine and atropine from *Atropa belladonna*, and the antitumor agent camptothecin from *Camptotheca acuminata*. (34). However, it has been difficult to draw up a general definition for alkaloids because of their chemical heterogeneity. As a result, not all compounds which are classified as alkaloids are basic in character nor do they contain nitrogen in a heterocyclic ring. Many widely distributed plant bases are not considered as alkaloids because they are simple aliphatic amines and are referred to as "biological amines" or "protoalkaloids" by some authors (30).

Alkaloids are found throughout the plant kingdom. They occur abundantly in the *Apocynaceae*, *Papaveraceae*, *Rubiaceae*, and *Solanaceae*. Though higher plants tend to have more alkaloids than lower plants, alkaloids have been found in the club mosses, horsetails and in certain fungi. Alkaloids have not been found in the Bryophytes but screening will probably reveal alkaloids in more plant families (34).

Although the particular function of alkaloids is unknown, the following suggestions have been made as to their role in plants:

(1) Alkaloids may be products of plant metabolism, i.e., nitrogen waste products.

(2) Alkaloids may serve as nitrogen reservoirs.

(3) Alkaloids may help to protect plants against attack by predators.

(4) Because alkaloids resemble plant growth regulators in structure, they may have some growth regulatory activity (42).

General Biosynthesis

The biosynthetic pathway for alkaloids was first proposed by Sir Robert Robinson in 1917. Tracer experiments with labelled compounds have confirmed this pathway wherein common amino acids and other small closely related molecules are the basic skeletons for alkaloids (34). Amino acids provide both of the major reactants needed for alkaloid biosynthesis, i.e., amines and aldehydes. These compounds undergo simple types of condensation reaction to form more complex molecules. Examples of precursor amino acids include ornithine, phenylalanine, and tryptophan (26).

Classification and Uses of Cocaine

Cocaine, the pharmacologically active alkaloid of Erythroxylon coca, is classified as a tropane alkaloid. The tropane alkaloids are divided into two main groups: those found in some genera of the Solanaceae or in the genus Erythroxylon. Solanaceous genera which contain tropane alkaloids include Atropa, Datura, Duboisia, Hyoscyamus, and Scopolia. Species of Erythroxylon are the only known sources of cocaine (33). The basic tropane skeleton is nortropane (Fig. 1) (azabicyclo-3,2,1 octane) (14) and can be regarded as a pyrrolidine ring with a C_3 or C_4 chain attached across the α-carbons (C-1 and

C-5) (33). In cocaine: $R_1 = CH_3$ $R_2 = COOCH_3$ $R_3 = OCOC_6H_5$ (Fig. 2)

Cocaine was the first local anesthetic to be discovered, but it has largely been replaced in medical usage by safer synthetics such as Novocaine (procaine) and Xylocaine (lidocaine). However, these synthetics do not possess the unique vascoconstrictive properties of cocaine which made it valuable as a local anesthetic in various types of surgery (6). For example, cocaine is still used as a local anesthetic in many procedures in which the eye, nasal or pharyngeal mucous membranes are to anesthesized (16).

Biosynthesis of the Tropane Skeleton

The biosynthesis of the tropane alkaloids was studied soon after the introduction of radiotracer techniques, confirming the hypothetical pathway proposed by Sir Robert Robinson in 1917 (33):

Several related biosynthetic pathways were found:

(1) The pyrrolidine ring could be derived from several closely related molecules such as ornithine, glutamic acid, putrescine (33), and succinicdialdehyde (34). Ornithine, an amino acid, is readily interconverted to glutamic acid. Putrescine and n-methyl putrescine are derived from ornithine through decarboxylation and methylation (33). Succinicdialdehyde may arise by the oxidative decarboxylation of ornithine or by the reduction of succinic acid. Several cyclic intermediates are possible but such intermediates are not known (34). One possibility is hygrine, which frequently occurs with tropane alkaloids (33).

(2) The C_3 or C_4 portion attached to the pyrrolidine ring is evidently derived from two molecules of acetate. In the Solanaceous tropane alkaloids, the carbon atom at the C_2 position is lost, while in the Erythroxylaceae, the carbon atom is retained as a carboxyl group (33) (Fig. 3).

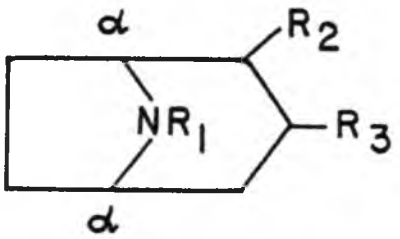

Figure 1. Tropane Skeleton.

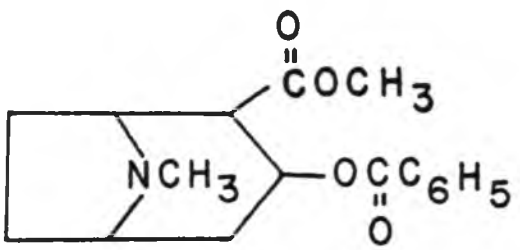

Figure 2. Cocaine.

Figure 3. Basic skeletons of tropane alkaloids from the genus Erythroxylon and the family Solanaceae.

In Vitro Synthesis of Alkaloids

Reports on alkaloid production in tissue culture have been diverse. Many factors have been shown to affect both the composition and level of alkaloids in culture such as the degree of differentiation of the cells (4, 43), addition of plant growth regulators as auxins and cytokinins to the culture medium (40), feeding of alkaloid precursors (9), variety or species used to establish the culture, (38) and the plant part used for callus initiation (36).

In cases where synthesis of secondary metabolites depends upon special biochemical and structural modifications of the cells, synthesis may not be possible unless these specialized conditions and structures can be induced in culture (10). Although cells contain all of the genetic information needed for biosynthesis of compounds found in the intact plant (25), this information may not be expressed unless the cells have reached a certain stage of biochemical or morphological differentiation (37).

Alkaloid production has been shown to be affected by the differentiation of callus tissue into organized structures such as roots or shoots, depending upon where the alkaloids are synthesized in the intact plant. In Atropa belladonna, atropine and other related alkaloids are synthesized mainly in the roots. Work on A. belladonna showed that alkaloids were synthesized only in cultured isolated roots, in callus derived from roots, or in seedling callus which had initiated roots (4, 43).

Morphological differentiation of callus may not be necessary for synthesis of alkaloids, but the alkaloid composition of the callus tissue in such cases often differs from that of the plant (23). Callus cultures derived from stem pith tissue of Nicotiana tabacum showed small amounts

of nicotine, but with the addition of kinetin and bud formation, nicotine production increased (40). However, high levels of nicotine were found in cultures of Nicotiana rustica in the absence of differentiation through the selection and establishment of a cell line capable of alkaloid biosynthesis in the absence of differentiation (38).

Alkaloid patterns in callus tissues may differ in several ways from that of the intact plant. Major alkaloids in the plant may be present in reduced amounts, be completely absent or be replaced by novel compounds in the callus tissue (2). Callus cultures derived from 11 species of Papaveraceae all showed similar alkaloid spectra, but upon regeneration of plantlets from the callus, the alkaloid patterns became more specific, resembling that of the intact plants. This seems to indicate that the cultured cells retain the ability to produce compounds found in the intact plant (22).

The addition of alkaloid precursors to the culture medium has been shown to increase alkaloid biosynthesis. The addition of tropic acid to rhizome callus of Scopolia parviflora increased the alkaloid content from 0.01 percent to 0.12 percent (39). Tropane alkaloid production was increased in Datura cultures by the addition of the amino acids ornithine and phenylalanine (9). In Datura innoxia suspension cultures, tropic acid, phenylpyruvate, and tropine increased the production of tropylesters (19). However, some precursors may be ineffective because particular biosynthetic enzymes are absent. For example, the addition of phenylalanine to Datura innoxia cultures did not stimulate alkaloid production because biosynthesis of tropic acid from phenylalanine seemed to be repressed (19).

The plant material or explant used to initiate cultures may have an effect on alkaloid content just after callus induction, but differences due to explant source are lost during successive transfers (36). It may be that in the early stages of culture different degrees of biochemical differentiation are retained in the cells. However, upon further subculture, this difference is lost as cells "de-differentiate" and return to a meristematic state (1).

In many cases, no variation in alkaloid composition was found in cultures derived from various plant parts (23, 38, 39). For example, callus cultures derived from petioles of Coptis japonica contained all of the alkaloids found in the rhizome of the intact plant (23). In Scopolia parviflora, cultures derived from stem and roots both produced the same low levels of alkaloids, although the alkaloid content in the rhizome is much higher in the intact plant (39). Cultures derived from the stem, root, capsule, and seedlings of eleven species of the Papaveraceae showed similar alkaloid spectra (22). The similarities in callus alkaloid spectrum may be due to de-differentiation whereby cells gradually return to similar states of morphological and biochemical differentiation (1).

Plant growth regulators have been shown to affect alkaloid biosynthesis. Auxins and cytokinins can regulate alkaloid production in tobacco callus tissue. For example, kinetin promoted nicotine production in tobacco callus in the absence of auxin (40). In another study, tobacco callus grown in the presence of 2,4-D showed better growth than callus grown in IAA media, but alkaloids were not detected in the 2,4-D callus. The IAA callus contained nicotine, anatabine, and anabasine. Callus which had been initiated and maintained on 2,4-D media and then

transferred to IAA media began to produce nicotine. Nicotine levels in the transferred callus rose with successive transfers (15). However, later work with the same tobacco variety showed that nicotine was produced at low levels of IAA, NAA, and 2,4-D. High levels of all three auxins inhibited alkaloid production. Hence both the quality and quantity of auxins affect alkaloid biosynthesis (36).

The regulatory effects of plant growth regulators on alkaloid biosynthesis may be direct or indirect. In cases where differentiation initiates or increases alkaloid production, the addition of growth regulators can promote biosynthesis by inducing cell differentiation (40). However, alkaloid production may not be directly linked to morphological differentiation but rather to biochemical differentiation since in some cases the need for organogenesis can be overcome by modification of the culture medium (15) or by selection of cell lines capable of producing alkaloids in the absence of differentiation (38).

Auxins control alkaloid biosynthesis in several ways. They may repress alkaloid production by promoting growth (13). In many cultures, increases in growth are often accompanied by decreases in alkaloid content (8). Alkaloid biosynthesis does not take place in many actively growing cells because precursors are diverted to other pathways involved in growth or because alkaloid biosynthetic enzymes are repressed during this stage (13). Auxins can also affect the pool of free amino acids available for alkaloid biosynthesis. In Nicotiana tabacum cultures, the addition of 2,4-D brought about changes in glutamic and aspartic acid, both of which are nicotine precursors (40).

MATERIALS AND METHODS

The coca plant, Erythroxylon coca Lam., belongs to the family Erythroxylaceae. It is extensively cultivated in South America, i.e., Peru, Bolivia, and Colombia, where the leaves are extracted for the pharmacologically active tropane alkaloid, cocaine, and other secondary alkaloids which are the main sources of cocaine (17).

Three varieties of E. coca were used in this experiment. Plants were grown under laboratory conditions. The three varieties used were: Trujillo (T), Cuzco (C), and Local (L). The L variety refers to E. coca collected at various sites in Honolulu: Lyon Arboretum, Foster Gardens, and the Marks Estate (27).

Establishment of Cultures

Terminal green stem pieces of E. coca were established under aseptic conditions for the production of callus. All visible leaves were removed from the stem pieces before they were surface sterilized. The sterilization procedure consisted of a 30 second dip in 10 percent Liquinox, followed by a wash in 95 percent ethanol. The explants were soaked in 15 percent Clorox for 15 minutes, followed by 10 percent Clorox for 10 minutes and were then rinsed several times with sterile distilled water. Both ends of the stem pieces were cut to remove dead tissue, and the explants were cut in one centimeter lengths. One explant was planted per tube, with about 1/4 - 1/2 of the explant remaining above the surface of the medium.

The medium used in all experiments was that of Murashige and Skoog (MS) at a 1/2 salt concentration (29). To the pre-mixed MS medium, the following organic constituents were added: sucrose (30 gm/liter), myo-inositol (100 mg/liter), nicotinic acid (1 mg/liter), pyridoxin·HCl (0.5 mg/liter), and thiamin·HCl (1 mg/liter) (Table 1). Agar was also added (8 gm/liter). In the establishment stage, the following hormones and growth regulators were used: indoleacetic acid (IAA), napthaleneacetic acid (NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D), each at a concentration of 5.0 μM. Since preliminary work showed that kinetin had little effect on callus formation, kinetin was kept at a constant level of 0.4 μM in all experiments.

The pH of the medium was adjusted to 5.7 - 5.8, and 20 ml portions were dispensed into 60 ml test tubes. The medium was sterilized by autoclaving for 15 minutes at 121°C. In the 2,4-D and NAA treatments, the growth regulators were added to the medium before autoclaving. The IAA treatments were prepared by filter sterilization.

All three varieties were established on the three auxins. Each treatment contained 20 - 25 test tubes. The cultures were kept in an environmental growth chamber for 8 weeks under dark conditions. The temperature was maintained at 25 ± 2°C. The design in all experiments was a completely randomized design (CRD).

First Transfer of Callus to Different Auxin Concentrations

The callus produced in the initial experiment was subsequently transferred to different levels of the same auxin used for establishment of the callus. In this manner, callus initiated on the 2,4-D medium at a concentration 5.0 μM was transferred to media containing 2,4-D at

Table 1

Modified Murashige and Skoog Medium (1/2 Salt Concentration)

Component	mg/l
$CaCl_2 \cdot 2H_2O$	220
$CoCl_2 \cdot 6H_2O$	0.0125
$CuSO_4 \cdot 5H_2O$	0.0125
$FeSO_4 \cdot 7H_2O$	13.9
H_3BO_3	3.1
KH_2PO_4	85
KI	0.415
KNO_3	950
$MgSO_4 \cdot 7H_2O$	185
$MnSO_4 \cdot 4H_2O$	111.5
$Na_2MoO_4 \cdot 2H_2O$	0.125
$Na_2 \cdot EDTA \cdot 2H_2O$	18.65
NH_4NO_3	825
$ZnSO_4 \cdot 7H_2O$	4.3
Sucrose	30,000
m-Inositol	100
Thiamin · HCl	1
Pyridoxin · HCl	0.5
Nicotinic acid	1
Agar	8,000

μM concentrations of 0.0, 1.25, 2.5, etc. This was also done for the NAA and IAA induced cultures. The concentrations used for all three auxins were: 0.0, 1.25, 2.5, 5.0, 10.0, 20.0, and 40.0 μM. Kinetin was kept at a constant level of 0.4 μM in all treatments.

In this experiment, the auxins were added to the media by cold sterilization using dimethyl sulfoxide (DMSO) (35). The hormone solutions were prepared by dissolving the proper amounts of each hormone in one ml of DMSO. The auxins were added to the autoclaved medium by means of a micropipette in 50 μl aliquots per 250 ml of medium. The medium was then poured into sterile test tubes.

The transferred callus pieces were approximately 2 x 2 mm in size. The cultures were kept in an environmental growth chamber under dark conditions, and the temperature was maintained at 25 ± 2°C. At the end of seven weeks, the cultures were rated for growth and color. The rating system for growth consisted of five classes, with 1 indicating superior growth, 3, average growth, and 5, poor growth. For the color ratings, an arbitrary scale was devised whereby 1 indicated little or no pigmentation, 3, average pigmentation, and 5, extensive pigmentation.

Second Transfer of Callus to Different Auxin Concentrations

The callus from the second experiment was transferred to fresh media with the same levels of salts, organic compounds, and hormones. The growth regulators were added to the autoclaved media by cold sterilization, and the size of the transferred callus pieces was 2 x 2 mm. In this experiment, the cultures were kept under two different light conditions: 24 hr dark and 12 hr light/12 hr dark. The lights used were cool-white fluorescent lights. The temperature was maintained at 25 ± 2°C. At the

end of seven weeks, the cultures were rated for color and fresh and dry weights were taken. The callus tissues were dried by freeze drying. The callus was also analyzed for the presence of alkaloids.

Alkaloid Analysis

The presence of the tropane alkaloid, cocaine, and other related alkaloids in the callus tissue of E. coca was studied. The alkaloids present in the leaf and green stem tissues of the plant were also analyzed.

Alkaloid Extraction

The procedure used for the alkaloid extraction was that of Holmstedt et al. (20), with modifications made in the organic solvents used for extraction and in preparation of the final solutions.

Two hundred mg (0.2 gm) of plant material was macerated with 20 ml of 95 percent ethanol. The mixture was heated at 75°C for 1.5 hours and left at room temperature overnight. The plant material was filtered off and washed with an additional 15 ml of EtOH. The EtOH solutions were combined and diluted with 60 ml of H_2O. The pH of the solution was adjusted to within a range of 9 - 10 by the addition of $NaHCO_3$. The EtOH solution was then immediately extracted three times with 20 ml of methylene chloride. The methylene chloride portions were retained and dried overnight over anhydrous Na_2SO_4. The methylene chloride was evaporated under a stream of nitrogen and ethyl acetate added. The ethyl acetate solution was concentrated with nitrogen to a volume suitable for analysis by gas chromatography. Figure 4 shows the flow diagram of sample preparation.

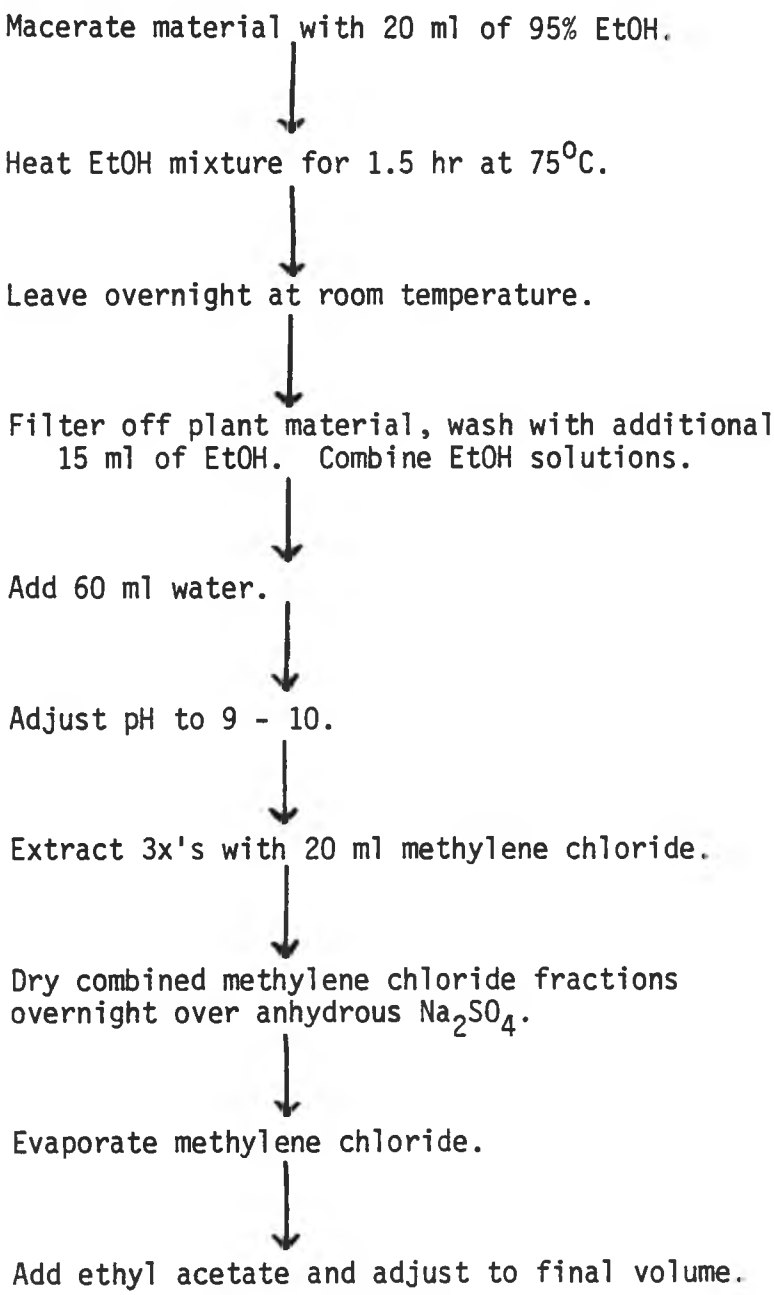

Figure 4. Diagram of extraction procedure.

Gas Chromatography

The procedure used for the alkaloid analysis by gas chromatography was that of Aynilian et al. (3), with modifications made in the column packing material and detector used.

A gas chromatograph (Varian Aerograph) equipped with a Coulson electrolytic conductivity detector (Tracor) was used for the alkaloid analysis. The detector was specific for nitrogen-containing compounds. Nitrogen is reduced in the detector furnace to ammonia, NH_4^+, before passing through the detecting cell. The presence of an ionized species such as ammonia in the detecting cell alters the current across two electrodes, thus producing a peak above the baseline signal.

A 3 meter coiled glass column with an inner diameter of 2 mm was packed with GP 3% SP-2250 DB on 100/120 Supelcoport (Supelco, Inc.) packing material. The column temperature was 175°C, and the injector port was heated to above 200°C. The furnace temperature was set at 800 - 820°C. The carrier gas was hydrogen at a flow rate of 40 ml/min. Before passing through the furnace, the effluent from the chromatograph was vented with hydrogen. The flow rate of the vent gas was 100 ml/min. The reactor gas was hydrogen at a flow rate of 30 ml/min. The sample size used was 5 µl.

For column packing, the column was cleaned before use by flushing with soap and water, followed by a tap water rinse. Dilute base (NaOH, 1:10, w/v) was run through the column, followed by distilled water. The pH of the column at this point should be either neutral or slightly basic. Acetone was flushed through the column, which was then dried under a stream of nitrogen. The column surface was further deactivated by silanization using trimethylchlorosilane (TMCS). A 1/10 strength

TMCS-toluene solution was used for this purpose. Silanization improves the performance of the column by tying up active silanol groups on the support surface (21). After drying, one end of the column was plugged with glass wool and attached to an aspirator. A small funnel was attached to the open end, and prepared packing material was poured slowly into the column. When the column appeared full, it was tapped manually with a metal rod and filled again. An electric drill was then used to gently vibrate the column to settle the packing material. After adding more packing, the open end of the column was plugged with glass wool. The column was conditioned for several days at 200 - 210^{o}C before use to remove volatile contaminants from the support surface and packing material.

The deionized water circulating through the detecting cell should be kept at a slightly basic pH (7.4). If the water becomes acidic, titration of the sample occurs and negative peaks result from decreased conductivity across the cell electrodes. The pH of the water may be affected by several factors. Halogenated compounds in the sample or solvent can cause the water to become acidic. The ion exchange resin may not be adequately purifying the water circulating through the cell. An acid scrubber in the detector may help to trap acidic compounds and keep the pH basic (41).

Quantitative Analysis

For quantitative analysis of the cocaine contained in the plant and callus samples, a standard curve was established by plotting the peak areas of cocaine standards versus concentrations (Fig. 5). Because samples were run on different days, peak areas were adjusted for changes in machine sensitivity before determining concentrations.

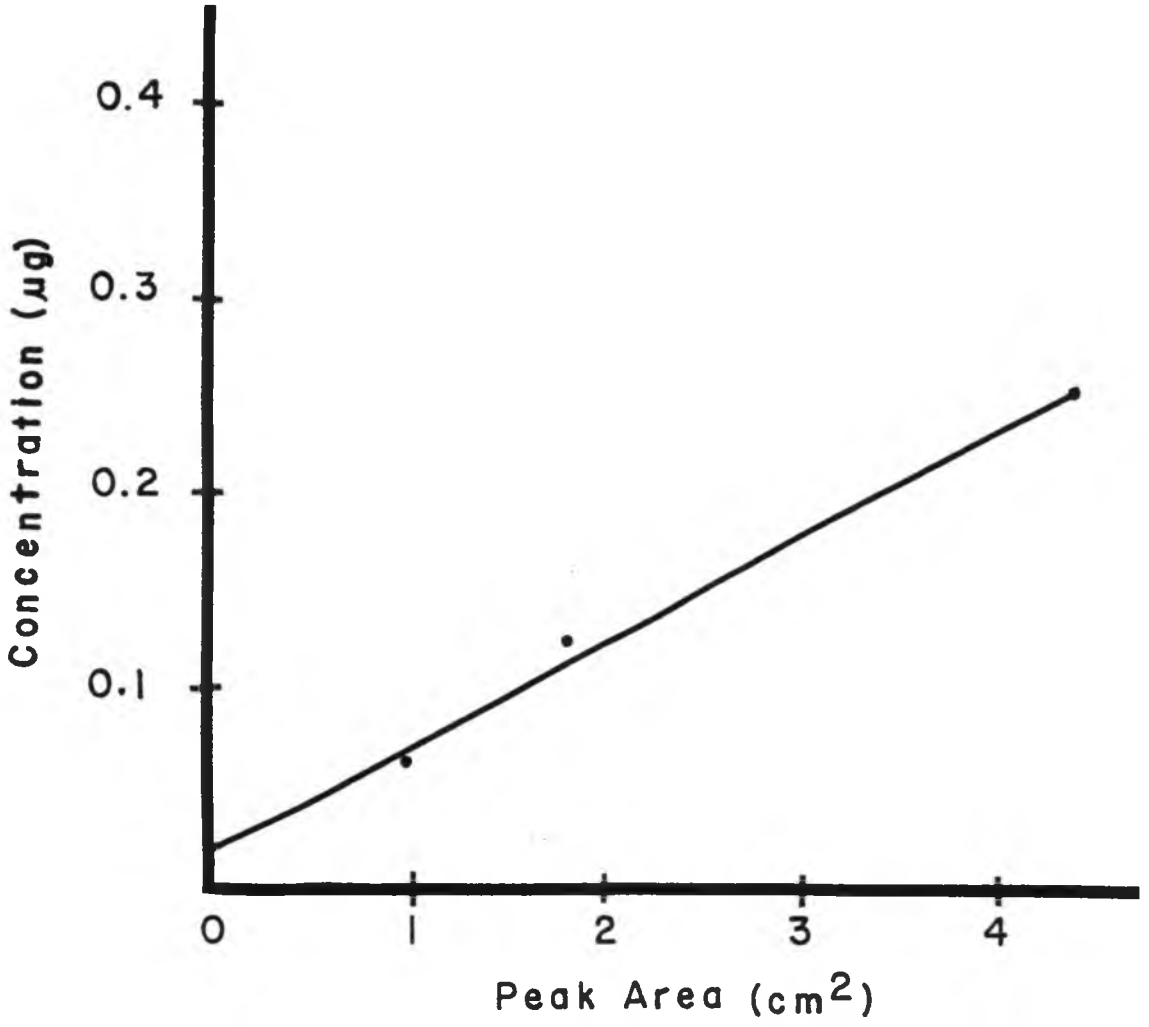

Figure 5. Plot of peak area versus concentration.

RESULTS

First Transfer of Callus to Different Auxin Concentrations

Analysis of the growth ratings of the callus produced from the first transfer showed that all of the main factors significantly affected callus growth (Table 2). Among the auxins, 2,4-D produced the best growth for all varieties over all auxin concentrations used. NAA and IAA produced significantly less callus growth. Variety L was the best overall variety, producing more callus growth than either variety C or T.

The only significant interaction in this experiment was that of auxin x variety (Table 3). The relative performances of the three varieties differed with the auxin used. In the 2,4-D treatments, varieties L and C were not significantly different and produced significantly more callus than variety T. In the NAA treatments, the performance of all three varieties was significantly different. In this case, variety L produced the most callus, followed by variety T. In the IAA treatments, L and C were the best varieties and were not significantly different. Variety C also did not differ significantly from variety T.

The IAA concentrations were probably too low to induce and support good callus growth since there were no significant differences in growth between the various IAA concentrations. However, there was a significant difference between the IAA treatments and the control. Higher concentrations of IAA should probably be used in both the establishment

Table 2

The Effect of Auxin, Auxin Levels, and Varietal Differences on Callus Growth

Main Effect		Growth Rating [y,z]
Auxin	2,4-D	2.7 a
	NAA	3.3 b
	IAA	3.8 c
Auxin level (μM)	0.0	4.6 d
	1.25	3.5 c
	2.5	3.3 bc
	5.0	3.1 ab
	10.0	3.0 a
	20.0	3.1 ab
	40.0	3.0 a
Variety	Local	3.0 a
	Cuzco	3.2 b
	Trujillo	3.6 c

[z]Mean separation by Duncan's multiple range test, 5% level.

[y]Growth ratings: 1 = excellent, 3 = average, 5 = poor.

Table 3

The Effect on Auxin x Variety Interactions on Callus Growth

Auxin	Variety[x]	Growth Rating[y,z]
2,4-D	L	2.8 a
2,4-D	C	2.9 a
2,4-D	T	3.2 b
NAA	L	3.2 b
NAA	C	4.1 f
NAA	T	3.5 c
IAA	L	3.6 cd
IAA	C	3.8 de
IAA	T	3.9 ef

[z]Mean separation by Duncan's multiple range test, 5% level.

[y]Growth ratings: 1 = excellent, 3 = average, 5 = poor.

[x]L = Local, C = Cuzco, T = Trujillo.

and growth stages, as insufficient material was available for transfer and 2,4-D induced callus was used for the IAA treatments.

The callus color ratings show that under 24 hr dark conditions, the 2,4-D cultures were less pigmented than the NAA and IAA treatments (Table 4). The increased pigmentation or browning in the NAA and IAA treatments was probably due to poor growth and cell death.

Second Transfer of Callus to Different Auxin Concentrations

The fresh weight data from the second callus transfer was transformed before analysis by a log transformation. All analyses and mean separations were on the transformed data but untransformed values are presented. The experiment was analyzed as a factorial in a completely randomized design.

All of the main factors significantly affected callus growth (Table 5). Among the auxins, 2,4-D produced better callus growth than either NAA or IAA. Auxin concentrations also significantly affected callus growth. There were significant differences in the responses of the three varieties. Variety L showed better callus growth over all treatments than either varieties C and T. Environmental factors were also important as callus maintained under 12 hr light/12 hr dark conditions grew significantly better than callus grown under 24 hr dark conditions.

The effect of auxin treatments on callus fresh weight is shown in Table 6. The 2,4-D treatments at concentrations of 10.0, 20.0, and 40.0 μM were significantly better than all NAA and IAA treatments. The IAA levels were probably not high enough to support callus growth, since all IAA levels did not differ significantly from the control. However, in the first callus transfer, significant differences were found between

Table 4

The Effect of Auxins and Varieties on Callus Color Under 24 Hour Dark Conditions

Auxin	Variety[x]	Color Rating[y]
2,4-D	L	2.8
2,4-D	C	2.9
2,4-D	T	3.2
NAA	L	3.2
NAA	C	4.1
NAA	T	3.5
IAA	L	3.6
IAA	C	3.8
IAA	T	3.9

[y]Color rating: 1 = little or no pigmentation
3 = average pigmentation
5 = extensive pigmentation

[x]L = Local, C = Cuzco, T = Trujillo

Table 5

The Effect of Auxin, Auxin Levels, Varietal Differences, and Environment on Callus Fresh Weight

Main Effect		Fresh Weight[z] (gm)
Auxin	2,4-D	0.2018 a
	NAA	0.0494 b
	IAA	0.0103 c
Auxin Level (μM)	0.0	0.0060 d
	1.25	0.0206 d
	2.5	0.0705 c
	5.0	0.0634 c
	10.0	0.1299 b
	20.0	0.1763 a
	40.0	0.1369 b
Variety	Local	0.1148 a
	Cuzco	0.0818 b
	Trujillo	0.0735 c
Environment	12 hr light/12 hr dark	0.1339 a
	24 hr dark	0.0525 b

[z]Mean separation by Duncan's multiple range test, 5% level.

Table 6

The Effect of Auxin Treatments on Callus Fresh Weight

Level (µM)	Auxin	Fresh Weight[z] (gm)
0.0	2,4-D	0.0070 f
1.25	2,4-D	0.0391 e
2.5	2,4-D	0.1733 c
5.0	2,4-D	0.1936 c
10.0	2,4-D	0.3443 a
20.0	2,4-D	0.3457 a
40.0	2,4-D	0.2728 b
0.0	NAA	0.0061 f
1.25	NAA	0.0125 ef
2.5	NAA	0.0187 ef
5.0	NAA	0.0201 ef
10.0	NAA	0.0308 ef
20.0	NAA	0.1545 c
40.0	NAA	0.0948 d
0.0	IAA	0.0041 f
1.25	IAA	0.0076 f
2.5	IAA	0.0084 f
5.0	IAA	0.0121 ef
10.0	IAA	0.0098 ef
20.0	IAA	0.0117 ef
40.0	IAA	0.0161 ef

[z]Mean separation by Duncan's multiple range test, 5% level.

the IAA treatments and the control. This may have been due to carry-over effects, as 2,4-D induced callus was used in some of the IAA treatments.

The auxin x variety interactions show that the relative performances of the three varieties was not the same when different auxins were used (Table 7). In the 2,4-D and NAA treatments, variety L performed significantly better than varieties C and T. However, in the IAA treatments, there were no significant differences in the callus production of the three varieties. There were also differences in the responses of varieties C and T to 2,4-D and NAA. In the 2,4-D treatments, varieties C and T were not significantly different, but in the NAA treatments, variety C produced significantly more callus than variety T.

In the environment x variety interactions, the relative performances of the three varieties differed under light/dark and dark conditions (Table 8). Under light/dark conditions, variety L produced significantly more callus than either variety T or C. However, under dark conditions, variety C performed significantly better than either variety L or T.

The auxin x environment interactions show that significant differences in growth between light/dark and dark cultures only occurred when 2,4-D was used (Table 9). In the NAA and IAA treatments, there were no significant differences in growth in cultures maintained under light/dark or dark conditions.

The callus color ratings show that the 2,4-D cultures were less pigmented than the NAA and IAA cultures under both light/dark and dark conditions (Table 10). Pigmentation was greater at lower concentrations of 2,4-D and in the NAA and the IAA cultures. The IAA callus under light/dark condtions was slightly more pigmented than the IAA callus under

Table 7

The Effect of Auxin x Variety Interactions on Callus Fresh Weight

Auxin	Variety[y]	Fresh Weight[z] (gm)
2,4-D	L	0.2537 a
2,4-D	C	0.1663 b
2,4-D	T	0.1937 b
NAA	L	0.0792 c
NAA	C	0.0528 d
NAA	T	0.0149 e
IAA	L	0.0094 e
IAA	C	0.0150 e
IAA	T	0.0063 e

[z]Mean separation by Duncan's multiple range test, 5% level.

[y]L = Local, C= Cuzco, T = Trujillo.

Table 8

The Effect of Environment x Variety Interactions on Callus Fresh Weight

Environment	Variety[y]	Fresh Weight[z] (gm)
L/D	L	0.1882 a
L/D	T	0.1246 b
L/D	C	0.0950 b
Dark	L	0.0540 d
Dark	T	0.0327 e
Dark	C	0.0703 c

[z]Mean separation by Duncan's multiple range test, 5% level.

[y]L = Local, T= Trujillo, C = Cuzco.

[x]L/D = 12 hr light/12 hr dark, Dark = 24 hr dark.

Table 9

The Effect of Auxin x Environment Interactions on Callus Fresh Weight

Auxin	Environment[y]	Fresh Weight[z] (gm)
2,4-D	L/D	0.3791 a
2,4-D	Dark	0.0889 b
NAA	L/D	0.0521 c
NAA	Dark	0.0468 c
IAA	L/D	0.0097 d
IAA	Dark	0.0110 d

[z]Mean separation by Duncan's multiple range test, 5% level.

[y]L/D = 12 hr light/12 hr dark, Dark = 24 hr dark.

Table 10

The Effect of Auxins and Varieties on Callus Color Under Two Different Light Conditions

Auxin	Variety[x]	Color Rating Means[y] 24 hr dark	12 hr light/12 hr dark
2,4-D	C	3.4	3.2
2,4-D	L	3.5	2.5
2,4-D	T	3.6	3.2
NAA	C	4.0	4.5
NAA	L	4.0	4.0
NAA	T	4.1	4.0
IAA	C	4.4	4.9
IAA	L	4.0	4.6
IAA	T	4.1	4.4

[x]Varieties: L = Local, C = Cuzco, T = Trujillo

[y]Rating scale: 1 = little or no pigmentation
3 = average pigmentation
5 = extensive pigmentation

dark conditions. This may be due to the increased breakdown of IAA under light conditions, resulting in decreased growth.

Summary of Growth Experiments

All of the main factors, auxins, auxin levels, environmental conditions, and varieties influenced callus growth. 2,4-D was the most effective auxin, producing significantly more callus fresh weight than either NAA or IAA. Light/dark conditions were better for callus production than dark conditions, and 2,4-D had the most significant effect on callus growth under both environmental conditions. Although variety L was the best overall variety, a breakdown of the analysis showed that the callus production of variety T at 2,4-D levels of 10.0, 40.0, and 5.0 μM under light/dark conditions did not differ significantly from that of variety L at 2,4-D concentrations of 20.0, 5.0, 10.0, and 40.0 μM under light/dark conditions. Thus, under the proper environmental conditions and with adequate auxin levels, variety T is able to perform as well as variety L.

The various interactions show that the relative performances of the three varieties differed with the auxin or the light conditions used. Because the relative performances of the varieties was not constant, the performances of the varieties under one set of cultural conditions cannot be used to predict their behavior under another set of conditions.

Alkaloid Analysis

Callus obtained from the growth experiments were analyzed for the presence of cocaine. The cocaine standard was obtained in the form of cocaine hydrochloride from Stepan Chemical Company. The retention time (R_t) of the cocaine standard was 6.5 minutes under the operating

conditions described. Besides cocaine, other volatile nitrogen-containing compounds were found in the callus and plant samples. Although standards were not available for comparison, these compounds were assumed to be other volatile alkaloids because a number of nitrogen-containing intermediates are possible in the biosynthesis of cocaine (33). Some of these compounds may also be artifacts caused by the extractive or chromatographic conditions.

To test the stability of the cocaine standard under the extractive and chromatographic procedures used, a known amount of cocaine (1.0 mg/10.0 ml) was extracted using the procedure described for plant material. The resulting chromatogram included three peaks with the following relative retention times (RR_t): Peak 3, RR_t = 0.23, Peak 8, RR_t = 1.00 (cocaine), and Peak 12, RR_t = 2.81 (Fig. 6). The relative peak percentages were also determined: Peak 3 = 13.6%, Peak 8 = 78.4%, and Peak 12 = 7.9%. The cocaine content of the plant and callus tissues was adjusted to correct for the degradation of cocaine with the analytical procedures used.

Besides degradation, the hydrolysis of cocaine by weak acids could affect both quantitative and qualitative results. Cocaine is readily hydrolyzed to ecgonine, benzoic acid, and methanol (14). Hydrolyzed nitrogen-containing derivatives of cocaine which are formed during the extraction process may not be detected because of the basic nature of the packing material. The packing would absorb acidic cocaine degradation products.

Alkaloid Content of Callus and Plant Tissues

Many of the volatile alkaloids found in the stem and leaf tissues of the plant were also found in the callus (Tables 11, 12). The callus

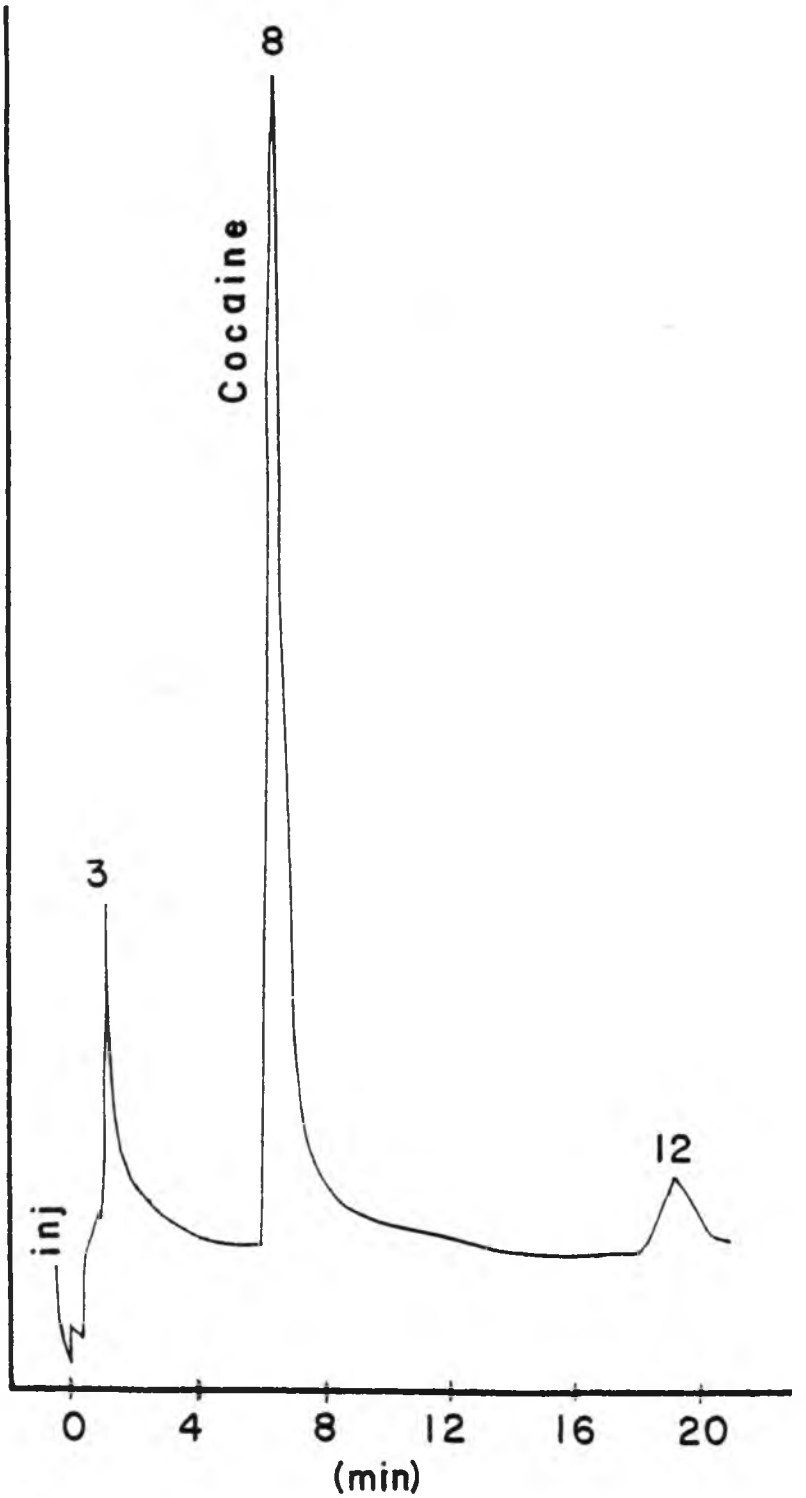

Figure 6. Chromatogram of extracted cocaine standard. Peaks other than cocaine peak were not identified. Peak numbers refer to Table of Relative Retention Times. Attenuation x2.

Table 11

Relative Retention Times of Nitrogen-Containing Compounds of *E. coca*.

Peak No.	Plant Samples[x] A	B	C	D	E	F	Std Cocaine
1					0.14		
2	0.16	0.17	0.16	0.16		0.17	
3	0.24	0.24	0.24	0.24	0.23	0.22	
4			0.28	0.28	0.27	0.26	
5	0.38	0.37	0.40	0.40			
8	1.00	1.00	1.00	1.00	1.00	1.00	1.00
11	2.70	2.67	2.68	2.76	2.62	2.70	
	n.s.[y]	n.s.	n.s.	n.s.	n.s.	n.s.	
13	4.70	4.68	4.65	4.74	4.54	4.58	

Retention times are averages of 3 runs, except for sample F, 2 runs.

[y]Peak not separated from previous peak.

[x]A = Variety T, leaf
B = Variety T, stem
C = Variety C, leaf
D = Variety C, stem
E = Variety L, leaf
F = Variety L, stem

Table 12

Relative Retention Times of Nitrogen-Containing Compounds of Callus Tissue of E. coca.

Peak No.	Callus Samples[y] A	B	C	D	E	F	Std Cocaine
1		0.15	0.15	0.15			
2	0.17	0.18			0.18		
3	0.23	0.22	0.23	0.23	0.22	0.23	
6	0.73						
7	0.85	0.79		0.81	0.81	0.81	
8	1.00	1.00	1.00	1.00	1.00	1.00	1.00
9	1.12						
10	2.43	2.41	2.54	2.51	2.50	2.53	
11	2.75	2.73					
12			2.88	2.86	2.86	2.86	

Retention times are averages of 3 runs, except for sample C, 2 runs.

All samples run on 02-11-81, except A on 02-05-81.

[y]Treatments:

A = 40.0 µM NAA, 12 hr light/12 hr dark (L/D), Variety L.
B = 40.0 µM 2,4-D, L/D, Variety L.
C = 5.0 µM 2,4-D, L/D, Variety L.
D = 10.0 µM 2,4-D, 24 hr dark (Dark), Variety C.
E = 40.0 µM 2,4-D, L/D, Variety C.
F = 40.0 µM 2,4-D, L/D, Variety T.

also contained compounds not found in the plant. However, while the callus was able to synthesize some of the alkaloids found in the whole plant, the concentration and relative proportions of the alkaloids differed between the plant and callus tissues. Cocaine was the major alkaloid in the leaf tissues, with concentrations ranging from 0.17-0.38% (Table 13 and Fig. 7) but was a minor alkaloid in the callus samples, with concentrations of 0.0004 - 0.003% (Table 14 and Fig. 9). The cocaine concentrations in the callus tissues were about 10 to 100 times less than in the original stem explants. The stems also contained less cocaine than the leaves (Table 13 and Fig. 8).

In the callus samples, the major peaks were found at: RR_t = 0.22 - 0.23 (Peak 3) and RR_t = 2.86 - 2.88 (Peak 12 and Fig. 9). These peaks were also present in the plant samples and correspond to the peaks found in the extracted cocaine standard but were not the major peaks in these samples. The large, early peak (Peak 3) may be composed of one compound or of several overlapping compounds. This peak cannot be entirely attributed to the formation of artifacts during the extraction process since the plant samples showed large cocaine peaks and relatively little degradation.

There were some differences in the cocaine content of callus derived from different varieties and maintained under varied cultural conditions (Table 14). Callus derived from variety L and maintained on 40.0 μM 2,4-D under light/dark conditions had about 10 times less cocaine than the other callus samples. However, none of the callus samples contained cocaine at levels approaching those found in the plant tissues. Different auxins and auxin levels also seemed to have no major effects on cocaine concentrations or on the alkaloid patterns of the callus tissues.

Table 13

Cocaine Content of *E. coca* Leaf and Stem Tissues

Variety	Tissue	Dry Weight (mg)	Total Cocaine (mg)	Cocaine[x] %
T	Leaf	378.0	1.43	0.38
T	Stem	475.6	0.23	0.05
C	Leaf	354.6	0.61	0.17
C	Stem	331.5	0.12	0.04
L	Leaf	411.0	1.21	0.29
L	Stem	380.0	0.18	0.05

[x]Cocaine concentrations are the averages of 3 runs. Concentrations were corrected for degradation.

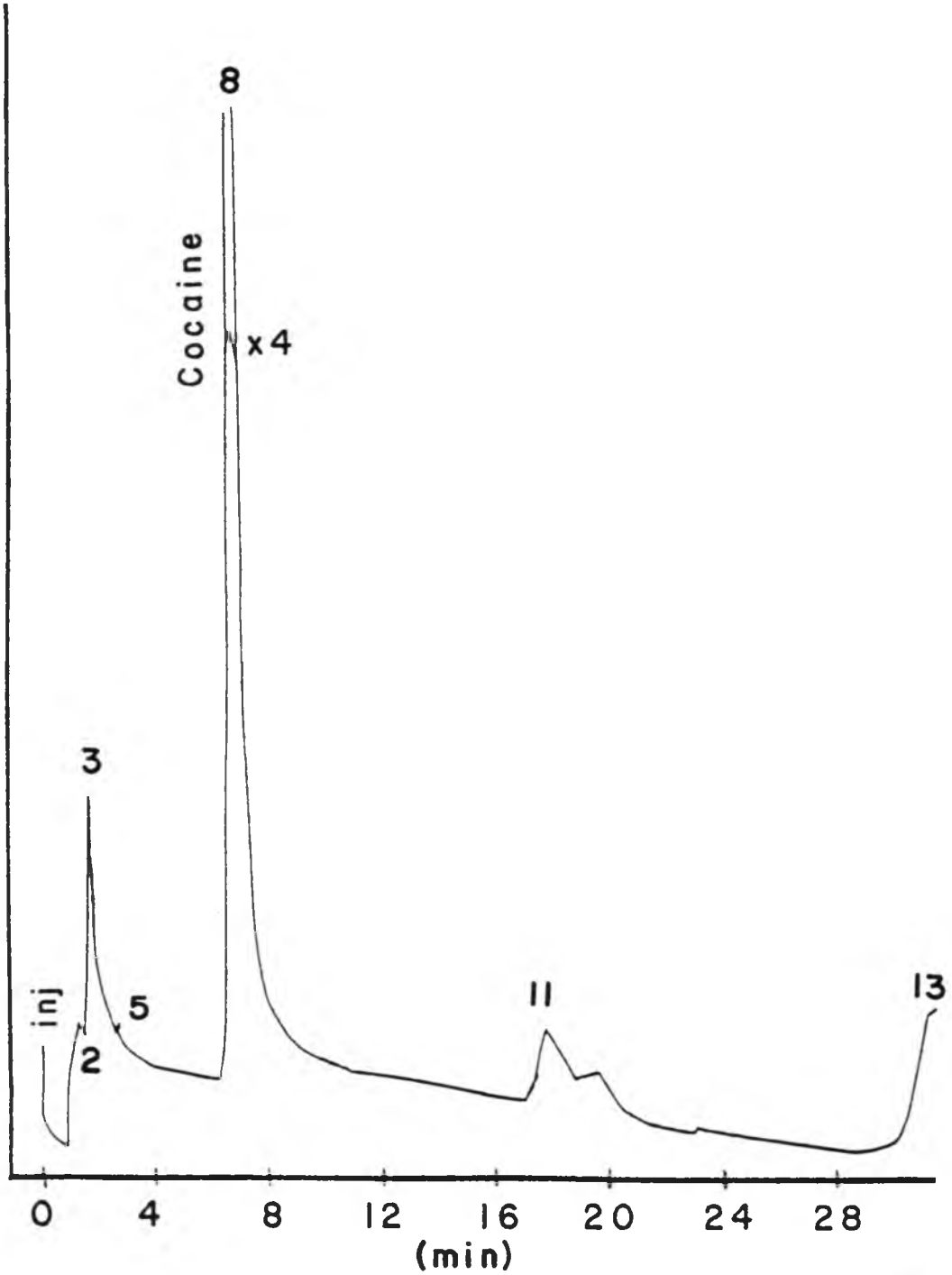

Figure 7. Chromatogram of nitrogen-containing compounds of E. coca 'Trujillo' leaf tissue. Peaks other than cocaine peak were not identified. Peak numbers refer to Table of Relative Retention Times. Attenuation x2.

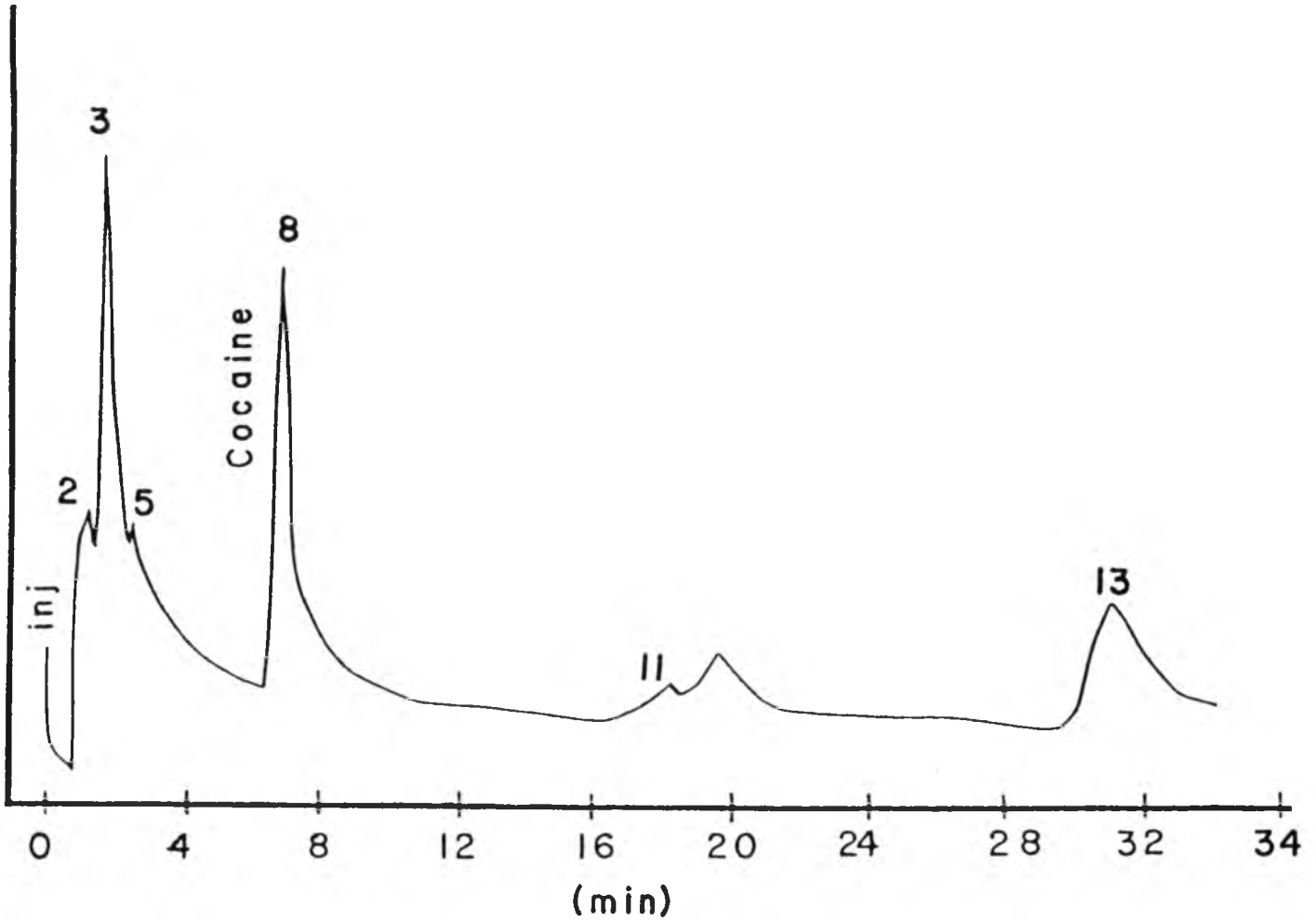

Figure 8. Chromatogram of nitrogen-containing compounds of E. coca 'Trujillo' stem tissue. Peaks other than cocaine peak were not identified. Peak numbers refer to Table of Relative Retention Times. Attenuation x2.

Table 14

Cocaine Content of E. coca Callus Tissue

Callus Source	Dry Weight (mg)	Total Cocaine (mg)	Cocaine[y], %
40 µM NAA, L/D[x], Variety L	242.3	<0.0013	<0.001
40 µM 2,4-D, L/D, Variety L	274.0	<0.0013	<0.0004
5 µM 2,4-D, L/D, Variety L	189.2	<0.0013	<0.001
10 µM 2,4-D, Dark, Variety C	160.4	≈0.0013	≈0.001
40 µM 2,4-D, L/D, Variety C	179.9	≈0.0051	≈0.003
40 µM 2,4-D, L/D, Variety T	454.7	≈0.0026	≈0.001

[y]Cocaine concentrations are the averages of 3 runs. Concentrations were corrected for degradation.

[x]L/D = 12 hr light/12 hr dark, Dark = 24 hr dark.

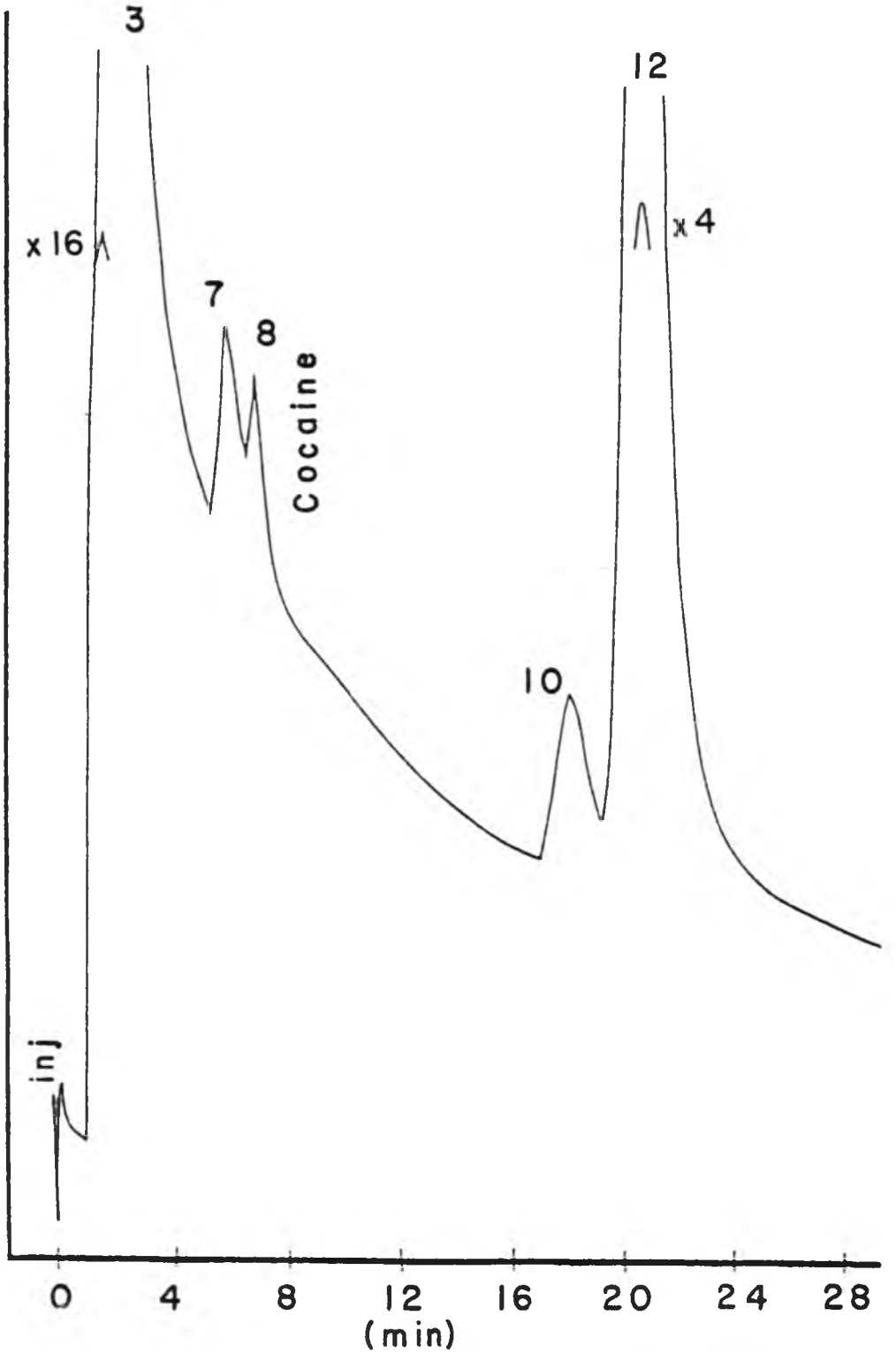

Figure 9. Chromatogram of nitrogen-containing compounds of callus derived from E. coca 'Trujillo' and cultured on 40.0 µM 2,4-D under 12 hr light/12 hr dark conditions. Peaks other than cocaine peak were not identified. Peak numbers refer to Table of Relative Retention Times. Attenuation x2.

DISCUSSION

The alkaloid composition of callus tissues may differ in several ways from that of the whole plant. Major alkaloids in the plant may be present in reduced amounts, be completely absent, or be replaced by new compounds in the callus (2, 5, 22). For example, callus cultures of Stephania cepharantha were not able to synthesize the main alkaloids of the plant but produced other alkaloids not found in the plant. This may have been due to the lack of specific enzymes controlling methylation and the formation of methylenedioxy groups in the callus tissue (2).

In the callus tissues of E. coca, cocaine (Peak 8) was not the major peak. The major peaks were Peaks 3 and 12. These peaks were also present in the extracted cocaine standard and may be artifacts resulting from the degradation of cocaine during extraction. However, these peaks cannot be entirely due to the degradation of cocaine since the plant samples had major cocaine peaks and relatively little degradation. Peaks 3 and 12 may be more prominent in callus tissues because biosynthetic enzymes found in the plant are either present in small amounts or are absent in the callus. Peak 3 may be a simpler, low molecular weight alkaloid related to cocaine which accumulates in callus tissues because the enzymes needed for either the carboxylation, oxidation, or esterification of the basic tropane skeleton are absent. Peak 12 may also be the result of the accumulation or diversion of cocaine precursors

into new biosynthetic pathways because of the lack of biosynthetic enzymes.

Alkaloid composition can also differ in that alkaloid patterns of callus tissues have been found to be simpler than those of the plant (22, 39). For example, callus derived from 11 species of the Papaveraceae all showed similar alkaloid patterns, although the original plants contained more specific alkaloids. Plantlets regenerated from the callus developed more specific alkaloid patterns which resembled those of the original plants (22).

Differentiation of callus into roots, shoots, or plantlets is necessary in some cases for the initiation of alkaloid production (4, 18, 43). In cultures of Atropa belladonna, atropine and other related alkaloids are only produced in cultured isolated roots, in callus derived from roots, or in seedling callus which had initiated roots (4, 43). Differentiation has also been shown to increase alkaloid levels in callus cultures (23, 40). In callus derived from tobacco stem pith tissue, nicotine levels rose with the differentiation of callus into buds (40).

In the case of E. coca, it appears that most of the alkaloids found in the plant can be synthesized when the cells are relatively undifferentiated, resulting in similar alkaloid patterns for the plant and callus tissues. However, qualitative and quantitative changes in alkaloid content would probably take place with differentiation of the callus into shoots since the leaves are the major source of cocaine.

Both the total alkaloid concentration and the cocaine content of the callus tissue were much lower than that of the plant leaf and stem tissues. The cocaine content of the leaves ranged from 0.17 - 0.38%,

while the callus contained cocaine at concentrations of 0.0004 - 0.003%. Reports of total alkaloid content of E. coca leaves in the literature vary from 0.5 - 1.5%, with about 50.0 - 80.0% of this being cocaine (3). The low alkaloid content of the E. coca callus tissue may be due to several factors. For example, although the undifferentiated callus was able to produce cocaine and other alkaloids found in the plant, differentiation may be needed to increase concentrations to levels found in the plant.

The analysis of the stem and leaf tissues showed that the leaves contained more cocaine than the stems. In this experiment, stems were used to initiate cultures because of the difficulty in surface sterilizing leaves and the high contamination rates which resulted when leaves were used. Cultures derived from leaf explants would probably have shown higher initial levels of cocaine than cultures derived from stems, but this difference would probably have disappeared during subsequent passages as cells de-differentiated (36). In tobacco callus cultures, the origin of the callus was important in the early stages of culture, but differences in nicotine content were lost between cultures during subsequent transfers (36). In other cases, no variation in alkaloid content was found in cultures derived from various plant parts (23, 38, 39). However, it would be advantageous to use leaves if high yielding cell lines were to be isolated and established from cultured cells, since cultures derived from leaves would probably contain more high yielding cells than cultures derived from stems (46).

Auxins have also been shown to regulate the alkaloid content of callus tissues. The addition of auxins may depress alkaloid biosynthesis by enhancing cell growth, thus causing the diversion of alkaloid

precursors into alternative, competing pathways (13), by the repression of alkaloid biosynthetic enzymes (13), or by affecting the free pool of amino acids available for alkaloid biosynthesis (40). In this experiment, there were some differences in the cocaine content of callus cultures maintained on different auxin concentrations. However, no conclusions can be drawn about the effect of auxin concentration on cocaine levels since cultures maintained on 40.0 μM 2,4-D showed a range of cocaine concentrations.

Although the callus samples did not contain cocaine at levels comparable to those found in the plant material, other secondary alkaloids may be present in the callus tissue which could be used for transformation into cocaine. The use of cell selection techniques could also increase alkaloid yields. Alkaloid production could be increased further by modifications of the media and cultural conditions (46).

SUMMARY

1. The cocaine content of E. coca leaves ranged from 0.17 -0.38%, while the stems contained cocaine at concentrations of 0.04 - 0.05%. The cocaine concentration of the callus was 10 - 100 times less than that of the original stem explants.
2. Many of the nitrogen-containing compounds detected in the plant were also found in the callus, indicating that the callus can synthesize some of the alkaloids found in the plant.

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