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ALKALOIDS OF CELL CULTURES DERIVED FROM STRAINS OF *PAPAVER BRACTEATUM*

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Key Word Index—*Papaver bracteatum*; Papaveraceae; tissue cultures; alkaloids; dihydrosanguinarine; oxysanguinarine; sanguinarine; thebaine.

Abstract—Growth and alkaloid production were studied in callus and cell suspension cultures derived from seeds of three strains of *Papaver bracteatum*. Traces of oxysanguinarine and sanguinarine were identified in the cultures. Dihydrosanguinarine was the major alkaloid produced by all cell cultures and its crystalline intracellular accumulation was observed using fluorescence microscopy. Suspension cultures developed from one seed strain produced more than 1% of dihydrosanguinarine on a dry weight basis. A time course analysis showed its maximum content (178 mg/l culture) on day 21; alkaloid synthesis appeared to be growth associated. This is the first report of a high dihydrosanguinarine-producing cell culture of *P. bracteatum*.

INTRODUCTION

Papaver bracteatum is an important source of thebaine (**1**), mature capsules containing up to 3.5% of this alkaloid [1]. Tissue cultures of *P. bracteatum* have been initiated by a number of workers with the aim of producing high yields of thebaine. However, little success has been achieved so far, with most workers reporting only small amounts of the alkaloid [2, 3] or its absence in undifferentiated cultures [4, 5]. Nevertheless a variety of isoquinoline alkaloids namely orientalidine [6], isothebaine [6], sanguinarine (**2**) [6, 7], stylopine [4], protopine [4, 7], norsanguinarine, oxysanguinarine (**3**), dihydrosanguinarine (**4**), chelyrubine and magnoflorine [7] have been identified in culture. Such a variation in alkaloid type may be associated with the different media used for culture growth, but could also be a reflection of the plant material used to initiate the cultures. Many cultures of *P. bracteatum* have been developed from seeds or seedlings where parent plant characteristics were unknown. Oriental poppies, *Papaver* section *Oxytona* have in the past been misidentified [8] and mixed collections of seed lots have been known to occur [9]. This study was undertaken in order to assess the influence of parent plant strain on alkaloid production in cell culture. Tissue cultures were initiated from three strains of *P. bracteatum* and their alkaloid production under controlled conditions was evaluated.

RESULTS AND DISCUSSION

Plant material

Plants grown by us from Arya II seed all showed features typical of *P. bracteatum*, i.e. non-spreading roots, a basal rosette of bristly, deeply-incised leaves, erect flower buds with bracts and large dark red petals [8, 9]. Other characteristics expected of *P. bracteatum* are shown in Table 1. The three plant strains separated by us differed only in petal markings; no markings (NB), a central black area (CB) and a black basal streak (BB). Only the latter should, according to Goldblatt [8] be considered as true *P. bracteatum*. Diameter measurements of 50 pollen grains from each flower type mounted in water, showed variations in size between the strains, average values being 31 μm for BB, 32.2 μm for CB and 33.6 μm for NB. These values differ from the size quoted in Table 1 for *P. bracteatum*.

Two separate extraction procedures showed thebaine (**1**) to be the only dominant alkaloid present in the capsules obtained from the three plant strains, with dihydrosanguinarine (**4**) and sanguinarine (**2**) occurring in trace amounts. The presence of (**1**) in major concentration, together with the typical plant features, led us to

Table 1. Characteristics of *Papaver* section *Oxytona* [8]

Species	Petal characteristics	Mean pollen diameter (μm)	Dominant alkaloid
P. bracteatum	Deep red black base streak	25.5	Thebaine
P. orientale	Orange no markings	27.4	Oripavine
P. pseudo-orientale	Scarlet black centre	28.8	Isothebaine

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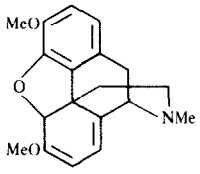

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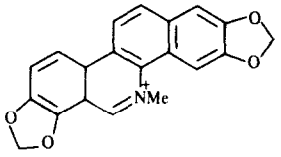

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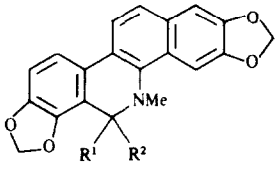

3 R^1, R^2 = O
4 R^1 = R^2 = H

Table 2. Comparison of dihydrosanguinarine content (% dry wt) in callus cultures developed from three strains of *P. bracteatum* grown in light or dark conditions over a two-year period

	Dihydrosanguinarine content			
	NB		BB	CB
Number of subcultures	Light grown	Dark grown	Light grown	Light grown
1	0.26	n.d.	0.30	0.24
8	0.52	0.22	n.d.	n.d.
16	0.49	0.92	0.22	n.d.
21	1.11	1.38	0.09	0.03

n.d.: not determined.

conclude that the plants were different strains of *Papaver bracteatum*.

Callus cultures

Seeds produced calli readily under the culture conditions employed, producing in all cases a beige coloured, friable callus which became brown after two weeks and necrotic after six. Growing cultures in the dark did not affect the degree of browning, which was therefore not due to the photodecomposition of catecholamine alkaloid precursors [10].

Alkaloid extraction of newly developed cultures initiated from all three seed sources produced the same spectrum and yield of alkaloids (Table 2). Compound **4**

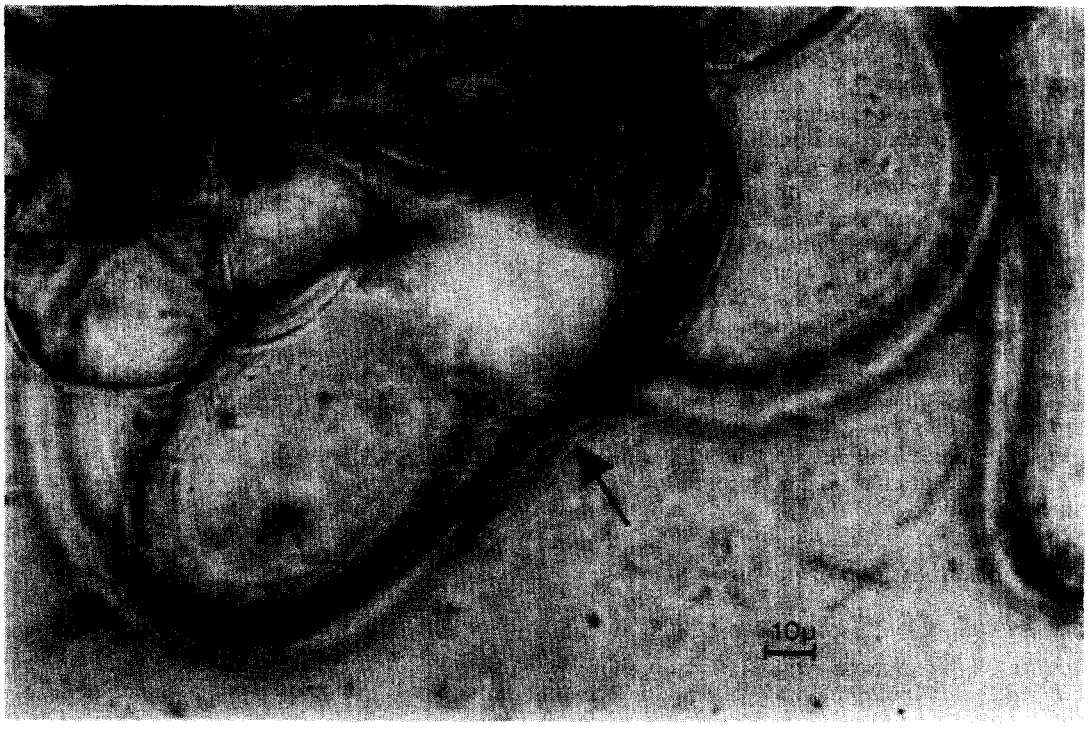

Fig. 1. Cultured cells of *P. bracteatum* showing crystalline intracellular deposits of dihydrosanguinarine visualized with fluorescence microscopy.

was the major alkaloid present and could be detected as blue fluorescent intracellular crystals using fluoromicroscopy (Fig. 1). Alkaloid **2** occurred only in trace amounts in the cultures. Alkaloid contents of callus cultures developed from the three seed strains grown in light or dark conditions and subcultured over a two-year period are shown in Table 2. On repeated subculturing light-grown BB callus produced decreasing amounts of **4** while dark-grown cultures began to exhibit organogenesis after 12 subcultures. Once tissue organization occurs alkaloid synthesis changes [5] and therefore these analyses are omitted. (Alkaloid production by regenerated plantlets of *P. bracteatum* will be published separately). CB cultures grew poorly but their dihydrosanguinarine (**4**) content also decreased with time, while their sanguinarine (**2**) production increased. The loss by tissue cultures of the capacity to produce secondary metabolites on continued culture without clonal selection is a known phenomenon [11]. NB cultures differed in showing enhanced alkaloid productivity with yields of (**4**) increasing to more than 1% of dry culture weight. The callus also changed colour from brown to grey.

General extraction and characterization of alkaloids

Dihydrosanguinarine (**4**), oxysanguinarine (**3**) and sanguinarine (**2**) were isolated from cell cultures of *P. bracteatum*. These alkaloids were characterized by spectroscopic and physical means and by comparison with literature data [12]. Authentic samples of (**4**) and (**3**) were prepared by chemical conversion of (**2**).

In alkaline solution quaternary isoquinolinium salts undergo a disproportionation reaction [(**2**)→(**3**)+(**4**)]. Acid/base extraction of alkaloidal material can frequently lead to the formation of artefacts [14] and therefore doubt exists as to the true nature of these metabolites. The isolation of (**4**) as a true metabolic product from the current cell lines of *P. bracteatum* was supported by fluoromicroscopy which showed the presence of blue fluorescent crystals of (**4**) in intact culture cells (Fig. 1).

Suspension cultures

Cell suspension cultures were developed from the high dihydrosanguinarine (**4**)-yielding callus (strain NB). Three cell lines were separated. The % yields of **4** in the cell lines, as well as the biomass and total content of **4** per litre of medium measured over a period of six months are in Table 3. From the results it is obvious that the cell lines are similar in their biomass and alkaloid production, but physically the NB1 cell line differed forming small dark brown, dense aggregates instead of friable, grey, loose cultures. These results also clearly show that although the overall content of **4** is high (*ca* 1% of dry wt) the cell lines are unstable with a declining alkaloid productivity becoming apparent during the six nonselective subcultures reported. Enhancement of alkaloid production by clonal selection [14] as well as stimulation of alkaloid formation by microbial and chemical means are being examined in current experiments.

Time course analyses

The pattern of growth and alkaloid accumulation by cell suspensions of cell line NB3 in batch culture is shown in Fig. 2. The cells showed an initial lag phase of growth until day 7. Growth measured as cell dry wt, reached a maximum at day 17 and then declined. Growth of the suspension cultures was accompanied by a decrease in sucrose content of the medium and by a concomitant increase in dihydrosanguinarine production. The maximum alkaloid content (day 21) coincided with the disappearance of sucrose from the medium. Alkaloid synthesis thus appears to be growth associated in this culture. The results presented are similar to those reported for the synthesis of another isoquinoline alkaloid berberine, by suspension culures of *Thalictrum rugosum* [15]. Data for the production of sanguinarine, oxysanguinarine and thebaine are not presented as these alkaloids were not produced in significant amounts.

The results of the present study indicate that parent plant strain does not affect the alkaloid spectrum formed in newly initiated callus cultures. Subsequent development of cultures without clonal selection can however lead to variations in alkaloid productivity (Table 2). The cultures of *P. bracteautum* initiated resulted in the separation of a high dihydrosanguinarine-yielding strain. Cell lines of this strain grown in suspension culture, although showing a high initial alkaloid productivity (*ca* 2% on a dry wt basis) proved to be unstable (Table 3).

EXPERIMENTAL

Plant material. Plants of *P. bracteatum* Lindl. were grown to maturity in open ground at the School of Pharmacy, Dublin, from seed supplied as Arya II. In succeeding years three different

Table 3. Biomass yield (g dry wt/l), dihydrosanguinarine (DHS) content (mg/l) and percentage DHS (dry wt) from three cell lines of *P. bracteatum* grown in batch suspension culture

Date of subculture 1986	Cell line NB1			Cell line NB2			Cell line NB3		
	Biomass	DHS	% DHS	Biomass	DHS	% DHS	Biomass	DHS	% DHS
27 June	12.5	254.8	2.04	13.0	170.2	1.30	12.5	218.7	1.75
18 July	7.7	127.8	1.66	15.6	170.7	1.09	11.9	177.9	1.49
25 Aug	9.9	185.3	1.87	9.8	153.1	1.56	9.4	97.7	1.04
26 Sept.	n.d.	n.d.	n.d.	12.8	86.3	0.67	n.d.	n.d.	n.d.
3 Nov.	13.6	118.0	0.86	n.d.	n.d.	n.d.	10.3	144.6	1.40
4 Dec.	8.2	91.4	1.11	15.6	54.3	0.35	12.7	137.9	1.09

n.d.: not determined.

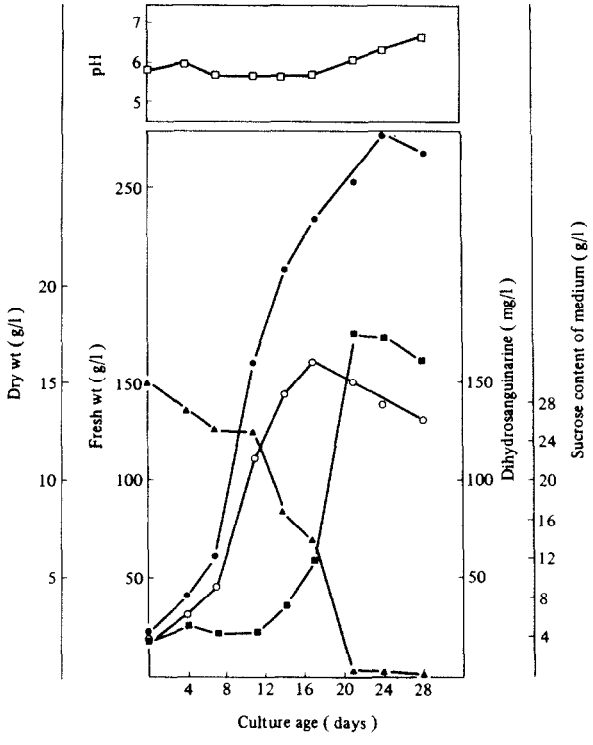

Fig. 2. Growth curve of *P. bracteatum* (cell line NB3) in batch suspension culture showing fr. wt ●—●, dry wt ○—○, dihydrosanguinarine content ■—■ of cell material, sucrose content ▲—▲ and pH □—□ of medium.

strains were sepd according to the presence of purple-black petal spots and designated NB (no black), CB (centre black spot) and BB (base black streak).

General procedures. TLC was performed on precoated silica gel plates ($60F_{254}$, Merck) using the solvent systems SI: EtOAc–MeOH–NH_4OH (17:3:1), SII: Me_2CO–toluene–EtOH–NH_4OH (200:200:60:9) and SIII: petrol–EtOAc–Me_2CO (8:2:1); detection UV and Dragendorff's reagent. IR spectra were run in KBr unless otherwise mentioned and UV in MeOH solns. ^1H NMR were recorded at 60 MHz and highfield NMR at 270 MHz with TMS as int std. MS were determined by EI at 70 eV. HPLC was carried out on a Spherisorb 10 ODS, 250 × 4.6 mm column; solvent system aq ammonium carbonate (0.3%) in MeOH (15% for thebaine and 25% for dihydrosanguinarine determinations), flow rate 2 ml/min for thebaine and 3 ml/min for dihydosanguinarine, with detection by UV at 285 nm. Fluorescence microscopy was carried out on a microscope fitted with a Ploemopak 2.4 fluorescent illuminator, with exciting filter BP 270–280 and suppression filter BP 410-580.

Tissue cultures. Cultures were initiated from seeds obtained from mature, field-dried capsules derived from plants which had shown the same flower colour characteristics through two generations. Seeds were surface sterilized by soaking in a 10% aq soln of a commercial NaOCl conc for 20 min and rinsing × 5 with sterile dist. H_2O.

Seeds germinated and formed calli readily on a Murashige and Skoog salt medium (M + S) [16] containing 2,4-D (0.22 mg/l), NAA (0.186 mg/l), glycine (2 mg/l), nicotinic acid (0.5 mg/l), pyridoxine HCl (0.5 mg/l), mesoinositol (200 mg/l), thiamine HCl (0.5 mg/l) and sucrose (30 g/l). Some cultures were grown in the dark, others in continuous dim fluorescent light, all at 25° (± 2°). Cultures were subcultured after 4 to 6 weeks. Suspension cultures were initiated by transferring callus portions into liquid medium (100 ml modified M + S in 250 ml conical flasks). Flasks were shaken on an orbital shaker (120 rpm, 1 cm throw). Suspensions were subcultured, using standard procedures, at monthly intervals. NB1 suspensions were initiated by transferring fifth generation callus portions, NB2 suspensions from ninth generation callus and NB3 suspensions were initiated from an NB2 subculture (fifth generation).

Time course studies. These were performed in triplicate with cultures developed with NB3 cell material (1 g fr wt in 50 ml medium, per 100 ml flask). Cultures were harvested at regular intervals over a monthly growth cycle. Fresh cell material was dried in a fan oven (below 40°). Alkaloid content was determined by quantitative HPLC. Sucrose concn was measured by a UV-method used for assaying sucrose in foodstuffs [Food Analysis-Boehringer Mannheim].

Alkaloid extraction and isolation. (a) *Thebaine* (**1**). Powdered, deseeded capsules and tissue cultures were extracted with 5% aqHOAc (2 × 50 ml). The extract was made alkaline with NH_4OH (conc) and extracted with $CHCl_3$ (3 × 50 ml). Combined extracts were dried (Na_2SO_4) and solvent was removed under vacuum. Residues (in MeOH) were subjected to TLC (systems SI and SII) and HPLC analyses. Thebaine (**1**), the major component of the ext was purified by prep. TLC (EtOAc MeOH–NH_4OH (conc); 17:3:1) and was identified by physical and chemical means and by comparison with an authentic sample. (b) *Sanguinarine* (**2**), *oxysanguinarine* (**3**), *dihydrosanguinarine* (**4**). Powdered, deseeded capsules and tissue cultures were extracted by refluxing in EtOAc followed by MeOH. Extracts were examined by TLC and like extracts pooled. Sepn of crude extract by CC over silica gel (hexane–EtOAc–Me_2CO, 8:2:1) yielded **2** together with **3** and **4** which were identified by physical and chemical means and by chemical transformations of **2**. Final acid–base extraction of the cell material did not lead to the recovery of any additional sanguinarine.

Thebaine (**1**). Mp 191–192° (colourless prisms, MeOH) (lit. [17] mp 193°). EIMS *m/z* 331 $[M]^+$.

Sanguinarine (**2**). Mp 273–275° (buff solid, MeOH–$CHCl_3$) (lit. [12] mp 275°). EIMS *m/z* 332 $[M]^+$.

Oxysanguinarine (**3**). Mp dec. 350° (cream solid, MeOH) (lit. [12] mp 347–349°). EIMS *m/z* 347 $[M]^+$. IR ν^{KBr} cm^{-1} 1655 cm^{-1}.

Dihydrosanguinarine (**4**). Mp. 193–194° (needles, MeOH) (lit. [12] mp 195–195.5°). EIMS *m/z* 333 $[M^+]$.

Conversion of **2** to **4**. **2** (10 mg) was stirred with $NaBH_4$ (1 eq) in MeOH (5 ml). The reaction mixture was hydrolysed and extracted with EtOAc to yield **4** which was identical (mp, IR, ^1H NMR, MS) with the isolate and with lit. values [12].

Conversion of **2** to **3**. **2** (10 mg) was stirred with $K_3Fe(CN)_6$ for 12 hr. The reaction mixture was hydrolysed and extracted with EtOAc to yield **3**. Physical and chemical data were identical with the isolated compound and lit. data [11].

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