Phytochemistry, Vol. 28, No. 8, pp. 2085–2088, 1989.
Printed in Great Britain.

0031-9422/89 $3.00 + 0.00

AGE AND TISSUE DISTRIBUTION OF ALKALOIDS IN *PAPAVER SOMNIFERUM*

ROBERT D. WILLIAMS and BRIAN E. ELLIS

Department of Chemistry and Biochemistry, University of Guelph, Guelph, Ontario N1G 2W1, Canada

(*Received in revised form* 18 *January* 1989)

Key Word Index—*Papaver somniferum*; Papaveraceae; cv Marianne; morphine; codeine; phthalidineisoquinoline alkaloids; narcotoline; noscapine; tissue distribution.

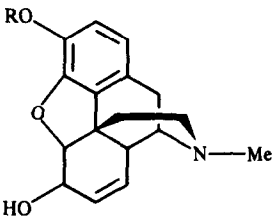
Abstract—The accumulation of alkaloids was studied in *Papaver somniferum* cv Marianne tissues from germination to post-petal-drop using analytical HPLC and TLC analysis. Morphinan and phthalidineisoquinoline alkaloids were found to accumulate in a time and tissue specific manner. The highest accumulation was observed in day 30 root tissue, where morphine and narcotoline reached concentrations of 313 μg/g and 490 μg/g fr. wt, respectively. Juvenile root tissue may be a useful tissue source for studies of the biosynthesis of these alkaloids.

INTRODUCTION

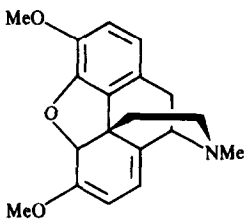
The opium poppy, *Papaver somniferum*, is an important medicinal plant because of its content of pharmacologically active benzylisoquinoline alkaloids including morphine (**1**), codeine (**2**), thebaine (**3**), papaverine (**4**) and noscapine (**5**). It is also being considered as an oilseed crop [1]. All known Papaveraceae species contain benzylisoquinoline alkaloids [2], but only a few closely related *Papaver* species have the capacity to produce morphinan alkaloids [3, 4]. Although the outlines of the biosynthetic pathway leading to the morphinan skeleton have been known for some time [5], the nature of several crucial reactions remains uncertain, and little is known about the overall regulation of morphinan synthesis.

An understanding of the age and tissue distribution of the morphinan alkaloids within *P. somniferum* plants is of importance when choosing a suitable tissue source for the study of morphinan biosynthesis and it may also provide insights into the regulation of this pathway. Previous studies have usually been restricted to older plants [5, 6], seedlings [3, 7], or redifferentiated tissues [8], with only a few studies of the complete life cycle [9, 10]. However, the latter studies used field-grown material where lack of environmental control complicates interpretation of the results.

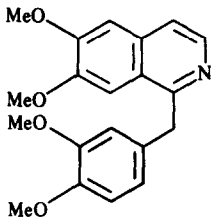
Detection and quantitation of the morphinan alkaloids can be carried out by PC, TLC, HPLC or radioimmunoassay (RIA). HPLC analysis is preferable for examination



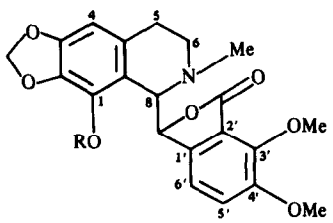
1 R = H
2 R = Me



3



4



5 R = Me
6 R = H

of the complete alkaloid spectrum, particularly with samples which may contain unknown compounds.

The present study describes the pattern of accumulation of the main papaver alkaloids over the life of the plant in different tissues of *P. somniferum* cv Marianne, when the plants were grown under controlled environmental conditions.

RESULTS AND DISCUSSION

Alkaloid profiles of the various tissue samples analysed by HPLC consistently showed the presence of morphine as well as a large unidentified peak at R_t 17.8 min. As the latter compound was the largest peak in the profile, its identification was undertaken. On the basis of ^{1}H NMR and mass spectra and by comparison with spectral data from hydrastine and other closely related compounds [11], the alkaloid was identified as narcotoline (**6**). Narcotoline has previously been reported to be present in opium at a concentration of 0.03% [12], as a minor alkaloid in some cultivars of *P. somniferum* plants (0.08% dry wt) [9], but not in others [10], and in young tissue at 0.016–0.018% (dry wt) [7]. In the cultivar Marianne narcotoline is clearly a much more prominent constituent of vegetative tissue.

Temporal patterns of alkaloid accumulation

The alkaloid profiles from aerial and root tissues of different ages showed marked differences both in magnitude and timing of alkaloid accumulation (Figs 1 and 2). No morphinan alkaloids could be detected in either tissue of seedlings until day 10, when a small amount of thebaine appeared. Codeine and morphine were not detected until days 15 and 20, respectively. This chronological pattern for the appearance of the major morphinan alkaloids parallels the sequence in which they are believed to be biosynthesized [5]. In a recent study of the accumulation of morphinan alkaloids in poppy seedlings using a very sensitive RIA method [4], the same alkaloids could be detected in even younger tissue and the same order of appearance was observed.

The alkaloid profile of the aerial tissue at different ages showed an increase in morphine accumulation from day

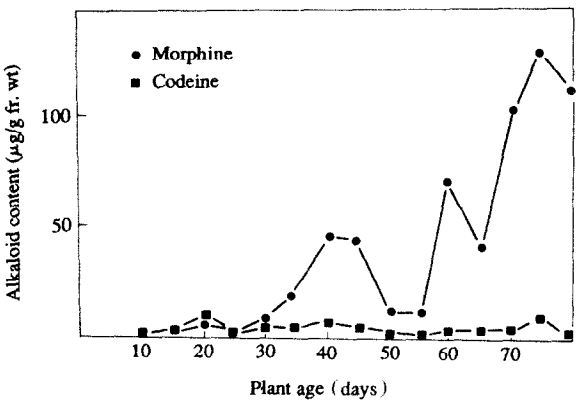


Fig. 1. Morphinan alkaloid content of the aerial tissue of developing *P. somniferum* plants. Each point represents the average from triplicate determinations for day 5 to day 65, and from duplicates for day 70 to day 80 (standard deviations not shown). For methods see Experimental.

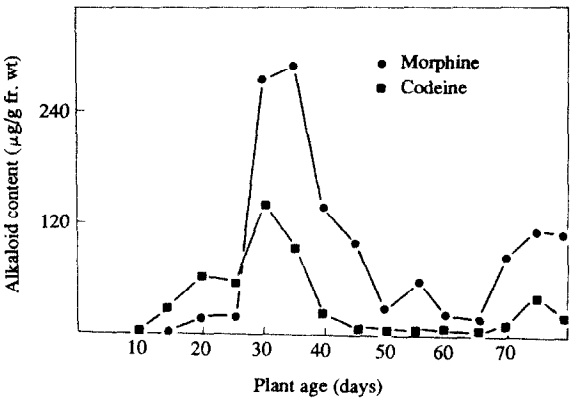


Fig. 2. Morphinan alkaloid content of the root tissue of developing *P. somniferum* plants. Points as indicated in Fig. 1.

35 to day 40, followed by a decrease at day 45 (Fig. 1). Since this change immediately preceded both bolting and floral development, it may be related to a shift in the developmental programme of the plant. The most striking aspect of the aerial tissue alkaloid content was the marked rise in morphine content between day 55 and day 80. This increase began during the bolting period and is correlated with extensive stem development followed by capsule formation. Both of these tissues contain an extensive laticifer network whose presence has been correlated with formation of morphinan alkaloids, but not with production of other benzylisoquinoline alkaloids [13].

In the root tissue, a remarkably high tissue concentration of morphine, and to a lesser extent, codeine, was attained on days 30 and 35, followed by a sharp decline (Fig. 2). This rise and fall seems to have been associated with a developmental shift within the roots, as extensive branching of the roots was observed in this same period. The loss of these alkaloids from the roots between days 40 and 50 was correlated with an increase in the alkaloid content of the aerial tissue. This shift in alkaloid accumulation between tissues has previously been observed in some studies of *P. somniferum* [14, 15] but not in others [9, 10]. Whether the observed increases in the aerial tissue alkaloid content are due to translocation from the roots, or to *in situ* degradation in the roots and synthesis in the aerial tissue, remains uncertain. As the Marianne root system matured, a steadily increasing accumulation of morphine was observed between days 65 and 80, corresponding to the similar increase in the aerial tissue.

The predominance of morphine in the juvenile root system has been observed in young field-grown plants of several other opium poppy varieties [14, 15], but in another case the timing and scale of codeine accumulation differed markedly from that of morphine in the same tissue [9].

In addition to the morphinan alkaloids, a number of other alkaloids were present throughout the life cycle of the plant. These included precursors of the morphinan alkaloids, (reticuline and salutaridine), phthalidineisoquinoline alkaloids, (narcotoline and noscapine) and the benzylisoquinoline alkaloid, papaverine. The salutaridine/reticuline content never exceeded 6 μg/g fr. wt, or 15 μg/g fr. wt, in the aerial tissue or root tissue, respectively, and was maximal in the root tissue on day 30, a

point which corresponds to the time of maximal accumulation of the morphinan (Fig. 2) and phthalidineisoquinoline alkaloids (Fig. 4).

Phthalidineisoquinoline alkaloids were detected in all tissues of the plant from day 15 onwards (Figs 3 and 4). The temporal pattern of accumulation of these alkaloids in the aerial tissue (Fig. 3) showed no peak of accumulation, but only a general increase from day 10 onwards. In contrast to the aerial tissue, the accumulation of noscapine and narcotoline in the root displayed a distinctive age dependence (Fig. 4). The peaks of accumulation at days 30 and 55 closely match the temporal pattern of morphine accumulation in Marianne roots (Fig. 2), a pattern which has also been observed in *P. somniferum* L. subsp. *eurasiaticum*, var. *glaucum* Rothm. [9]. In the latter case, however, the amounts of narcotoline observed were much lower than those found in the present study.

When the aerial tissue was further separated into various tissue types, tissue-specific differences in the concentrations of various alkaloids were readily appparent (Table 1). In general, the younger tissue (e.g. top leaves, in contrast to rosette leaves) contained greater quantities of alkaloids. The highest total alkaloid content was found in stem, root and capsules. The proportions of individual alkaloids detected in the capsules compares well with those previously reported [9, 10, 14, 16] except for the large amounts of narcotoline detected in the present study.

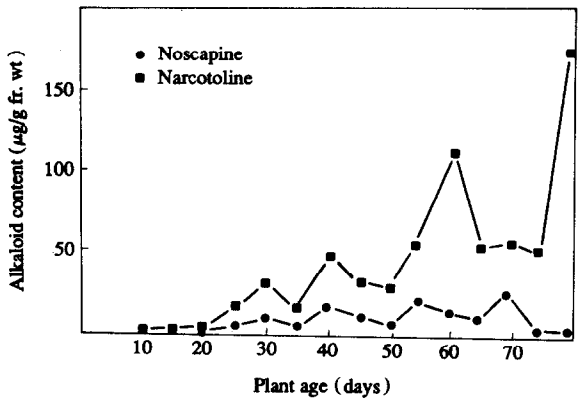


Fig. 3. Phthalidineisoquinoline alkaloid content of the aerial tissue of developing *P. somniferum* plants. Points as indicated in Fig. 1.

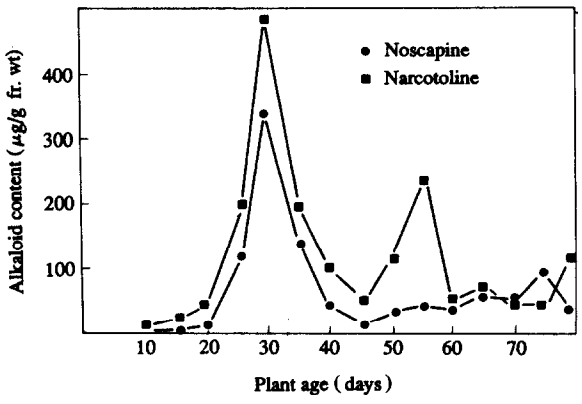


Fig. 4. Phthalidineisoquinoline alkaloid content of the root tissue of developing *P. somniferum* plants. Points as indicated in Fig. 1.

The high concentration of alkaloids detected in juvenile root tissue of the opium poppy poses an interesting problem with respect to the site of biosynthesis. It is not known whether the synthesis of this pool of morphinan alkaloids occurs *de novo* in the roots or takes place in the aerial tissue followed by transport to the roots [14, 15]. It is perhaps relevant that cultured roots of *P. bracteatum* have been reported to synthesize small amounts of thebaine [17], which would imply that no absolute requirement for aerial tissue involvement exists in morphinan synthesis.

The apparent high biosynthetic capacity of juvenile root tissue of *P. somniferum* offers an attractive opportunity for future investigations. If the problems associated with soil/microbial contamination could be overcome and sufficient tissue were readily available, roots of the appropriate age could be a valuable source of the relevant enzymes. To this end, root cultures and 'hairy root' cultures of *P. somniferum* are presently being investigated.

EXPERIMENTAL

Plant material. Papaver somniferum L. cv Marianne seeds (obtained from P. J. van der Have Seeds Rilland, Netherlands) were sown on sterilized soil in a growth chamber under a 16 hr light/8 hr dark regime, watering when required. Plants were always harvested 12 hr into the light cycle to avoid diurnal variation in alkaloid content [9], immediately sepd into different tissue types and used for alkaloid extraction.

Alkaloid extraction. Plant tissue (0.5–25 g fr. wt) was homogenized in 80% aq MeOH (10 ml/g tissue), allowed to stand 24 hr at 4°, filtered and the filtrate concd *in vacuo*. The aq. residue was adjusted to pH 1 (1 M HCl) and washed (3 × 25 ml) with petrol. The aq phase was adjusted to pH 8.5 (1 M NH_4OH) and extracted (3 × 25 ml) with $CHCl_3$–*iso*-PrOH (3:1). The organic phase was evapd and the residue taken up in 95% EtOH (2 ml) for TLC and HPLC analysis. Refluxing the tissue in aq. MeOH did not appreciably increase yields (data not shown). Extraction of tissue with either 1 M HCl, or with concn HCl after MeOH extraction, did not recover any additional alkaloid material, indicating that acid-labile storage forms of these alkaloids [18] were either not present or were already extracted by aq. MeOH.

Alkaloid profiles. Alkaloid analysis was carried out on tissue frs beginning 5 days post-germination and continuing at 5-day intervals until plants were 80-days-old, *ca* 10 days after petal-drop. For each replicate up to day 60, more than 10 plants were combined, from days 65–70, 3 plants were combined and for days 75 and 80 single plants were used. The plants were divided into aerial and root tissue frs until day 60, at which time bolting of the plant was clearly evident. Day 60 plants were divided into leaves, root and stem, while day 65 and older plants were further divided into top leaves, rosette leaves, stem, floral tissue and capsules (when present). The alkaloid profiles from two separate life cycles were very similar (results not shown).

Materials. Authentic morphine, codeine and thebaine were provided by Health and Welfare Canada (Ottawa). Reticuline, *nor*-reticuline, salutaridine, and salutaridinol-I were the gift of Dr K. Rice (NIH, Bethesda, MD). Papaverine, noscapine, and hydrastine were obtained from Sigma.

TLC analysis. Whatman silica gel K6F of the alkaloid fractions, used solvent systems A [$CHCl_3$–MeOH (7:1)] and B [toluene–Me_2CO–EtOH–NH_4OH (20:20:3:1)] [19]. Alkaloids

Table 1. Tissue distribution of morphinan (M) and pthalidineisoquinoline (P) alkaloids in maturing *P. somniferum* plants

Plant age (days)		Tissue alkaloid content (μg/g fr. wt)* Root	Stem	Rosette leaves	Top leaves	Floral	Capsules
60	M	35.5	95.2	57.8	NP†	NP	NP
	P	73.0	140.3	137.5	...	...	...
65	M	41.9	70.8	11.5	57.6	NP	NP
	P	101.8	43.3	31.8	120.3	...	...
70	M	116.9	218.1	13.0	32.6	145.0	NP
	P	75.8	105.8	39.0	62.8	68.5	...
75	M	182.6	277.9	31.7	35.1	167.0	300.1
	P	140.0	104.7	41.5	79.0	27.0	163.8
80	M	162.1	90.0	45.1	168.0	27.9	187.3
	P	73.3	167.0	78.5	92.3	106.8	390.3

*Result from duplicate determinations.
†NP = not present.

were visualized by UV, iodoplatinate [20], Marquis [21] and Frodhe [21] spray reagents and identified by comparison with standard alkaloids.

HPLC analysis. This was carried out [18] on a μBondapak C-18 column (5 μm, 30 cm × 3.9 mm i.d.). The elution programme consisted of 8% MeCN in 0.1 M Pi buffer pH 3.5 (4 min), increasing to 25% MeCN in 0.1 M Pi buffer pH 3.5 over 6 min and maintained for 25 min. Alkaloid samples were filtered (0.45 μm) prior to inj. UV detection (286 nm) was used to prepare standard curves for each alkaloid. The integrated peak areas were normalized to μg alkaloid/g fr. wt tissue. R_ts (min) and detection limits: morphine, 6.0, 500 pmol; codeine, 11.8, 500 pmol; salutaridinol-I, 12.8, 200 pmol; *nor*-reticuline, 16.8, 150 pmol; reticuline/salutaridine, 17.3, 120 pmol; salutaridine, 17.3, 120 pmol; thebaine, 21.1, 65 pmol; noscapine, 27.7, 300 pmol; and papaverine, 28.1, 120 pmol. Narcotoline and papaverine were both analysed as noscapine equivalents. The variability between identical inj was less than 5% of the area of any given peak and re-analysis of the same samples 10 weeks later resulted in less than 5% variation from the previous analysis values. The HPLC method permitted complete sepn of the morphinan alkaloids, but not of some of the other benzylisoquinoline alkaloids. Co-elution of reticuline and salutaridine, and of papaverine and noscapine, required confirmation of the peak identity by TLC using solvent systems A and B, respectively.

Identification of narcotoline. Alkaloid exts were combined and concd. The residue was subjected to prep. TLC (solvent B; R_f 0.7), the compound of interest identified by comparison with a sample isolated by gradient HPLC and eluted from silica gel with $CHCl_3$–*iso*-PrOH (3:1). The eluate was repeatedly chromatographed by isocratic RP-HPLC (23% MeCN in 0.1 M Na-Pi buffer pH 3.5; R_t = 9.8 min) and the purified product was analysed by ^{1}H NMR (400 MHz) and mass spectrometry. CIMS (NH_3) *m/z* 400 $[M+1]^+$ (100), 212 (12), 206 (61), 195 (28); ^{1}H NMR ($CDCl_3$): 2.1, 2.4, 2.7 (4H, *m*, H-5, H-6), 2.55 (3H, *s*, NMe), 3.87 (3H, *s*, OMe), 4.10 (3H, *s*, OMe), 4.39 (1H, *d*, *J* = 4.1 Hz, H-8), 5.66 (1H, *d*, *J* = 4.1 Hz, H-9), 5.97, 5.99 (2H, *d* of *d*, *J* = 1.3 Hz, O–CH_2–O), 6.21 (1H, *d*, *J* = 8.4 Hz, H-5'), 6.28 (1H, *s*, H-4), 6.98 (1H, *d*, *J* = 8.4 Hz, H-6').

Acknowledgements—Our work was made possible by the financial support of the Natural Sciences and Engineering Research Council of Canada.

REFERENCES

1. Nyman, U. and Hall, O. (1974) *Hereditas* **76**, 49.
2. Preininger, V. (1985) in *The Chemistry and Biology of Isoquinoline Alkaloids* (Phillipson, J. D., Roberts, M. F. and Zenk, M. H., eds), p. 23. Springer, New York.
3. Phillipson, J. D. (1983) *Planta Med.* **48**, 187.
4. Wieczorek, U., Nagakura, N., Sund, C., Jendrezejewski, S. and Zenk, M. H. (1986) *Phytochemistry* **25**, 2639.
5. Battersby, A. R., Dobson, T. A., Ramuz, H., Barton, D. H. R., Kirby, U. W., Steglich, W. and Thomas, G. M. (1965) *J. Chem. Soc.* 2423.
6. Fairbairn, J. W. and Steele, M. J. (1981) *Phytochemistry* **20**, 1031.
7. Miram, R. and Pfeifer, S. (1958) *Naturwissenschafen* **45**, 573.
8. Yoshikawa, T. and Furuya, T. (1985) *Planta Med.* **50**, 110.
9. Miram, R. and Pfeifer, S. (1959) *Sci. Pharm.* **27**, 34.
10. Miram, R. and Pfeifer, S. (1960) *Sci. Pharm.* **27**, 15.
11. Sariyar, G. and Shamma, M. (1986) *Phytochemistry* **25**, 2403.
12. Wrede, F. (1937) *Arch. Exp. Pharmakol. Pathol.* **184**, 336.
13. Rush, M. D., Kutchan, T. M. and Coscia, C. J. (1985) *Plant Cell Rep.* **4**, 237.
14. Wegner, E. (1951) *Pharmazie* **6**, 409.
15. Poethke, W. and Arnold, E. (1951) *Pharmazie* **6**, 420.
16. Vincent, P. G. and Engelke, B. F. (1979) *J. Assoc. Off. Anal. Chem.* **62**, 310.
17. Zito, S. W. and Staba, E. J. (1981) *Planta Med.* **45**, 53.
18. Wold, J. K. (1978) *Phytochemistry* **17**, 832.
19. Engelke, B. F. and Vincent, P. G. (1979) *J. Assoc. Off. Anal. Chem.* **62**, 538.
20. Ono, M., Engelke, B. F. and Fulton, C. C. (1969) *Bull. Narcotics* **XXI**, 32, 39.
21. Fulton, C. C. (1969) *Modern Microcrystal Tests for Drugs*, p. 335. Wiley, New York.
22. Wu, C. Y. and Wittick, J. J. (1977) *Anal. Chem.* **49**, 359.