ALKALOID PRODUCTION FROM *PAPAVER* TISSUE CULTURES

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ABSTRACT.—*Papaver bracteatum* was established as cell suspension culture, as suspension culture with callus grown roots, and as suspension culture containing embryoids and shoots. Alkaloid analysis using thin layer chromatography and reverse phase high pressure liquid chromatography detected the presence of thebaine (0.007%) in shoot cultures. *Papaver somniferum* cell suspension cultures and suspension cultures containing callus grown roots were established also. Cryptopine was isolated and identified by its mass spectrum.

Papaver somniferum L. is the most important source of the opium alkaloids codeine and morphine. *P. bracteatum* Lindl. is another important source of these alkaloids because the mature capsule contains up to 3.5% thebaine at a purity of 95% (1, 2). Thebaine is readily converted to codeine by standard chemical procedures. Tissue cultures of *Papaver* may provide an alternative source for these alkaloids (2). Tissue cultures of *Papaver* have been grown in modified Murashige and Skoog Medium (revised tobacco medium) and Prairie Regional Laboratory B–5 Medium (4). *Papaver* callus grown in vitro produced alkaloids in low concentrations, phenanthrine-type (morphine) alkaloids were produced infrequently (5). However, the spectrum of alkaloids accumulated in the cells included aporphine (6), protopine (7), benzophenanthrine (8), phthalideisoquinoline (5, 9) and benzylisoquinoline types (9). Absence of enzymes for the conversion of (−)-R-reticuline (see fig. 1) may be responsible for the deficiency in phenanthrine alkaloid production (10). Some cultures have a limited capacity to biotransform thebaine to codeine (11) or codeinone to codeine (10), while the cultures themselves do not produce any phenanthrine alkaloids.

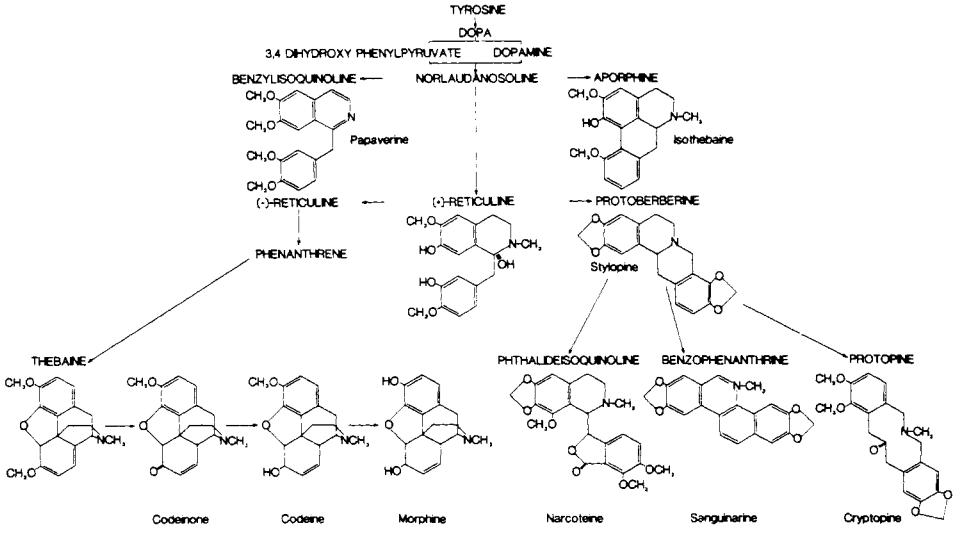

FIG. 1. Biosynthetic relationship between the alkaloids of *Papaver* species.

Furuya *et al.* (6) studied callus cultures of *P. somniferum* and found that the origin of the explant (petiole, root, seedling, stalk and capsule) and the type of medium used did not affect the alkaloid patterns observed. Kamimura and

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Akutsu (12) made a similar observation with *P. bracteatum* callus. Furthermore, Kamimura *et al* (13) observed that buds or plantlets of *P. bracteatum* induced by growth regulators had different alkaloid patterns from those of the plant. Supplying precursors (L-ornithine, L-phenylalanine, DL-tropic acid, L-dopamine and L-tyrosine) to these cultures did not stimulate thebaine production (13).

P. bracteatum callus cultures which initially contained a relatively high amount of thebaine gradually produced less thebaine and more protopine type alkaloids over a two-year period (14). Tetraploid, tracheid-containing callus of *P. somniferum* reportedly produced codeine, morphine and thebaine (15). Tam *et al.* (16) have isolated codeine from diploid *P. somniferum* cell suspensions that contained tracheids and giant cells.

The objective of this study was to establish *Papaver* cell suspension cultures, embryoid cultures and shoot cultures *in vitro* and to make a comparative analysis of their alkaloid content.

EXPERIMENTAL

TISSUE CULTURES.—Seeds of *P. bracteatum* Lindl. (Arya II, P.I. 381605) (2) and *P. somniferum* L. (Kek Duna, Hungary, 1975 harvest)[2] were sterilized with 3.0% sodium hypochlorite for two minutes. The seeds germinated in approximately 5 days. Whole seedlings were aseptically transferred onto solid revised tobacco (RT) medium (9) supplemented with 5 ppm benzyladenine (BA) or 1 ppm 2,4 dichlorophenoxyacetic acid (2,4-D) to establish shoot and callus cultures, respectively. Shoot and callus cultures were subcultured every 4–6 weeks. Following 4 subcultures on solid RT medium, shoot and callus cultures were transferred into 125 ml Erlenmeyer flasks containing 25 ml of liquid RT medium supplemented with either 5 ppm BA for the growth of shoots or 0.1 ppm 2,4-D for the development of cell suspensions. Cells of *P. bracteatum* (inoculum 0.37 gm fresh wt.) and *P. somniferum* (inoculum 0.87 fresh wt.) were grown in five different embryo induction media (table 1 and 2, I–V). Cultures were examined microscopically and analyzed for alkaloids at specific intervals (table 1 and 2).

All cultures were grown at 28°. Shoot and embryo cultures were grown on a gyrotory shaker (78 rpm) in the light (15 h day, Fluorescent Plant-Grow Bulbs, Sears, 40W, Cool-light, 500 f.c.). Callus cultures were grown in the dark.

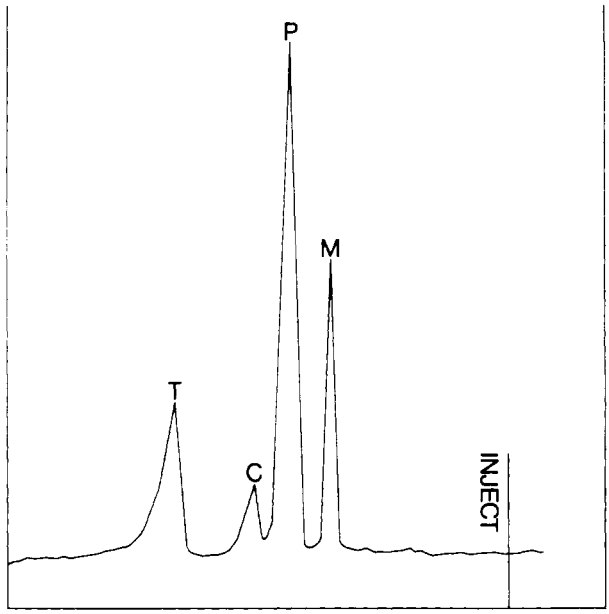

FIG. 2. Hplc chromatogram of a mixture of thebaine (T; 8.2 min.), codeine (C; 6.3 min.) papaverine (P; 5.5 min.) and morphine (M; 4.5 min.). Operating conditions: column, μBondapak C_{18}; mobile phase, methanol:0.3% ammonium carbonate in water (75:25); ambient temperature; flow rate, 1 ml/min; detector fixed wavelength (254 nm).

[2]Very kindly provided by Dr. Quinton Jones, U.S. Department of Agriculture, Washington, D.C.

TISSUE CULTURE ANALYSIS.—Cultures were extracted by a modification of the procedure of Wu and Dobberstein (17). The cultures were homogenized in a volume of 5% acetic acid corresponding to the fresh weight of cells. The mixture was filtered, washed with 5% acetic acid and the filtrates made alkaline with conc. ammonium hydroxide. The alkaline filtrate was then extracted three times with chloroform and the chloroform extracts combined and dried over anhydrous sodium sulfate. After filtration, the chloroform was evaporated *in vacuo*, yielding a residue suitable for chromatographic analysis.

The residue from the chloroform extracts was dissolved in one milliliter of methanol+ 0.3% ammonium carbonate in water (75:25) for thin layer chromatography (tlc). Reference samples (1 μg/μl) were dissolved in the same solvent. Samples and standards (10 μl) were applied to silica gel GF plates (Analtech, Newark, DE 19711) and developed with chloroform-methanol (9:1) to a solvent height of 10 cm (18). After development, the plates were air dried and examined for alkaloids by uv light and by spraying with Dragendorff's reagent (19). Preparative layer chromatography on silica gel GF plates (1 mm) with the same solvent system. The bands corresponding to co-spotted reference alkaloids were scraped and eluted with methanol.

The samples were also analyzed by high pressure liquid chromatography (hplc). The solutions were millipore filtered and injected (20 μl) onto a μBondpak C_{18} column (1 foot long, $\frac{3}{8}$ inch O.D.: Waters, Milford, MA 01757). The operating conditions were: ambient temperature; eluting solvent was methanol:0.3% ammonium carbonate in water (75:25), flow rate was 1 ml/min; wavelength of uv detector, 254 nm. Under these conditions a suitable separation of thebaine, codeine, morphine and papaverine could be accomplished within 15 minutes (fig. 2). For preparative isolation of an alkaloid, multiple injections (20 μl each) were made of samples previously purified by preparative layer chromatography. Samples corresponding to uv absorbance peaks were collected automatically (Gilson, C.P.R. peak collector, Gilson, Middleton, WI 53562).

RESULTS AND DISCUSSION

TISSUE CULTURE DIFFERENTIATION AND ORGANIZATION.—*P. bracteatum* and *P. somniferum* seedlings used as explants formed callus on solid RT medium supplemented with 1 ppm 2,4D within 2 to 4 weeks. Callus cultures readily formed suspension cultures when transferred to liquid RT medium supplemented with 0.1 ppm 2,4D. Similar *Papaver* cultures had been established previously (20) and had been subcultured for several years on either solid or liquid RT media. The suspension cultures consisted of light and dark cell aggregates (fig. 3A). The solid medium supporting growth of callus became dark brown in approximately 4 weeks. Shoot cultures of *P. bracteatum* (fig. 3B) were subcultured every 3 weeks and were maintained for over one year.

The cells grown as suspension were subcultured on 5 different media in order to induce the formation of embryoids and/or organized structures (21, 22). After 58d in media I & IV, cells of *P. bracteatum* reorganized into root structures (table 1; fig. 3C). Green embryoid-like structures developed in media II, III and V (table 1, fig. 3D). In medium VI (table 1) roots and embryoid were not developed. All cell material grown in media II and III formed shoots (fig. 3E) when transferred to solid RT medium without growth regulators. Plantlets did not form on any of the media used.

The Growth Index values obtained for *P. bracteatum* at 38d were approximately twice that of *P. somniferum* at 20d (tables 1 and 2). This relationship is partially explained by *P. bracteatum's* smaller weight inoculum and longer growth period. A relatively lower *P. bracteatum* growth rate was observed in medium III— as compared to the other media. The lowest growth rate observed was that of *P. somniferum* on medium IV.

ALKALOID ANALYSIS OF *P. bracteatum* CULTURES.—The *P. bracteatum* Lindl. plants (Arya II, P.I. 381605) used in this study were reported to contain 309.2 μg/100 mg dry weight of thebaine in the aerial parts and 680.1 μg/100 mgs dry weight of thebaine in the roots (2). Extracts of 38d cultures grown in the five different embryo induction media (table I, I–V) were all positive with Dragendorff's reagent. Thebaine was in all the extracts when analyzed by tlc and hplc (R_f 0.35, R.T. 8.2 min). Hplc analysis revealed no other alkaloids. However, tlc showed several alkaloid spots which did not correspond to reference compounds.

Extracts from 58d cultures grown on five different embryo induction media (table I, I–V) were all positive with Dragendorff's reagent. No correlation

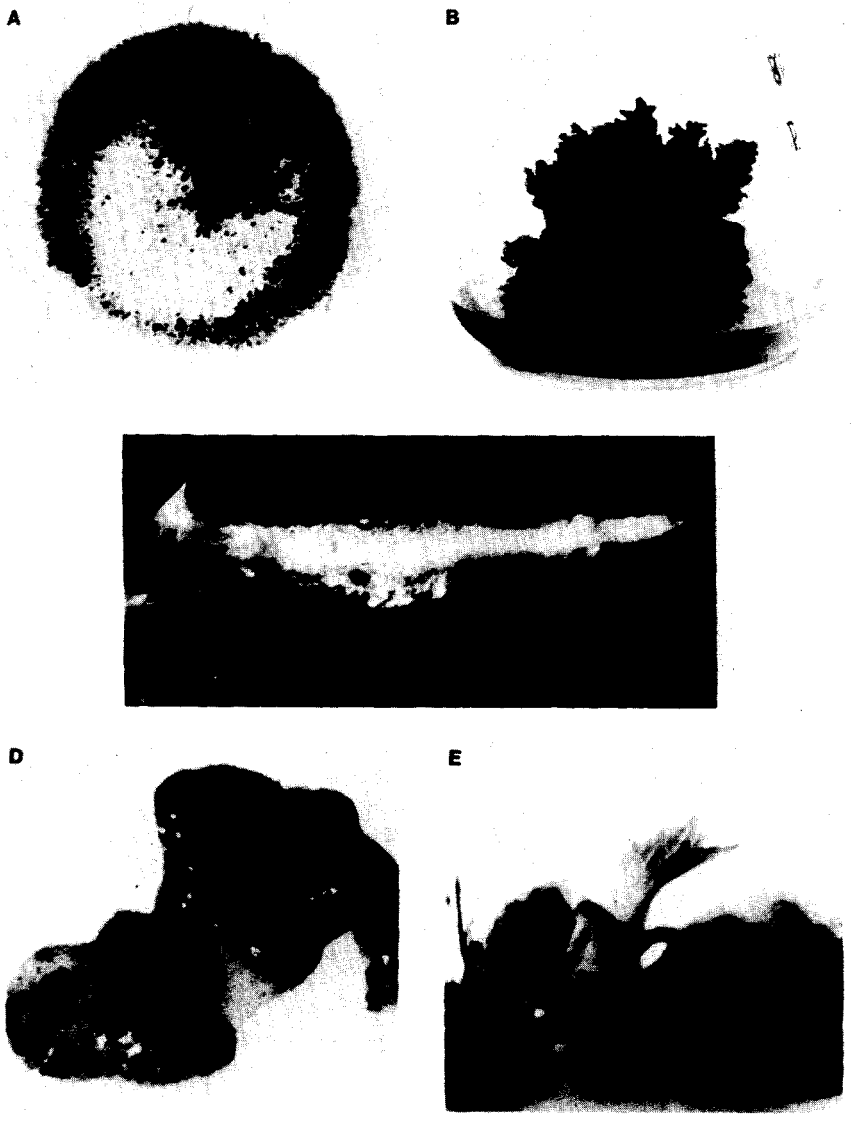

FIG. 3. *Papaver bracteatum* tissue cultures. A. Light and dark cell clusters on bottom of 250 ml flask—17 days old. B. Shoot culture—21 days old. C. Root organ (10 mm) from unorganized cells growing in Medium IV containing galactose. D. Embryo-like structure (1 mm) from Medium II. E. Shoot-like structures evolving from embryo-like structures from Medium II or Medium III. (See table 1 for medium growth regulator concentrations).

between the many alkaloid spots on tlc and hplc could be established for the phenanthrine alkaloids. The media from the experiments were analyzed for alkaloids and none were detected by Dragendorff's reagent, tlc or hplc. Tlc and hplc analysis of control cultures grown for 38 or 58d on RT medium supplemented with 1.0 ppm 2,4-D (table 1, VI) showed no morphine alkaloids.

Leaf shoot cultures of *P. bracteatum* (2.4 gm dry weight) grown 30d on RT medium supplemented with 0.1 ppm 2,4-D and 0.5 ppm BA were extracted and analyzed for phenanthrine type alkaloids. Tlc of the extract revealed 2 Dragendorff positive spots corresponding to codeine (R_f 0.23) and thebaine R_f 0.35). These two alkaloids were also detected by hplc (R.T. 6.3 min. codeine; R.T. 8.2

TABLE 1. Growth, morphogenic response and alkaloid content of *P. bracteatum* cultures grown in 5 different media.

Medium modification[1]	Growth index[2]		Morphogenic response	Tissue extracted (g. fresh wt.)		Alkaloids identified by hplc, and tlc
	38d	58d		38d	58d	
I. NH_4(10mM)/ NO_3(30mM).....	12.5	17.5	Reorganization into root structures	37.7	27.9	Thebaine
II. BA(0.5 ppm)/ IBA(1 ppm).....	11.4	14.7	Green embryoid-like structures	21.2	27.4	Thebaine
III. BA(3.0 ppm)/ IBA(1 ppm).....	5.4	8.5	Green embryoid-like structures	7.1	9.2	Thebaine
IV. Galactose (7.0%).....	18.6	19.5	Reorganization into root structures	28.9	48.1	Thebaine
V. Azaindole (3 ppm).....	13.8	12.9	Green embryoid-like structures	28.7	31.5	Thebaine
VI. 2,4-D (0.1 ppm)...	22.5	24.6	Dark, cell aggregates	24.2	51.4	—

[1]Modified Murashige and Skogg's medium (15): inorganics—half strength, sucrose (3.0%) inositol (0.2%), vitamins (2). Modifications: NH_4/NO_3 supplied as ammonium nitrate and potassium nitrate. BA=benzyl adenine; IBA=indole butyric acid; 2,4-D=2,4-dichlorophenoxyacetic acid.

[2]Growth Index=Final fresh wt./inoculum fresh wt. determined at day (d) stated.

min., thebaine). Substantiation of the presence of thebaine was accomplished by isolation by preparative layer chromatography which yielded 160 μg (6.67 μg/100 mg dry weight). The mass spectrum of the isolated thebaine was consistent with the mass spectrum of authentic thebaine (23): *m/e* 311[M^+], 296 [M^+-15], 281[M^+-30), 268[M^+-43], 255[M^+-56), 176[M^+-137], 148[M^+-163] and 117[M^+-194].

TABLE 2. Growth, morphogenic response and alkaloid content of *P. somniferum* cultures grown in 5 different media.

Medium[1] modification	Growth index[2]		Morphogenic response	Tissue extracted (g. fresh wt.)		Alkaloids identified by hplc, tlc and mass spec.
	20d	34d		20d	34d	
I. NH_4(10mM)/ NO_3(30mM).....	6.6	5.9	Callus and root structures	29.2	15.5	codeine Cryptopine
II. BA(0.5 ppm)/ IBA(0.5 ppm).....	5.7	5.6	Callus and root structures	25.5	19.5	Codeine Cryptopine
III. BA(3.0 ppm)	6.2	5.2	Callus and root structures	21.7	13.7	Codeine Cryptopine Thebaine
IV. Galactose (7.0%).....	2.6	3.6	Callus and root structures	9.2	9.5	Codeine Cryptopine Thebaine
V. Azaindole (3 ppm)..... (Sigma Chem. Co. St. Louis, MO)	6.2	4.7	Callus and root structures	21.6	20.7	Codeine Cryptopine Thebaine

[1]Modified Murashige and Skoog's medium (15): inorganics—half strength, sucrose (3.0%), inositol (0.2%), vitamins (2). Modifications: NH_4/NO_3 supplied as ammonium nitrate and potassium nitrate. BA=benzyl adenine; IBA=indole butyric acid.

[2]Growth Index=Final fresh wt./inoculum fresh wt. determined at day(d) stated.

ALKALOID ANALYSIS OF *P. somniferum* CULTURES.—Extracts of 20d cultures from the five different embryo induction media (table 2, I–V) all gave a positive reaction with Dragendorff's reagent. All appeared to contain codeine (tlc and hplc; R_F 0.32, R.T. 6.2 min.). Thebaine was detected in the 20d cells from media III, IV and V (tlc, hplc; R_F 0.35, R.T. 8.2 min.). Media from cultures I–IV (20d) did not contain any alkaloids.

Extracts from 34d cultures grown on five different embryo induction media (table 2, I–V) all reacted positively with Dragendorff's reagent. These samples also appeared to contain codeine. Thebaine was not detected by tlc. However, trace levels were detected in all samples by hplc. Several other alkaloid spots on tlc did not correspond to reference compounds.

To confirm the presence of codeine, all extracts were combined and the putative codeine isolated by preparative layer chromatography. Final purification was by repetitive hplc. The mass spectrum of the alkaloid isolated in this manner did not correspond to that of codeine. Co-chromatography using both tlc (R_F 0.26) and hplc (R.T. 6.6 min.) with reference codeine (R_F 0.23, R.T. 6.2 min.) indicated a strong similarity. However, the alkaloid was identified as cryptopine from its characteristic mass spectral fragmentation pattern: *m/e* 369[M^+], 354[M^+-15], 325[M^+-44], 297[M^+-72], 283[M^+-86], 267[M^+-102], 190-[M^+-179], 179[M^+-190] and 148[M^+-221] (24). Protopine-type alkaloids have been observed in other *P. somniferum* cultures (5).

In conclusion, *P. somniferum* showed only the formation of callus and root structures on the 5 different media. Growing the cultures for 20 or 34d had no significant effect on the alkaloid content.

P. bracteatum cultures containing callus with roots were not significantly higher in alkaloids than cell suspension cultures. Thebaine was detected in tissues grown for 38d on the five different media but not in tissue grown for 58d. Shoot cultures contained 0.007% dry wt. thebaine. Since neither cultures containing callus with roots nor shoot cultures produced large amounts of thebaine, it may be necessary to have both organs present in a culture for high thebaine production. Finally, it appears that the production of phenanthrine-type alkaloids by *P. bracteatum* cultures is related principally to the development of shoots.

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