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ENHANCEMENT OF DIHYDROSANGUINARINE PRODUCTION IN SUSPENSION CULTURES OF *PAPAVER BRACTEATUM*, I. MEDIUM MODIFICATIONS

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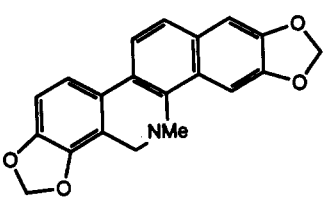
ABSTRACT.—A suspension culture of *Papaver bracteatum* was developed, which initially yielded high levels (>2.55% of dry wt) of the benzophenanthridine alkaloid dihydrosanguinarine [**1**]. This productivity has been lost on repeated subculture. In an attempt to improve alkaloid yields modifications in the concentrations of various minor constituents present in the culture medium were evaluated. The vitamins thiamine and pyridoxine and the microinorganic constituents cobalt, manganese, zinc, and copper were examined at 0.5, 2.0, and 4.0 times their concentration normally present in the modified Murashige and Skoog medium. Only copper when added at 2.0 and 4.0 times the control concentration significantly enhanced alkaloid yields. A fourfold increase was noted when twice the copper concentration was included in the medium at subculture, while a doubling of yields resulted from a fourfold copper concentration added during active cell growth, on day 14 of the growth cycle.

Suspension cultures developed from seeds of *Papaver bracteatum* Arya II Lindl. (Papaveraceae) were found to produce the benzophenanthridine alkaloid dihydrosanguinarine [**1**] as major secondary metabolite (1). Benzophenanthridine alkaloids are known to have many pharmacological activities (2–5), with sanguinarine [**2**] showing antifungal, antiprotozoal, antibacterial, and cytotoxic effects (2) and dihydrosanguinarine having antibacterial activities (3). Compound **2** is of current commercial interest as an effective antiplaque agent for use in various oral healthcare products (6). Several recent publications have evaluated the commercial production of **2** from cell cultures of *Papaver somniferum* (7–9). However, as sanguinarine can also be formed from dihydrosanguinarine (10), cell cultures able to give high yields of **1** may have potential for commercial exploitation.

Newly established cultures of *P. bracteatum* (cell line NB[2]*) were initially found to produce dihydrosanguinarine [**1**] in yields frequently in excess of 2.55% (dry wt). Alkaloid production was found to show considerable variation during the first 18 months of culture but since then has been relatively stable at ca. 0.55% (Figure 1). Similar variabilities and losses in alkaloid productivity have been reported with other nonselected plant cell cultures on repeated subculture (11). In order to enhance the production of **1**, the concentrations of several minor constituents of the culture medium were modified and their effects on cell growth and alkaloid formation assessed. The constituent concentrations examined were half, double, and 4 times those normally present in the modified MS medium used as control.

The effects of the vitamins thiamine and pyridoxine were evaluated. Our re-



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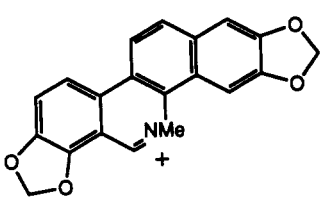

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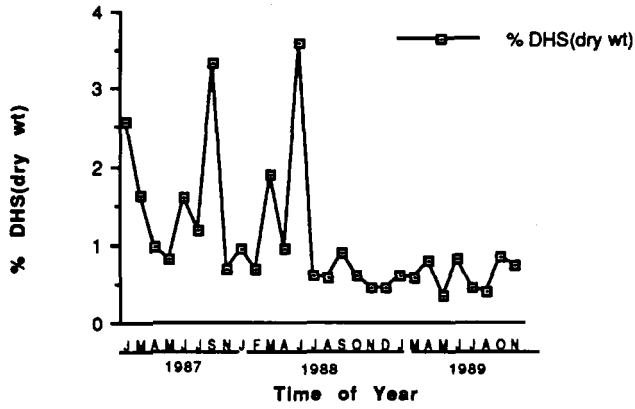

FIGURE 1. Dihydrosanguinarine [1] production by suspension cultures of *Papaver bracteatum* (cell line NB[2]*) during a 3-year period of serial subculture in a modified MS medium. Cells were subcultured at 28-day intervals by transferring ca. 2 g (fresh wt) of cells into 100 ml medium.

sults show that their presence in the culture medium at varying concentrations does not significantly affect culture growth or alkaloid yields. The requirements of plant cell cultures in general for these vitamins have not been clarified (12).

The following secondary mineral elements were also examined: cobalt, manganese, zinc, and copper. The results obtained indicate that varying the medium concentrations of cobalt, manganese, and zinc does not significantly affect culture growth of *P. bracteatum* or alkaloid yields. Although these metal ions are all known to be important catalysts in a variety of enzyme reactions, our results underline the fact that their exact requirements for plant cell culture growth and secondary metabolite production are uncertain (12,13). Copper was the only microelement for which increased concentrations in the nutrient medium significantly enhanced alkaloid yields (% dihydrosanguinarine) without affecting growth (dry wt). Doubling the concentration of copper present in the nutrient medium at the beginning of the growth cycle (day 0) produced a 4-fold increase in dihydrosanguinarine yield at harvest (day 28) (Figure 2a). Assessment of the optimal time for the addition of extra metal ions to the culture is crucial for maximizing alkaloid formation. For this a separate experiment was carried out where copper at two concentrations was added to the cultures at two stages of the growth cycle: on day 14 (representing exponential phase) and day 21 (representing stationary phase). From results shown in Figure 2 it is obvious that for enhanced alkaloid yields copper must be present at the beginning of the growth cycle or during the period of active cell division and growth (up to day 14) (1). Once the culture is in its stationary phase (after day 21) additional copper does not affect alkaloid production.

In some plant cell cultures addition of various heavy metals (14,15) including copper (16,17) to the growth media have been reported to induce secondary metabolite formation. This enhancement has been referred to as "elicitation" and the metals involved as "abiotic elicitors." The precise molecular signal involved in elicitation is not yet clear. However, in the biosynthesis of various benzophenanthridine alkaloids the addition of biotic elicitors of microbial origin have been found to increase the activity of some key enzymes involved in their formation, including tyrosine decarboxylase (18), DOPA decarboxylase (9),

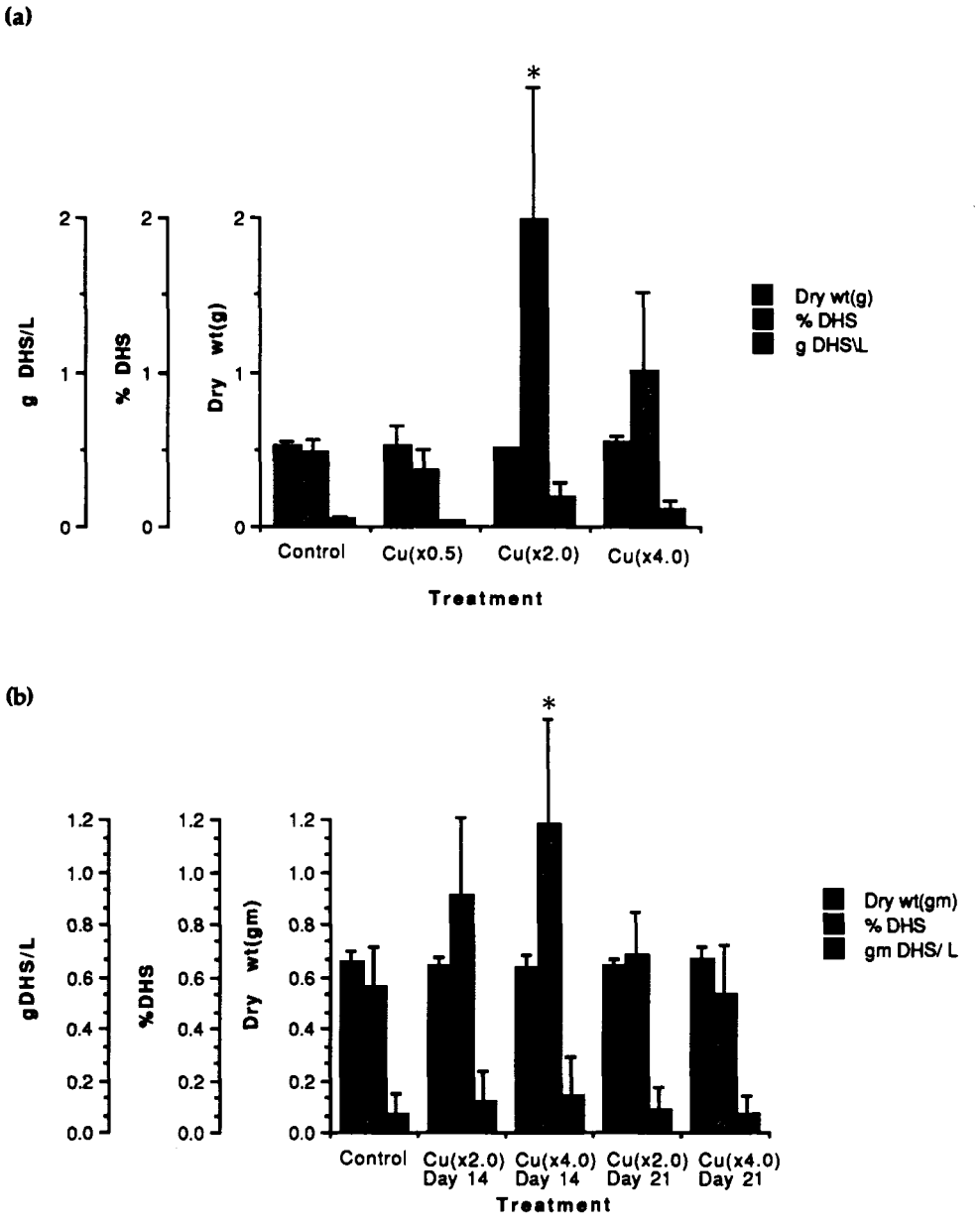

FIGURE 2. Effects on growth and dihydrosanguinarine [**1**] yields produced in *Papaver bracteatum* suspension cultures by various concentrations of copper added (a) at subculture (day 0) and (b) on day 14 or 21 after subculture. Cells were harvested on day 28 (dry wt g/50 ml medium; values shown are the mean of four replicates ± SD $*p<0.05$).

and protopine-6-hydroxylase (19). This latter enzyme catalyzes the formation from protopine of 6-hydroxyprotopine which then undergoes a spontaneous non-enzymatic rearrangement to **1**. The effect on the copper-requiring enzyme dopamine hydroxylase (20) has not been examined. From the results it can be concluded that addition of extra copper to the nutrient medium of actively dividing and growing *P. bracteatum* suspension culture leads to the enhanced production of **1**. This most likely represents an example of medium optimiza-

tion and not elicitation. Berberine alkaloid production in cultures of *Coptis japonica* can similarly be maximized by cultivation in a Linsmaier and Skoog medium supplemented with 10 times the normal concentration of copper (21).

EXPERIMENTAL

CULTURE CONDITIONS.—Suspension cultures were derived from seeds of *P. bracteatum* and grown in a modified Murashige and Skoog (MS) medium as described by Hook *et al.* (1). Seeds were obtained from Division of Narcotic Drugs, United Nations, Geneva, Switzerland. A voucher specimen is deposited in the Herbarium, Botany Department, Trinity College, Dublin, Dublin 2, Ireland. For medium modification experiments, cultures were grown in 100-ml conical flasks containing 50 ml of medium and harvested after 28 days by suction filtration. Fresh cells were dried in a fan oven at below 40° for 48 h prior to weighing and extraction.

PREPARATION OF VARIED MEDIA.—All trace metals and vitamins used as variants were normal components of the modified Murashige and Skoog medium. The variations of concentrations used in the experiments were 0.5, 2.0, and 4.0 times the normal concentration of each. Only one constituent was varied at a time. Cells were added to varied media at subculture (day 0) and grown for 28 days. Only in a follow-up experiment for copper were additional metal ions (×2.0 and ×4.0 the concentration originally present in the medium) added on days 14 and 21 after subculture. All tests were carried out in quadruplicate and compared to controls of the normal modified MS medium.

PREPARATION OF ALKALOID-CONTAINING EXTRACTS.—Dried, powdered cell material (0.5 g) was extracted twice with EtOAc (for 2 h with 50 ml and 1 h with 25 ml) followed by MeOH (for 2 h with 50 ml). Extracts were filtered and the filtrates evaporated to dryness under vacuum. The EtOAc and MeOH residues were dissolved in MeOH (hplc grade) and used directly for hplc analysis.

HPLC PROCEDURE.—The samples were analyzed on a Spherisorb 10 ODS column (250 × 4.6 mm i.d.) with a Kontron 600 hplc instrument fitted with a BIORAD UV monitor. The samples were eluted with a solvent system of 25% aqueous $(NH_4)_2CO_3$ (0.3%) in MeOH at a flow rate of 2 ml/min, with detection at 285 nm. Dihydrosanguinarine present in the extracts was quantified by comparison against calibration curves prepared daily from pure reference alkaloid. Amounts present in EtOAc and MeOH extracts were summed to give a total alkaloid **1** content.

ALKALOID **1** IDENTIFICATION.—Dihydrosanguinarine was previously identified as the major alkaloid produced by these cell cultures (1). As this alkaloid cannot be purchased it had always to be isolated and identified according to the methods indicated by Hook *et al.* (1) prior to its use as the hplc reference. Its identity in culture extracts was confirmed for each set of experiments by tlc and hplc retention data as well as cochromatography of the pure alkaloid with a sample extract.

STATISTICAL ANALYSIS.—A complete randomized block was the experimental design. Data were subjected to analysis of variance and Student's *t*-test.

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