PERGAMON

PHYTOCHEMISTRY

Phytochemistry 52 (1999) 1229–1234

Diurnal fluctuations of the alkaloid concentration in latex of poppy *Papaver somniferum* is due to day–night fluctuations of the latex water content

Kim Itenov[a,b,*], Per Mølgaard[b], Ulf Nyman[b]

[a]*Department of Plant Biology, Research Centre Flakkebjerg, Danish Institute of Agricultural Sciences, DK 4200 Slagelse, Denmark*
[b]*Department of Medicinal Chemistry, Pharmacognosy Section, Royal Danish School of Pharmacy, Universitetsparken 2, DK 2100 Copenhagen, Denmark*

Received 21 October 1998; received in revised form 30 April 1999

Abstract

In this paper we show the diurnal variation in latex water content in the laticifer system of the opium poppy and relate this variation to the observed variation in alkaloid concentration in the latex. A pronounced, opposed variation to the diurnal water fluctuations are shown for the major opium alkaloids, morphine, codeine and noscapine in the poppy cultivar Parmo under dry weather conditions in Denmark in 1994. Diurnal fluctuations of the alkaloid concentration in the opium poppy latex have previously been explained as a result of rapid metabolism in the biosynthetic sequence leading to morphine. Our results, however, disagree with this explanation as a significant correlation between the diurnal fluctuation in the latex water content and that of the alkaloids morphine, codeine and noscapine can be demonstrated. It is concluded that the observed diurnal fluctuations in the concentration of the major alkaloids are not reflections of enzymatic processes but the result of water transport between the laticifers and the surrounding vascular tissue. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Papaver somniferum; Papaveraceae; Alkaloids; Laticifer; Osmoregulation; Stomata control; Transpiration

1. Introduction

Investigations of the ontogenetic variation of secondary metabolites in plants have been made over several decades, e.g. alkaloid changes during fruit development in *Papaver somniferum* L. (Miriam & Pfeifer, 1959) and in *Conium maculatum* (Fairbairn & Challen, 1959). Diurnal variations of alkaloids have been reported for *Datura stramonium* (Hemberg & Flück, 1953), *Lupinus sp*. (Birecka & Zebrowska, 1960), *C. maculatum* (Fairbairn & Suwal, 1961), *Atropa belladonna* (Fairbairn & Wassel, 1967) and for *P. somniferum* (Heidenreich & Pfeifer, 1962).

Diurnal fluctuations in the concentration of the major alkaloids morphine, codeine and thebaine in the latex from immature fruits of *P. somniferum* was first reported in 1964 (Fairbairn & Wassel, 1964). These observations altered the perception of the opium alkaloids from being waste products immobilised in the latex vacuoles into a theory of rapidly interchanging metabolites. Fairbairn and Wassel gave figures for the latex dry matter, defined as the methanol insoluble residue of the extraction (Fairbairn & Wassel, 1964), but its composition is poorly defined and must be less than the content based on true latex dry matter.

Recently, similar investigations have been repeated on plant material cultivated in India (Shukla, Khanna & Singh, 1996) showing a deviating pattern of fluctuations with double maximum of concentrations in the daytime. The authors explain these observations by referring to the theory of rapidly interchanging metabolites influenced by the tropical climate, and doubt the alkaloids as accumulated products. However, in both

* Corresponding author. Tel.: +45-58113300; fax: +45-58113302.

PII: S0031-9422(99)00420-3

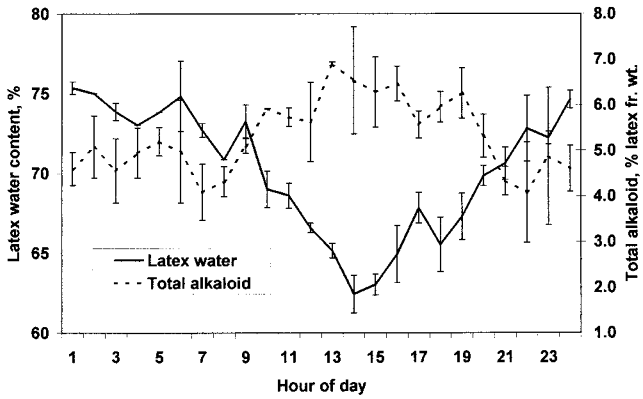

Fig. 1. Diurnal fluctuations of latex water content and total alkaloid concentration (major alkaloid) in latex samples from incised capsules of *P. somniferum*. Values based on latex fresh weight, hour of day average (means ± sd) from a 48 h sampling period.

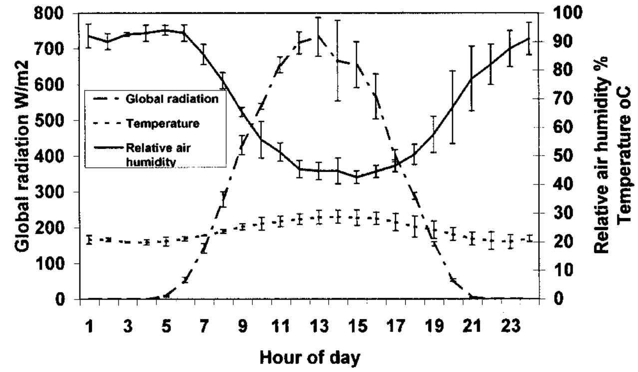

Fig. 2. Diurnal fluctuations of the topoclimatic parameters during a 48 h sampling period (means ± sd) global radiation, temperature and relative air humidity. Hour of day average.

investigations, the alkaloid content is based on latex fresh weight and no test for correlation between alkaloid and water content have been attempted.

The biosynthesis from tyrosine passes norcoclaurine (Loeffler, Stadler, Nagakura & Zenk, 1987; Stadler, Kutchan, Loeffler, Nagakura, Cassels & Zenk, 1987) to (S)-reticuline which is the branching point metabolite of the phthalidisoquinoline pathway to noscapine and via (R)-reticuline to the morphinanes. The biosynthesis of the morphinanes has two pathways: thebaine → neopinone → codeinone → codeine → morphine (Battersby, Martin & Brockmann-Hansen, 1967; Blaschke, Parker & Rapoport, 1967; Parker, Blaschke & Rapoport, 1972) and thebaine → oripavine → morphinone → morphine (Brockmann-Hansen, 1984), both having enzymatic demethylation of 3-O- and 6-O-positions, but with different emphasis on the intermediate codeine.

The major alkaloids of the cultivar Parmo used in this investigation are the morphinane alkaloids morphine, codeine, thebaine and the phthalideisoquinoline alkaloid noscapine which, chemotaxonomically, place the cultivar as the chemoprovariety phthalidisoquinoline (IQ : Phthalid) (Nyman & Hansson, 1978). The alkaloids are stored in latex vesicles, which like the vacuole are tonoplast covered and act as storage compartment of the alkaloids (Fairbairn, Hakim & El Kheir, 1974; Nessler & Mahlberg, 1976; Nessler & Mahlberg, 1977; Wink, 1993) and of inorganic cations and sulphate and meconate (Pham & Roberts, 1991).

In this paper we present the results of a reinvestigation of the diurnal fluctuation of alkaloids in the latex of *P. somniferum* (Itenov, 1999).

2. Results

The averages of all observations of the content of major alkaloids in the latex based on dry weight are (mean ± SD): morphine 13.80% ± 1.72, codeine 3.41% ± 0.90, thebaine 0.25% ± 0.11 and noscapine 0.39 ± 0.13. No papaverine could be detected in this cultivar (Parmo) on any occasion. The average of all observations (mean ± SD) of latex water is 71.02% ± 3.85 and the alkaloid content based on latex fresh weight are morphine (MF) 4.15% ± 0.73, codeine 1.03% ± 0.30, thebaine 0.08% ± 0.03 and noscapine 0.12 ± 0.04%. The simultaneous variations in latex water content and the fresh weight concentration of major alkaloids show pronounced reverse phase fluctuations with high daytime levels of alkaloids coinciding with low latex water levels (Fig. 1). During day time (9.00 to 20.00 h) the level of alkaloids is 5.9% ± 0.67 and during night time (21.00 to 9.00 h) 4.7% ± 0.39. The shift from day time to night time level takes two hours as does the shift in the morning. The diurnal fluctuations of latex water content (LW) attain a night time level of 73.7 ± 1.2 between 22.00 and 8.00 h and a day time average of 66.7% ± 2.6 with a minimum of 62% at 13.00 h. The average day and night level of latex water deviates 3.5% units below and above the daily mean of 70.2%.

In Fig. 2 the relative air humidity is overlayed by the controlling meteorological parameters global radiation and air temperature. The relative air humidity shows a fairly stable night-time level of 92% between midnight and 7.00 h, and an afternoon level of 46% between noon and 17.00 h. The coincidence of maxima and minima together with the increase and decrease of the opposing parameters is clearly shown.

To test the influence of environmental control of the diurnal alkaloid fluctuation, correlation analyses were performed using a dataset of simultaneous measurements of morphine based on freshweight, latex water content, and the climatic parameters global radiation (S_i), relative air humidity (RH) and air temperature.

The positive and negative correlations between these

Table 1
Matrix of correlation coeficients r- and p-values for the latex water content, morphine concentration based on fresh weight and the climatic parameters global radiation, air humidity and air temperature

	Global radiation, W/m²	*Air temperature, °C*	*Air humidity, RH%*	*Latex water, LW%*	*Morphine, MF%*
Global radiation W/m²	**1.0**				
Air temperature °C	**0.81**	**1.0**			
	0.0001				
Air humidity RH%	**−0.87**	**−0.94**	**1.0**		
	0.0001	0.0001			
Latex water LW%	**−0.85**	**−0.85**	**0.89**	**1.0**	
	0.0001	0.0001	0.0001		
Morphine MF%	**0.74**	**0.65**	**−0.67**	**−0.75**	**1.0**
	0.0001	0.0001	0.0001	0.0001	

parameters as well as their level of significance may be derived from Table 1, which also shows the correlation matrix of latex water and morphine with the climatic parameters.

Latex water shows a high negative correlation with the climatic observations (S_i: $r = -0.85$, $p = 0.0001$), (Temperature: $r = -0.85$, $p = 0.0001$), but positive correlation to humidity (RH: $r = 0.89$, $p = 0.0001$). Morphine in contrast is positively correlated with S_i ($r = 0.74$, $p = 0.0001$) and temperature ($r = 0.65$, $p = 0.0001$), but negatively correlated with humidity ($r = -0.67$, $p = 0.0001$). Hence, the maximum morphine concentration occurs in the afternoon when water content is low.

The relation between latex water content (LW) and the latex fresh weight morphine content (MF) shows a significantly negative correlation ($r = -0.75$, $p = 0.0001$). The linear dependency shown in Fig. 3 has a parametric regression fit $Y = 13.131 - 0.1285 \times X$, $r^2 = 0.5664$ (anova: $F = 54.8575, p = 0.0001$), where latex water is the independent variable (X) and morphine content the dependent variable (Y).

Also the fresh weight content of the other main alkaloids codeine and noscapine correlates negatively with latex water (Table 2), for codeine $r = -0.41$ ($p = 0.0054$) and noscapine $r = -0.52$ ($p = 0.0003$), while there is no correlation of thebaine $r = -0.17$ ($p = 0.2783$) with latex water.

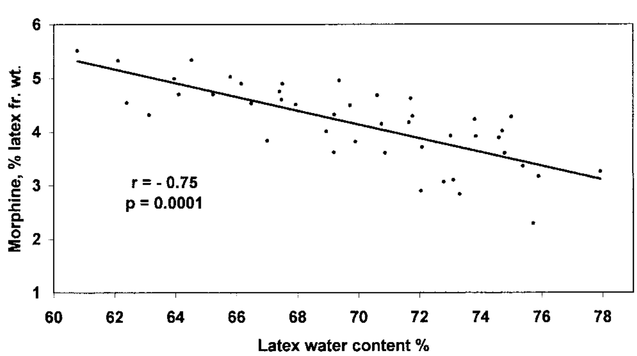

Fig. 3. Correlation of morphine concentration (latex fresh weight) with latex water content in samples from incised capsules of *P. somniferum* from a 48 h sampling period.

Fig. 4 shows all hourly observations of the latex fresh weight content of morphine, codeine, thebaine and noscapine together with smoothed average (3 h) of the major alkaloid content over a 48 h sampling period.

Morphine fluctuates about the 4% level with daytime maxima passing the 5% line.

Codeine fluctuates in a similar way about the 1% level with maximum in the daytime and minimum at night. The content of noscapine and thebaine are orders of magnitude lower. The fluctuation in individual alkaloids is rapid, especially for the minor ones. However, the smoothed total gives evidence for the diurnal cycle.

3. Discussion

The high water content of the latex in the night and the low content in the daytime compared with the occurrence of the higher level of morphine in day time indicates that the water content perform a controlling function in the fluctuation and the high negative correlation between the latex water content and the morphine concentration is a reliable support for the proposed hypothesis, that changes in latex water content are the background for fluctuations in morphine concentration. The high correlation between the cli-

Table 2
Matrix of correlation coefficients r- and p-values for the correlation of latex water (LW) content with the concentration, % based on fresh weight of morphine, codeine, thebaine and noscapine

	Morphine (%)	Codeine (%)	Thebaine (%)	Noscapine (%)
r	**−0.75**	**−0.41**	**−0.17**	**−0.52**
p	0.0001	0.0054	0.2783	0.003

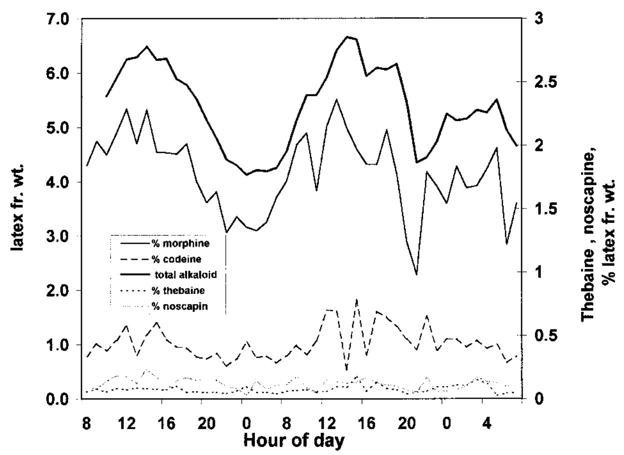

Fig. 4. Hourly observations of concentration major alkaloids morphine, codeine, thebaine, noscapine and the 3 h smoothed average of the total alkaloids in latex samples from incised capsules of *P. somniferum*. Concentration based on latex fresh weight during the 48 h period.

matic parameters and the latex water content frame the theory that it is the environmental control of water status in the plant that causes the diurnal fluctuation in the alkaloid concentration in the latex.

The influence of water as a controlling factor in the diurnal fluctuations was never tested in the previous papers (Fairbairn & Wassel, 1964; Shukla et al., 1996). However, the authors related the alkaloid content to an insoluble fraction which is not equivalent to the dry matter content, but called so because it was the fraction of exudate which was not dissolved during the extraction procedure.

We analysed the data of Fairbairn & Wassel (1964) by correlation analysis of morphine concentration to water content from the two hourly observations of July 10th, 1962 for 19 samples (series T5) and found a correlation coefficient $r = -0.68$ ($p = 0.0013$), and those from the three hourly measures of July 13th–14th for nine samples (series T2) gave a correlation coefficient $r = -0.84$ ($p = 0.0045$). These results are very similar to our observation of $r = -0.75$ ($p = 0.0001$) and strongly support the hypothesis that the variation in the latex water content results in dilution, respectively, concentration of the latex dry matter and the morphine, irrespective to the amount of undissolved dry matter.

The report of two maxima of alkaloid content in the diurnal fluctuations found in India (Shukla et al., 1996) could be explained as stomata control of the transpiration by closing stomata between noon and 16.00 h.

Stomata control of the ability of plants to regulate the water content in their tissue is used to classify plants as isohydric or anisohydric. In the latter case, the water content in the plant body may fluctuate substantially in response to microclimatic variation in the environment (Tardieu, Lafarge & Simonneau, 1996).

The water transport through plants normally follows a diurnal cycle governed by the osmotic potential in the soil and the relative air humidity (Scholander, Hammel, Bradstreet & Hemmingsen, 1965). The resulting water potential in the xylem influences the turgor pressure in the associated vascular tissue, of which the laticifers of *P. somniferum* are integrated parts (Fairbairn & Kapoor, 1960; Kapoor, 1973). The relationship between climatic factors and the osmotic status of latex water was first described in *Hevea brasiliensis* L. (Buttery & Boatman, 1966), which has the same type of articulated, anastomosing laticifers as *P. somniferum* (Kapoor, 1973; Fahn, 1979), although placed in the secondary phloem in this tropical tree.

The existence of water channels, aquaporines, in the plasma membranes and the tonoplast (Daniels, Mirkov & Chrispeels, 1994; Chrispeels & Maurel, 1994; Fray, Wallace, Grierson & Lycett, 1994) in combination with ion-channels (Schroeder, 1988) and proton pumps (Martinoia, 1992) emphasises the active role of water in regulating the osmotic pressure in the laticifers, whereby the stored alkaloids are diluted or concentrated in the latex vesicles. Evidence of rapid metabolism of alkaloids in the latex of *P. somniferum* previously shown with half-lives of 10–20 h (Fairbairn, Paterson & Wassel, 1964) should rather be explained as catabolic processes in the cytoplasm or in the young vesicles, and not necessarily as turnover in the major alkaloid containing vesicles (Fairbairn & Djote, 1970; Pham & Roberts, 1991).

Osmoregulation alone offers the necessary and sufficient explanation for the fluctuations observed. The day and night levels of latex water and morphine are explained as water transport processes according to our proposed hypothesis, and not as a rapid biosynthetic turnover as hitherto believed (Fairbairn & Wassel, 1964).

4. Experimental

4.1. Plant material

The opium poppy, *Papaver somniferum* L. cv. Parmo, was grown under field conditions in 1994 at the Agricultural Experimental Station Roskilde, Danish Institute of Agricultural Sciences. The plants were raised in 22 m^2 plots with 45 cm row distances, fertilised with 100 kg N/ha. Plant density of 30 plants/m^2 was obtained by thinning.

4.2. Meteorological data

The field plots were located in a distance of 50–100 m from an automatic weather station (AWS) with data collection every 10 min including air temperature (°C), relative air humidity (RH%), global radiation (S_i, W/m^2), precipitation (mm), wind speed (m/s), soil temperature and surface humidity.

4.3. Sampling techniques

Samples were taken in a 48 h period starting 10 days after petal fall 27th July at 8.00 h. Each hour 10–15 green uniform main capsules labelled at petal fall were incised with a scalpel to give two parallel tracks 1 cm long and 0.5 cm apart. The latex was collected after 5–10 s by suction into a tared 1 ml syringe. Collection of latex from 15 to 20 capsules resulted in a yield of 100–500 µl.

The syringe was placed in a tared beaker and after weighing (latex fresh matter weight) it was split and quantitatively washed into the beaker with methanol. After evaporation at 90°C for 12–16 h followed by cooling in excicator, the weight of latex dry matter was obtained and the latex water content (LW%) and latex dry matter content (LD%), LW + LD = 100, were calculated.

4.4. Extraction of alkaloids

The dry latex was dissolved in 35 ml 5% acetic acid by ultrasonic treatment for 10 min. After adjusting pH to 8.2 (10 N ammonia) the solution was quantitatively transferred to a separation funnel. The aqueous phase was extracted two times with 20 ml CH_2Cl_2 (2×100 ml) and the organic phase was evaporated at 40°C under a stream of nitrogen. The remainder was dissolved in 6 ml HPLC eluent (Anon., 1987).

4.5. HPLC analysis

The column was Nucleosil 10 CN (Macherey–Nagel & Co. mbH) 250×4 mm I.D. The mobile phase (NH_4Ac, pH 5.8 : acetonitrile : dioxane (80 : 10 : 10) was maintained at a flow rate of 1.0 ml/min and the alkaloids were detected at 254 nm except for thebaine (188 nm). The ammonium acetate was prepared by dissolving 200 g NH_4Ac in 600 ml water and adding 8.2 ml acetic acid, the final volume being adjusted to 1000 ml. A stock solution of alkaloids was prepared by dissolving morphine sulphate, codeine chloride, thebaine, noscapine hydrochloride and papaverine (all obtained from Sigma) in a concentration of 1 mg/ml mobile phase. Standard solutions were prepared from the stock solution by mixing volumes of morphine, codeine, thebaine, noscapine and papaverine in the proportion 4 : 4 : 2 : 0.5 : 0.5 and diluting to appropriate concentrations.

4.6. Statistical treatment

The correlation of latex water content with major alkaloids was tested by Pearson correlation coefficients, parametric regression fit and analysis of variance (SAS, 1990).

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