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Tissue Culture and Alkaloid Production of *Erythroxylum coca* var. *coca*

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Ingrid M. Fordham
William R. Lusby

**ABSTRACT.** A tissue culture method was developed to study the biosynthesis of alkaloids in *Erythroxylum coca* var. *coca*. Shoot cultures were established from excised embryos of seed from *Erythroxylum coca* var. *coca* and grown on a semi-solid medium. Alkaloids extracted from leaves of shoot cultures and the parent plant were identified by gas chromatography-mass spectrometric (GC/MS) analysis and quantified by gas chromatography. Shoot cultures that were developed from one *Erythroxylum coca* var. *coca* plant produced the major and minor alkaloids common to the species. The
cocaine levels of the shoot cultures were 50 percent of that produced by the parent plant but within the range reported for the species. Maximal levels of cocaine were produced in the leaves of shoot cultures within eight days after transfer to fresh media. The production of alkaloids in the shoot cultures demonstrates that roots are not necessary for tropane alkaloid production in *Erythroxylum coca* var. *coca*.

**KEYWORDS.** Cocaine, *trans*-cinnamoylcocaine, *cis*-cinnamoylcocaine, shoot culture, explants, tropane.

**INTRODUCTION**

Tropane alkaloids possess a tropane ring (a fused bicyclic amine combining pyrrolidine and piperidine rings). Several medicinally important plant alkaloids, such as scopolamine (hyosine), atropine (a racemic mixture of (R)- and (S)-hyoscyamine), and cocaine, are members of this class of compounds. Most of the recent advances in the enzymology of the tropane alkaloids hyoscyamine and scopolamine have been achieved through the combined use of tissue culture of solanaceous species, biosynthetic precursors, and metabolic inhibitors (2, 5, 10, 11, 15-17). While a chemical pathway for cocaine biosynthesis has been proposed, surprisingly little is known of the enzymology of this tropane alkaloid (2). As with other tropane alkaloids, progress on determining the enzymology of cocaine biosynthesis might be facilitated if a suitable *in vitro* system were available. We report here a protocol for the tissue culture of *Erythroxylum coca* var. *coca*, the major cocaine-producing species cultivated in South America (6), that can be used for *in vitro* studies of cocaine biosynthesis. Prior to this report, no methods for the tissue culture of cocaine-producing *Erythroxylum* species have been published (12).

**MATERIALS AND METHODS**

*Plant Material.* An *Erythroxylum coca* var. *coca* plant of Peruvian origin was started from seed in a 1.2-l pot filled with commercial
potting media (Promix BX®, Premier Brands, Inc., Stamford, CT). The plant was grown in a greenhouse, watered as needed, and fertilized (after seedling establishment) with 6 g of 19-6-12 (N-P₂O₅-K₂O) controlled-release fertilizer (Osmocote®, Grace-Sierra) every 3 months.

The in vitro cultures were established from seed taken from ripe fruit of this plant. The pulp was removed from the fruit and the seed surface disinfected in an aqueous solution of 1.05 percent sodium hypochlorite (20% Chlorox®) and 1% Tween 20®. The embryos were removed from the seeds under a stereomicroscope in a sterile laminar flow hood and immediately explanted vertically into 25 × 95 mm shell vials containing 10 ml of semi-solid medium, such that the cotyledons were above the medium surface. The medium was prepared by adding 50 g liter⁻¹ corn starch and 0.5 g liter⁻¹ Gelrite® (Kelco, San Diego) to modified Murashige and Tucker lemon fruit callus medium (4) containing half-strength MS salts (3) supplemented with 0.28 mM myo-inositol, 14.8 μM thiamine-HCl, 13.3 μM glycine, 20.3 μM nicotinic acid, 24.3 μM pyridoxin-HCl, 2.22 μM benzyladenine, 0.25 μM indolebutyric acid, 0.65 μM gibberellic acid (autoclaved, 75% K salt), and 58.4 mM sucrose. The mixture was heated with vigorous stirring until translucent, dispensed into shell vials, capped (Magenta Corp., Chicago, IL), and sterilized at 121°C and 138 kPa for 20 min.

When the second pair of true leaves emerged (approximately 10 to 14 days in culture) radicles and cotyledons were excised to promote shoot proliferation from cotyledonary and leaf axils. The proliferation of shoot tissue from these axes occurred within 5 weeks. Thereafter, tissue consisting of 15 to 20 shoots was transferred to fresh medium every 4 weeks. Cultures were grown at 27 ± 2°C under a 12-h photoperiod with a photosynthetic photon flux density of 100 ± 10 μmol m⁻²s⁻¹. Using this protocol, 4 shoot cultures (referred to as C₁, C₂, C₃, and C₄), each established from a separate seed, were produced. After transfer to fresh media, shoot cultures were grown from 4 to 12 days. At harvest, tissue from ten vials was combined, rinsed in deionized water, blotted dry, dipped into liquid nitrogen, freeze-dried, weighed, and stored no longer than one week at −20°C over silica gel. Leaves were separated from shoots prior to analysis for alkaloids.

For comparative studies of leaf alkaloids between the parent
plant and tissue cultures, the parent plant was stripped of its leaves and grown at a constant temperature of 27 ± 2°C and a 12-h photoperiod with a photosynthetic photon flux density of 100 ± 10 µmol m⁻² s⁻¹. Fully expanded leaves that developed under these growth conditions were harvested, frozen in liquid nitrogen, freeze-dried, weighed, and stored no longer than one week at −20°C over silica gel.

Preparation of Extracts. The freeze-dried leaf tissue was powdered in an A-10 analytical mill (Tekmar, Cincinnati, OH) and extracted with 20 ml HPLC grade methanol (MeOH) per 100 mg dry weight of tissue for 1 h at room temperature with shaking. For identification of alkaloid constituents, the extract was filtered through a Whatman No. 3 filter paper, diluted with 10 ml of deionized-distilled water, and placed under vacuum at room temperature to evaporate the methanol. The remaining aqueous solution was made acidic with 0.1 N sulfuric acid and washed with methylene chloride (MeCl₂). The aqueous phase was made basic with dilute ammonium hydroxide and extracted with MeCl₂. The MeCl₂ was evaporated to dryness under vacuum at room temperature and the residue resuspended in 1 ml MeOH containing 1 µg µl⁻¹ of 4-androstane-3,17-dione (internal standard).

Identification and Quantification of Alkaloids. For quantification of alkaloids, freeze-dried leaf tissue was prepared and extracted with MeOH as above, filtered, evaporated to dryness under vacuum at room temperature, and resuspended in 0.5 or 1 ml MeOH (for explant and parent plant leaf tissue, respectively) containing 1 µg µl⁻¹ of 4-androstane-3,17-dione.

Alkaloid identification was performed using GC/MS analysis. The electron impact (70 eV) mass spectra were obtained with a Finnigan-model 4510 gas chromatograph mass spectrometer operated at an indicated source temperature of 150°C, using helium as the carrier gas. Samples were introduced via an on-column injector and chromatographed on a 30 m by 0.25 mm (i.d.) DB-1 (dimethylsilicone) capillary column with a film thickness of 0.25 µm. Column temperature was maintained at 70°C for 2 min, then heated at 25°C min⁻¹ to 260°C for the duration of the analysis. Spectra from m/z 50 to 450 (1.0 second scan cycle) were collected by means of
an Incos data system. Background-subtracted spectra were compared with spectra of pure standards by means of the data system.

Quantitative analysis of extracts was performed on a Hewlett-Packard 5890A gas chromatograph equipped with a hydrogen flame-ionization detector, a 30 m by 0.25 mm (i.d.) dimethylsilylcone capillary column with a film thickness of 0.25 μm, and helium as the carrier gas. Inlet and detector temperatures were 285°C, column temperature was 70°C initially, increased 25°C min⁻¹ for 8.4 min, and held at 280°C for the final 8 min. The system was calibrated using varying concentrations of pure standards dissolved in MeOH containing 1 μg μl⁻¹ 4-androstane-3,17-dione.

The alkaloid standards used were cocaine hydrochloride (Sigma Chemical Co.), cis-cinnamoylcocaine (Department of National Health and Welfare, Ontario, Canada), and trans-cinnamoylcocaine (U.S. National Institute on Drug Abuse). Data were analyzed by single-factor analysis of variance, and significantly different means were separated at the 95 percent level or greater by Tukey’s Range Test.

**RESULTS**

While the genus *Erythroxylum* was initially observed to be incompatible with many standard and modified tissue culture media and also intolerant of several commercial gelling agents, the modified Murashige and Tucker lemon fruit callus medium (4) proved successful when gelled with corn starch and Gelrite for the establishment and maintenance of the proliferating-shoot cultures (Figure 1).

As determined by retention times and comparison with mass spectral data of pure standards and sample components, the 4 *in vitro* cultures that were derived from separate seeds from 1 *Erythroxylum coca* var. *coca* plant all produced the major and minor cocaine alkaloids common to the species (Figures 2 and 3). No significant differences among the 4 different shoot cultures as to the quantity of cocaine, cis-cinnamoylcocaine, or trans-cinnamoylcocaine produced in the leaf tissue were noted (Table 1). When averaged over all cultures, the cocaine content of the cultures was approximately 50 percent of that in the parent plant leaf tissue. Levels of cis-cinnamoylcocaine were equal to or less than trans-
Figure 1. Proliferating-shoot culture. Established from an excised embryo of an *Erythroxylum coca* var. *coca* seed grown on modified Murashige and Tucker medium (4).

cinnamoylcocaine levels in both the parent plant and shoot cultures. Shoot cultures, however, produced significantly higher levels of cinnamoylcocaines than the parent plant. While a trend in increased biomass continued for the 12 days in culture, cocaine levels in the cultures were maximal after 8 days of growth (Figure 4).


**DISCUSSION**

Cocaine levels in leaves of field and greenhouse-grown *Erythroxylum coca* var. *coca* have been reported to range from 0.13 to 0.68 percent and 0.23 to 0.96 percent on a dry weight basis with a mean of 0.63 and 0.73 percent, respectively (1, 7). The higher level of cocaine in the leaves of the parent plant (1.05%) over those of these previous studies may be due to differences in drying, press-
Cocaine

![Cocaine Mass Spectrum](image)

Tissue Culture
RT = 9.61

![Cis- or trans- Cinnamoylcocaine Mass Spectrum](image)

Tissue Culture
RT = 10.53 & RT = 11.37

Figure 3. Mass spectra of methanol extract. From peaks exhibited in Figure 2; RT = retention time.

Observation, and/or duration of storage before analysis. For example, in one study, preparation of leaf material and duration of storage was not reported (1), while in the other study the leaf material was stored for longer than a year before analysis (7). When averaged over all explants, the cocaine content of the cultures was 0.51 percent of dry weight, less than the parent plant, but well within the ranges reported for the species. The reason for the differences in cocaine content between leaves of the parent plant and those of the shoot cultures is unknown, but may be due to differences in leaf
Table 1. Alkaloid levels in leaves from *Erythroxylum coca* var. *coca*.

<table>
<thead>
<tr>
<th>Alkaloid</th>
<th>Parent</th>
<th>C₁</th>
<th>C₂</th>
<th>C₃</th>
<th>C₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cocaine</td>
<td>1.05 ± 0.01*</td>
<td>0.58 ± 0.04</td>
<td>0.52 ± 0.07</td>
<td>0.53 ± 0.01</td>
<td>0.42 ± 0.03</td>
</tr>
<tr>
<td><em>cis</em>-cinnamoyl-cocaine</td>
<td>0.08 ± 0.01*</td>
<td>0.24 ± 0.02</td>
<td>0.32 ± 0.05</td>
<td>0.26 ± 0.01</td>
<td>0.26 ± 0.02</td>
</tr>
<tr>
<td><em>trans</em>-cinnamoyl-cocaine</td>
<td>0.22 ± 0.04*</td>
<td>0.43 ± 0.02</td>
<td>0.36 ± 0.04</td>
<td>0.46 ± 0.01</td>
<td>0.37 ± 0.01</td>
</tr>
</tbody>
</table>

1. Parent plant and 4 separately established, 12-day-old tissue cultures.
2. Mean of 4 replicates ± s.e.; * = significantly different at the 0.01 level from other values in the same row.
development. Nevertheless, cultures produced maximum levels of cocaine within eight days of growth, demonstrating that the protocol described here would be useful for short-term studies of cocaine biosynthesis.

The level of trans-cinnamoylcocaine in the explant leaf tissue was higher than that reported for leaves of field- and greenhouse-grown *Erythroxylum coca* var. *coca* (7, 13). In addition, trans-cinnamoylcocaine was the predominant cinnamoylcocaine in all the leaf tissue of this study, opposite of what Plowman and Rivier (7) reported for leaves of field- and greenhouse-grown *Erythroxylum coca* var. *coca*. Rivier (9), however, demonstrated that young leaves contain higher levels of *cis-* than *trans*-cinnamoylcocaine and higher total cinnamoylcocaines than older leaves of *Erythroxylum coca* var. *coca*. Thus, differences in cinnamoylcocaines in the parent plant tissue and the culture tissue may represent differences in the development of the leaves. Whether both cinnamoylcocaines are biotically produced in the plant is in question. The exposure of an ethanolic
solution of *trans*-cinnamoylcocaine to sunlight resulted in the same ratio of isomers as that measured in the plant (2). Thus, alternatively, differences in light quality and quantity may account for the different cis:trans ratios reported in this and previous studies.

The site of tropane alkaloid biosynthesis in solanaceous plants is generally acknowledged to be in the roots with the shoots and leaves possessing the potential of further processing the alkaloids (14, 16). In fact, cultures of excised roots have become the preferred specimens used in studying the biosynthesis of tropane alkaloids (8, 16). The explants in this study were rootless; nevertheless, the leaves contained cocaine levels comparable to leaves of intact plants. Thus, unlike other plant species, roots are not required for the biosynthesis of tropane alkaloids in *Erythroxylum* spp.

The tissue culture method described here for *Erythroxylum coca* var. *coca* results in shoot cultures with uniform alkaloid production. Based on preliminary results with the metabolic inhibitor glyphosate, by introducing precursors and inhibitors of tropane synthesis into the media, this tissue culture method should prove useful in confirming the proposed biosynthetic pathway and establishing the enzymes that control cocaine biosynthesis in *Erythroxylum* spp.

REFERENCES


