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MORPHINANE ALKALOIDS IN CULTURED TISSUES AND REDIFFERENTIATED ORGANS OF *PAPAVER SOMNIFERUM*

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Key Word Index—*Papaver somniferum*; Papaveraceae; callus culture; differentiated organs; morphinane alkaloids.

Abstract—The capacity of alkaloid synthesis was examined in cultured tissues of *Papaver somniferum*. Callus, derived meristemoids, redifferentiated roots and redifferentiated shoots grown on nonamino acid-containing media containing natural (indoleacetic acid and isopentenyl adenine) and synthetic (kinetin, naphthaleneacetic acid and 2,4-dichlorophenoxyacetic acid) hormones were analysed for their capacity to synthesize thebaine, codeine, and morphine. Callus tissues produced thebaine and, on certain hormones or their combinations, also produced codeine. Derived meristemoids and roots synthesized only thebaine, whereas redifferentiated shoots synthesized morphine as well as thebaine and codeine. These studies emphasize the importance of the level or degree of cell and tissue differentiation for synthesis of particular alkaloids in the morphinane pathway.

INTRODUCTION

Papaver somniferum, opium poppy, is known for its capacity to synthesize and accumulate the morphinane alkaloids, thebaine, codeine, and morphine, in the laticifer cell of the normal plant. Recently, several investigators have examined cultured tissues of *P. somniferum* for production of these alkaloids. Two groups [1, 2] have reported that all three alkaloids, including morphine, were synthesized in suspension cultures grown on Murashige and Skoog's (MS) medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) in the absence [1] or presence [2] of amino acids. Another group [3] identified codeine in suspension callus cultures grown on media supplemented with casein hydrolysate. However, in other studies of cultured tissues and regenerated plantlets, these investigators were unable to detect any morphinane alkaloids, although other alkaloids were present [4, 5]. Subsequent work reported that suspension cultures converted (−)-(s)-reticuline to (−)-(s)-scoulerine and (−)-(s)-cheilanthifoline, but not to thebaine, codeine, or morphine [6]. In another study [7] it was demonstrated that suspension cultures grown on MS medium supplemented with 2,4-D and zeatin or kinetin contained no detectable morphinane alkaloids.

The bases for these diverse reports on the alkaloid composition of cultured tissues is unclear, but may relate, in part, to the level of differentiation present in the cultured tissues. It has been shown in an ultrastructural study of cultured tissues of *P. somniferum* that laticifer cells were absent from callus [8]. The same work further reported the presence of two recognizable levels of re-organization within the callus cultures. One level was represented by the formation of meristemoids or organ-like structures arrested at an early stage in re-organization and lacking laticifers. The other was represented by redifferentiated shoots and roots possessing tissues including laticifers typical of the normal plant.

The purpose of this study was to examine the capacity of callus tissues, meristemoids, and redifferentiated roots and shoots to synthesize morphinane alkaloids when grown on media containing different combinations of natural [indoleacetic acid (IAA) and isopentenyl adenine (IPA)] or synthetic [2,4-D, kinetin (K) and naphthaleneacetic acid (NAA)] plant hormones. No amino acids were added to the media. Thus, the occurrence of alkaloids would reflect the presence in the cells or tissues of the complete biosynthetic complex for the production of morphinane alkaloids.

RESULTS

Callus alkaloids and growth. Callus tissues contained 0.59–33.1 μg total morphinane alkaloids/g dry wt tissue. The quantity of alkaloid in callus varied for different media (Table 1). The total morphinane alkaloid content in callus grown on different combinations of IPA was relatively low. However, combinations of IPA with IAA (0.1 ppm) typically resulted in a relative increase in total alkaloid in callus tissues. The highest amount of alkaloid (31.2 μg) in callus occurred on IPA (4 ppm) and IAA (0.1 ppm).

Alkaloid formation on media containing different concentrations of K was relatively low; NAA alone (0.2 ppm) yielded a higher total alkaloid concentration (10.3 μg) than any level of K employed in this study. When K was combined with NAA (0.2 ppm) there was an appreciable increase in total alkaloid content on two media. The highest level of alkaloid production (33.1 μg) in callus tissues was present in a combination of K (4 ppm) and NAA (0.2 ppm) (Table 1). In contrast, there was a relatively small alkaloid production (1.1 μg) for tissues on either 2,4-D (0.1 ppm), or with a combination of 2,4-D and kinetin (0.1 ppm), although these callus tissues grew well (Table 1).

Table 1. Tissue weight and alkaloid composition of callus from *P. somniferum* grown on different hormones

Hormone (ppm)	Callus (mg dry wt)	Morphinane alkaloids Total (μg/g dry wt)	Thebaine (%)	Codeine (%)
IPA				
0.2	17.7	6.4	85	15
0.5	33.0	1.4	100	0
1.0	29.5	2.8	100	0
2.0	36.9	2.1	100	0
4.0	35.0	0.8	100	0
IPA + IAA				
0.0–0.1	9.0	6.6	100	0
0.2–0.1	13.0	6.0	100	0
0.5–0.1	11.3	16.0	22	78
1.0 0.1	6.5	3.1	81	19
2.0–0.1	12.6	0.8	100	0
4.0–0.1	3.8	31.2	15	85
K				
0.1	7.9	6.9	100	0
0.5	14.8	3.2	100	0
1.0	27.7	3.4	83	17
2.0	42.1	8.5	32	68
4.0	25.1	3.9	2	98
K + NAA				
0.0 0.2	31.4	10.3	100	0
0.1 0.2	15.3	13.1	100	0
0.5–0.2	15.5	2.9	100	0
1.0 0.2	15.9	1.9	100	0
2.0 0.2	22.9	0.6	100	0
4.0–0.2	6.9	33.1	1	99
2,4-D + K				
0.1 0	29.0	1.1	91	9
0.1 0.1	19.0	1.1	100	0

Thebaine and codeine were determined in callus by TLC and GC, and morphine by HPLC analyses. The alkaloids were identified further by mass spectrometry. Thebaine was the predominant alkaloid and was detected in callus grown on all 24 selected hormone concentrations employed in this study (Table 1). Codeine was also synthesized in callus grown on 15 media combinations and was the predominant alkaloid (over 50% of total) in five media (Table 1). The highest percentages of codeine (98–99%) compared with thebaine occurred on a medium containing a combination of K (4 ppm) and NAA (0.2 ppm), and on K (4 ppm).

Callus tissue growth (dry wt) differed for each hormone concentration and reflected differences for alkaloid concentrations in the callus (Table 1). The range in tissues grown on the synthetic hormone combinations of NAA and K (6.9 22.9 mg tissue/callus piece) was greater than that for tissues grown on the natural hormone combinations of IAA and IPA (3.8–13.0 mg tissue/callus piece). Growth was consistently greater for tissue grown on concentrations of IPA alone (17.7 36.9 mg tissue/callus piece) and most K concentrations (7.9–42.1 mg tissue/callus piece) as compared with the respective combinations of auxin and cytokinin.

Shoots. Shoots redifferentiated in greatest abundance on media containing K (2 ppm) than on other selected hormone concentrations employed in this study. Shoots which developed on IPA or IAA media were normal in appearance whereas they appeared abnormal when redifferentiated on the callus grown on media containing 2,4-D (0.1 ppm), or K and 2,4-D. Shoots did not develop roots.

Shoots removed from callus and analysed for alkaloids were found to contain all morphinane alkaloids, thebaine, codeine, and morphine. Total morphinane alkaloid content of these shoots averaged 187 μg/g dry wt, an amount lower than that found for shoots of intact seedlings (Table 2). The percentage of each alkaloid was 40% thebaine, 12% codeine and 48% morphine. Thebaine and codeine were identified by GC; morphine; by HPLC. The thebaine, codeine, and morphine peaks from HPLC were collected and confirmed to be these respective alkaloids by GC/MS using derivatized samples [9].

Meristemoid alkaloids. Dense white nodular masses, or meristemoids, which enlarged typically to *ca* 2 mm, differentiated on callus grown on all hormone concentrations. These nodules were removed from the callus and analysed for their alkaloid composition. Thebaine was the only morphinane alkaloid detectable in meristemoids (Table 2).

The white nodular meristemoids when isolated from callus and cultured on media either maintained the white nodular morphology or differentiated into buds. The

Table 2. Morphinane alkaloid content of different tissues of *P. somniferum*

Tissue	Total (μg/g dry wt)	Alkaloid (%) Thebaine	Codeine	Morphine
Intact seedling				
Shoot	1040	12	2	86
Root	999	43	6	51
Callus-induced organs				
Meristemoids, removed from callus (no buds)	13.2	100	0	0
Cultured meristemoids with buds	Trace	100	0	0
Shoots	187	40	12	48

latter upon analysis were found to contain traces of thebaine (Table 2). Only a few of these buds developed into shoots *ca* 1 cm tall under our culture conditions.

Roots. Roots frequently redifferentiated on callus tissue, although they typically remained short, less than 1 cm long. The largest number of roots developed on callus grown on media supplemented with NAA (0.2 ppm), or NAA and K (0.2 and 0.1 ppm, respectively). Root formation at these hormone concentrations often was so profuse as to cover the callus piece completely. Roots did not commonly redifferentiate from callus grown on media supplemented with the natural hormones, IAA or IPA, as employed in this study. Callus possessing roots, as when grown on NAA and K (0.2 and 0.1 ppm, respectively), had a somewhat higher thebaine concentration (20.4 μg/g dry wt tissue) than callus which lacked roots but was harvested from medium at the same time (18.9 μg thebaine/g dry wt tissue).

DISCUSSION

Alkaloid analyses showed that short-term callus tissues synthesized small quantities of thebaine and codeine when grown on a culture medium without added amino acids. Alkaloid concentration and composition of cultured tissues was a reflection of hormone composition of the medium, callus growth rate, and extent of organ redifferentiation on the cultures. Thebaine was detectable in nearly all cultures. No clear basis for codeine production was evident, however, since it was detectable in callus grown on several hormone combinations including IPA and IAA, or K, or K and NAA.

Alkaloid synthesis in tissues appeared to be related to the growth rate of tissues. Fast-growing tissues, such as those grown on IPA or K, had relatively low alkaloid concentrations compared to slow-growing tissues cultured on combinations of K and NAA, or IPA and IAA. The highest percentages of codeine compared with thebaine occurred in callus with a low growth rate. These cultures, grown on combinations of IPA and IAA, or K and NAA, typically contained the highest total alkaloid content.

Organ redifferentiation influenced alkaloid synthesis in that morphine, as well as codeine and thebaine, occurred in shoot organs redifferentiated on callus. Shoots derived from the callus produced greater amounts of total alkaloids than callus indicating that the shoot controlled both the quality and quantity of alkaloids. It is possible that the synthesis of morphine in these shoots may be related to the presence of laticifers known to be present in regenerated shoots on *P. somniferum* [8]. Redifferentiated shoots contained less alkaloid than shoots of intact seedlings. Roots on callus, and meristemoids with and without buds, produced only thebaine. Thus, thebaine and codeine can be synthesized by callus cells as well as organs, but redifferentiated shoots appear necessary for the production of morphine. One exception to be noted was that cultured meristemoid samples which contained buds and one or two small shoots possessed no detectable codeine or morphine. Although these organs were present, the relative amount of alkaloids in them was probably too low to be detected.

HPLC is preferred over GC for the analysis of morphine when performed as described in this report. Morphine peaks were absent in GC analyses, but were present in analyses performed by HPLC. The identity of morphine in shoot samples was confirmed following isolation and GC/MS identification using derivatized samples.

These experiments and others [1–3] have demonstrated that short-term static callus tissues and suspension cells in culture have the capacity to synthesize morphinane alkaloids. These studies, like others [1–3], have shown that alkaloid synthesis will occur on media supplemented with 2,4-D as well as on media containing natural hormones. Cultures supplemented with [2, 3] and without [1] amino acids have been reported to contain morphinane alkaloids indicating that these compounds are not prerequisite for alkaloid synthesis in culture. In this study we observed that tissue and organ redifferentiation into shoots is a major factor that influences alkaloid synthesis, and that morphine was synthesized only in redifferentiated shoots.

EXPERIMENTAL

Tissue culture. Seeds of *P. somniferum* L. (gift of Dr. O. Braenden, United Nations) were surface sterilized with Clorox for 10 min, washed with sterile deionized H_2O and germinated to seedlings possessing the first primary leaves on 1% non-nutrient agar in 125 ml conical flasks capped with polypropylene film. Callus cultures were developed either from leaf, hypocotyl, and root segments derived from seedlings placed on Murashige and Skoog medium [10] without amino acids supplemented with 2,4-D (0.1 ppm) and K (0.1 ppm), or NAA (0.2 ppm) and K (0.1 ppm) for callus induction. Callus, after its establishment and separation from parent organs, was transferred to media containing a selected hormone concn. All transfers were made at 28-day intervals. Inocula consisted of four pieces of callus, each *ca* 75 mg fr. wt or 2 mg dry wt, placed on 40-ml media in flasks. All cultures

were grown in a culture room under a 12 hr light (700 lx)–12 hr dark cycle at 25°.

The selected hormone concns and combinations employed in this study are listed in Table 1. Calli were grown for two 28-day periods on each test medium and then harvested for alkaloid analysis. Analyses were performed on callus tissues after the second and third 28-day period of culture on the test media.

Harvested callus was lyophilized and a 200 mg dry wt quantity of lyophilized tissue of each sample was extracted for alkaloid analysis [11]. The final residue was dissolved in EtOH. 75% of the EtOH extract was used for prep. TLC and the remaining 25% for HPLC. Thebaine and codeine were analysed by GC but morphine was analysed by HPLC.

TLC. Samples were applied to heat-activated (100° for 1 hr) Si gel sheets (Eastman Kodak No. 6061) and run in a freshly prepared solvent system of EtOAc–MeOH–NH_3 (17:2:1) [12]. Morphinane alkaloid R_f values were determined using authentic alkaloids and spots were visualized using Dragendorff's reagent [13] and UV. Prep. TLC was then performed and the specific alkaloid spots were scraped from the sheets for elution. The adsorbent was placed in 2 ml 0.0625 M Tris buffer, pH 9, to which was added 2 ml $CHCl_3$–*iso*PrOH (3:1). The mixture was vortexed and centrifuged 10 min at 1000 g, and the $CHCl_3$–*iso*PrOH fraction collected. This extraction procedure was repeated ×3 and the combined fractions dried down using N_2. This residue was resuspended in EtOH and an aliquot of the sample injected into the GC.

GC. Alkaloid analysis was performed with a FID instrument. The temp. was programmed from 200 to 290°, 8°/min, with a N_2 flow rate of 50 ml/min. The injection port temp. was 250° and the detector 300°. Glass columns (2 m × 2.43 mm i.d.) were packed with 3% OV-17 on 100/120 Supelcoport. Cholesterol (chromatography grade, 0.25 mg/ml) and antipyrine (0.25 mg/ml) were used as int. standards.

HPLC. For morphine analysis, the HPLC was equipped with a 5-μm porous Si gel (Perkin–Elmer) stainless steel column (30 cm × 3.9 mm i.d.) and a syringe-loading injector with 100-μl capactity. The solvent system was nanograde hexane $CHCl_3$ EtOH Et_2NH (60:6:8:0.1). The flow rate was 2 ml/min; the wavelength setting on the UV detector was 285 nm. Standard alkaloid curves were prepared daily to quantitate alkaloids.

GC/MS. The desired compound for GC/MS analysis was collected by HPLC, dried down under N_2, and then resuspended in EtOH. Derivatized samples were prepared. Standards (200 μg morphine, 200 μg codeine, 100 μg thebaine) also were derivatized for comparison. Standards were dried down and Tri Sil Z containing pyridine (50 μl) (Pierce) was added to each and then mixed. The mixture was kept for 30 min at room temp. and subsequently injected into the GC/MS. This same procedure was used for the tissue culture samples, except that 20 μl Tri Sil Z containing pyridine were added to the residue [14]. A quadrupole low-resolution mass spectrometer was employed. Glass columns (6 ft × 2 mm i.d.) were packed with 3% OV-17 on 100/120 Gas Chrom Q (Applied Science Co.). An interface temp. between GC and MS of 250°, an ionizing voltage of 70 eV, and a flow rate of 25 ml/min were utilized. The scan speed was set at 330 μm/sec.

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