MORPHINAN ALKALOIDS IN CALLUS CULTURES OF *PAPAVER SOMNIFERUM*

CRAIG C. HODGES and HENRY RAPOPORT

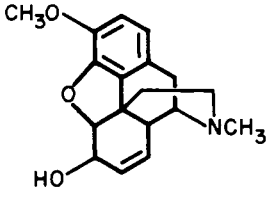
Department of Chemistry, University of California, Berkeley, California 94720

ABSTRACT.—Radio immuno assays were used to screen callus cultures of *Papaver somniferum* for codeine, **1**, morphine, **2**, and thebaine, **3**. Many cultures contained morphinan alkaloids, and six with high contents were analyzed with quantitative hplc techniques. In contrast to the alkaloid distribution found in whole plants, five cultures accumulated a predominance of codeine and one accumulated thebaine. As a function of hormone supplements to the media, trends were found with callus growth but not with alkaloid production. After repeated subculturings, calli no longer accumulated morphinan alkaloids.

Morphinan alkaloids have been detected in callus tissues and cell suspensions of *Papaver somniferum* (1, 2). However, chromatographic analyses used in these studies have required substantial quantities of cultured cells and have thus limited protocols involving extensive screening. Consequently, there is little evidence for judging which aspects of culture conditions promote morphinan alkaloid production, or with what frequency alkaloid-producing lines occur.

The need for sensitive and rapid analytical methods is frequently limiting in projects to produce secondary metabolites from plant cell cultures. In response to this need, the effectiveness of radioimmunoassay (RIA) techniques has been described for the analysis of trace quantities of digoxin in cell cultures of *Digitalis lanata* (3).

In our studies, several types of media with a variety of hormone supplements were tested for ability to initiate or promote subsequent growth of cultures from hypocotyls of opium poppy seedlings. Cultures showing reasonable growth were screened by RIA for morphinan alkaloids, and those giving high positive responses were analyzed by high performance liquid chromatography (hplc) to determine contents of codeine (**1**), morphine (**2**), and thebaine (**3**).



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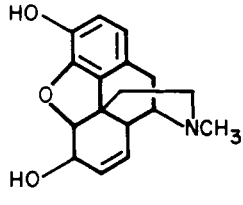

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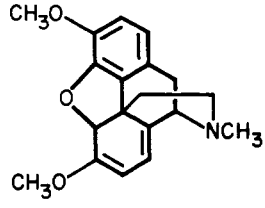

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RESULTS

Surveys were conducted to identify the optimal hormone supplements to media both for callus initiation and for subsequent callus growth. Two basal media reported for poppy culture (4) were tested; Murashige and Skoog's (M&S) (5) without glycine, and Miller's 1968 medium (6). In our experiments, both were supplemented with 10% coconut water. The 1-B5C medium used in a recent report on codeine-producing cultures (1) was not considered for the first experiments. This medium has a relatively high potassium content and was avoided since greenhouse experience showed that the plants of our particular lineage grew better with relatively low potassium nutrients. With promising results from the initial callus cultures, reinvestigation of the basic medium was not pursued.

Nearly 200 hormone combinations were tested for ability to initiate callus from sterile seedlings. These surveys systematically tested combinations of indole-3-acetic acid (IAA), naphthyleneactic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin, 6-*N*-(2-isopentenyl)aminopurine (2-iP), and gibberellic acid in concentrations ranging up to 3 mg/liter. Twenty to thirty seedlings were applied to each medium containing a particular hormone combination.

Substantial variation among tissue responses to the same medium and hormone regime was observed. Formation of callus was occasionally observed from the leaves or epicotyl, but more frequently from the hypocotyl. Calli in later studies were derived from excised pieces of this tissue.

Callus initiation was poor when hormones were used in large quantities (0.5 to 3 mg/liter). Eventual tissue darkening and death were apparent. In all cases, the initial and subsequent growth were slow. With regimes in which auxin concentrations were in the range of 0.02 to 0.2 mg/liter, callus induction from the hypocotyls was frequent. Slow initiation occurred if no cytokinins were added. Slightly faster growth was obtained with the addition of 0.03 or 0.3 mg/liter of 2-iP or kinetin.

Most rapid induction and subsequent callus growth were promoted by 0.1 mg/liter of kinetin and 0.2 mg/liter of 2,4-D. Addition of NAA from 0 to 0.2 mg/liter to these levels of kinetin and 2,4-D had no effect. Either of the basic media, M&S or Miller, gave good results. Callus induction has been reported (4) from seedling hypocotyls of *P. somniferum* under similar medium conditions: Miller's (1968) medium with 0.1 mg/liter kinetin, 0.2 mg/liter 2,4-D, and 0.2 mg/liter NAA. Callus for our further experiments was also derived on this induction medium, supplemented with 10% coconut water.

Preparing callus for further experiments involved inoculation with hypocotyl and subsequent growth for four weeks. This included one subculturing at the end of the second week. The search was then begun for conditions which would encourage reasonable further growth. Small portions of callus, prepared on the induction medium as above, were transferred to these new growth-promotion media. Using 2,4-D and NAA at 0, 0.02, and 0.2 mg/liter and kinetin and 2-iP at 0, 0.03, 0.1, and 0.3 mg/liter, 63 hormone combinations in the M&S medium (without glycine but with 10% coconut water) were tested. These calli were grown for three weeks without subculturing and observed for growth characteristics. Portions of the calli were then collected for alkaloid analyses.

Many of the hormone combinations, 38 of the 63, were favorable to reasonable growth. For our selection, any culture showing some definite and stable growth was of interest. Those showing very rapid expansion were not necessarily of primary interest, since cells devoting substantial effort to production of alkaloids might be expected to have fewer resources available for division and growth.

A complex variety of growth was observed in these callus cultures. Rapid growth was apparent on medium which had no added hormones, only cytokinins, or the particular combination of 0.2 mg/liter 2,4-D, 0.2 mg/liter NAA, and 0.3 mg/liter 2-iP. In the last combination, calli were light-colored and appeared to be of uniform structure by macroscopic inspection. Calli grown on medium with no added hormones, however, showed local dark areas and frequent shoot and root differentiation. As a typical example of the mass increase observed in these fast growing cultures, the inoculum of one culture weighed 53 mg, fresh (3.1 mg, dry) and increased to 854 mg, fresh (38 mg, dry) after three weeks.

Unlike the hormone regimes above, others gave quite variable growth. Calli on the same medium sometimes differed by a factor of three in mass. Complex effects within the inoculation callus may have occurred from residual hormones absorbed from the initiation medium. Slower growing calli often occurred on media containing 0.20 or 0.22 mg/liter total auxin with any of the cytokinin concentrations. These calli usually contained both light and dark regions, and only

a few showed organ differentiation. The three-week old cultures showing reasonable growth were then surveyed for alkaloid production.

A commerically available RIA system designed for clinical analyses of morphine in urine was readily adapted for use on plant callus samples. Large quantities of callus subcultured only on the induction medium were homogenized. Portions of the homogenate were extracted and shown by hplc to contain no significant quantity of morphinan alkaloids. The radioimmunoassays gave negative responses to 100 μliter samples of this homogenate. Positive responses of the RIA system to morphine, codeine, and thebaine were obtained and calibrated; homogenate samples spiked with known quantities of these alkaloids were used. Assays could be used to estimate the combined quantity of morphinan alkaloids in the range of 0.1 to 100 ng. The sensitivities to morphine and codeine were nearly the same, while the RIA response to thebaine was less by a factor of two.

Tissues from the growth-promotion media were then tested for alkaloid content. Portions of calli grown on the same hormone regime were collected, combined, homogenized, and tested for morphinan alkaloids by RIA. Of the 38 samples, each from a different medium sustaining reasonable callus growth, 17 gave RIA responses at least 10 times higher than the 0.1 ng detection limit.

Of the samples giving positive RIA responses, six registered in the high range of 10 to 100 ng of morphinan alkaloids. Larger quantities of these callus homogenates were extracted and the extracts analyzed by hplc to determine the contents of codeine, morphine, and thebaine separately. The results of these analyses are given in table 1.

TABLE 1. Alkaloid analyses of callus cultures of *Papaver somniferum* by hplc.

Culture No.	Hormone Regime (mg/liter)	μg Alkaloids/g tissue, fresh wt		
		Thebaine	Codeine	Morphine
1	2-iP (0.03)	3.9	8.7	2.6
2	2-iP (0.1)	45.0	0	0
3	Kinetin (0.03)	2.9	30.2	1.3
4	NAA (0.2)	19.0	21.7	12.5
5	2,4-D (0.02)	12.7	34.4	1.4
6	2-iP (0.03) +NAA (0.2) +2,4-D (0.2)	13.5	28.4	3.7

High codeine concentrations were found in calli grown with several of the hormone regimes. Ten calli grown with the hormone regime of No. 6 (table 1) showed high overall growth and reasonable homogeneity. These calli were therefore subcultured and the lines kept separate through three subculturings. In some cases, heterogeneous regions within a single callus developed, so these were subcultured as separate lines. At the end of nine weeks, with subculturings on the first, third, and sixth week, 42 colonies had been derived. These were then separately tested for morphinan alkaloids by RIA with the expectation that some of the colonies would be stable, high-codeine producing lines. Surprisingly, morphinan alkaloids were not detected in any of the callus samples.

DISCUSSION

Earlier reports gave the impression that poppy cell cultures generally did not accumulate morphinan alkaloids. A number of workers reported the growth of poppy cells *in vitro*, but few mentioned the detection of morphinan alkaloids in the cultures. For those cases where alkaloids were found, the observations were made on single lines, providing no comparison of alkaloid contents among different cul-

tures (1). This suggested that extensive screening would be necessary to locate a productive callus. Thus our finding of positive RIA responses for morphinan alkaloids in 45% of the healthy cultures was quite unexpected. The contrast of our results with our expectations suggests that the previous work has been limited by analytical methods. For our studies, the immediate advantage of the radioimmunoassay system was, therefore, its extreme sensitivity.

Morphinan alkaloid analysis by hplc of callus extracts revealed that the normal ratio of alkaloids found in the mature plants were not mimicked in the callus extracts. The *P. somniferum* variety from which the calli were derived produces alkaloids with a morphine/codeine/thebaine ratio of approximately 90/5/0.7, which is typical of commercial plants. In contrast, none of the high-alkaloid producing cultures developed a predominance of morphine.

The appearance of codeine as the major morphinan in five of six callus samples, and thebaine in the other, indicated significant changes from normal alkaloid biochemistry as it occurs in the plants. This invites comparative studies of plants and cell cultures to provide insights into this complex field. The accumulation of codeine also suggests the possible biotechnological application of cell cultures, since it would also obviate the present necessity of codeine synthesis from morphine.

Callus initiation and growth promotion were observed with certain hormone additions to the basic media. Although these responses showed some variability, methodology for poppy callus culture was established. Morphinan alkaloid accumulation in these cultures must also be considered a response to the transfer of tissue and its subsequent growth on media of new hormone supplements. Tissue before the transfer was shown not to contain significant concentrations of morphinans. However, alkaloid accumulation had no apparent correlation with the addition of particular types and quantities of hormone supplements to the basic medium.

The failure to maintain alkaloid production through successive subculturings on the same medium may occur as the nature of the alkaloid-producing cells changes. However, this may instead simply reflect the enrichment of the cultures with cells devoting resources to replication in preference to secondary metabolism. Methods are needed to identify and select those cells which synthesize morphinan alkaloids. Pure cultures can then be studied to differentiate between these hypotheses.

EXPERIMENTAL

CALLUS INDUCTION AND GROWTH OPTIMIZATION.—Seed of *Papaver sominferum L.*, of origin previously described (5), were surface sterilized in 3% sodium hypochlorite by swirling for 30 sec under 15 mm pressure followed by gentle agitation at atmospheric pressure for 12 min. The seeds were separated and washed with several rinses of sterile deionized, distilled water and transferred to moist sterile filter paper for germination in the dark for 10 days.

For the experiments to determine induction conditions, the whole seedlings were placed directly on media of either Murashige and Skoog (M&S) (5) without glycine addition, or that of Miller (1968) (6). Both were supplemented with 10% coconut water. The media were adjusted to pH 5.8 before autoclaving. Hormones tested are described in the text. The cultures were subjected to 24 h light and 12 h light-12 h dark conditions at 23° to 28°.

For subsequent experiments, standard conditions for inducing callus involved excising 3 to 5 mm sections of the seedling hypocotyls and placing these on Miller's 1968 medium with 10% coconut water and the hormones (mg/liter): kinetin (0.1), 2,4-D (1.2), NAA (0.2). Cultures were grown on a 12 h light-12 h dark photo-period under Sylvania Gro-Lux fluorescent lamps in a growth chamber at 24° to 27°. Callus was subcultured every 2 to 3 weeks.

Experiments to promote growth of induced callus were done with material initiated as above and subcultured after 14 days onto fresh induction medium. After 14 more days, portions of calli were transferred onto various growth-promotion media as described in the text. Samples of the calli were collected 23 days after the last subculturing for alkaloid analyses.

RADIOIMMUNOASSAYS (RIA) OF MORPHINAN ALKALOIDS.—In an all glass tissue homogenizer, 0.12 to 0.3 g of fresh callus was homogenized without buffer and the resulting crude homogenate used immediately or fast frozen on dry ice for storage. A 100 μliter portion of the crude homogenate was analyzed directly using a commercially available radioimmunoassay kit for

morphine.[1] A callus homogenate had been previously analyzed on a larger scale by hplc and found to contain no detectable quantity of morphinan alkaloids (<0.1 μg/200 g callus). Samples of the homogenate were spiked with known quantities of thebaine, codeine, or morphine in phosphate buffer at pH 6.5 and tested by RIA to provide a calibration of the assay system for each alkaloid from 0.1 to 100 mg per sample.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF MORPHINAN ALKALOIDS.—For hplc analysis, a 1.5 to 3 g callus sample was mixed with 20 ml of methanol, ground in a small blender, diluted to 40 ml with methanol, centrifuged, and the supernatent removed and evaporated under vacuum. The residue was partitioned between 5 ml of 0.5M H_3PO_4 and 5 ml of chloroform. The aqueous layer was separated, cooled in ice-H_2O, basified to pH 8.3–8.8 with NH_4OH, and extracted twice with 5 ml of chloroform/isopropanol (3/1). The combined organic extracts were dried with anhyd. Na_2SO_4 and evaporated.

Extremely sensitive analyses were possible only after careful attention to instrumental conditions.[2] The alkaloid samples were dissolved in hplc-grade chloroform/methanol/distilled triethylamine (90/10/0.1). Alkaloids in 10 μliter injections were separated with the same solvent system flowing at 1.0 ml/min through a normal phase Altex Ultrasphere-Si column (5 μm particles, 4.6 mm id x 250 mm). The alkaloids were detected at 285 nm and had the following retention times (min): thebaine, 4.5; codeine, 5.3; morphine, 8.2. Detection limits were highest for morphine: 0.06 μg per injection.

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LITERATURE CITED

1. W. H. J. Tam, F. Constabel, and W. G. W. Kurz, *Phytochemistry*, **19**, 486 (1980), and references cited therein.
2. A.-F. Hsu, *J. Nat. Prod.*, **44**, 408 (1981).
3. E. W. Weiler in "Plant Tissue Culture and Its Bio-technological Application", W. Barz, E. Reinhard, and M. H. Zenk, Eds., Springer-Verlag, New York, 1977, p. 266.
4. C. L. Nessler, and P. G. Mahlberg, *Can. J. Bot.*, **57**, 675 (1979).
5. T. Murashige, and F. Skoog, *Physiol. Plantarum*, **15**, 473 (1962).
6. C. O. Miller in "Biochemistry and Physiology of Plant Growth Substances", F. Wightman and G. Setterfield, Eds., The Runge Press, Ottawa, Canada, 1968, p. 33.
7. C. C. Hodges, J. S. Horn, and H. Rapoport, *Phytochemistry*, **16**, 1939 (1977).

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[1]Abuscreen Radioimmunoassay for Morphine by Roche Diagnostics, available in an 100 assay kit, was used here. The protocol specified in the test kit was applied to analysis of callus homogenates directly with the exception that the volume of sat. $(NH_4)_2SO_4$ for antibody precipitation was increased to 140% of the kit value.

[2]A Spectra-Physics 8000 Liquid Chromatograph utilizing a microprocessor-controlled mode to reduce pressure fluctuations in pumped solvents was used in combination with a Schoeffel 770 Spectrophotometric Detector.