Diurnal Fluctuation of Quinolizidine Alkaloid Accumulation in Legume Plants and Photomixotrophic Cell Suspension Cultures

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Z. Naturforsch. **37 c**, 369–375 (1982); received January 1, 1982

Lupinus, Baptisia, Sarothamnus, Diurnal Rhythm, Photomixotrophic Cell Suspension Cultures, Quinolizidine Alkaloids

Fluctuations of quinolizidine alkaloid content in leaflets of *Lupinus polyphyllus, L. hartwegii, Baptisia australis,* and *Sarothamnus scoparius* were studied over a 36 h period. The alkaloid contents reached their maximum at noon or early afternoon, and their minimum during the night. The amplitudes of diurnal variations in alkaloid levels lay within a range of 60 to 470% of the early morning contents of alkaloids (= 100%) in leaves. In photomixotrophic cell suspension cultures of *L. polyphyllus* and *S. scoparius* cultured under day-night regime, a similar increase of the alkaloid levels was observed within 5 to 8 h after onset of illumination. A subsequent excretion of the alkaloids produced, which also followed a diurnal rhythm, was found. *S. scoparius* cells excreted lupanine, *L. polyphyllus* cells 13-cinnamoyloxylupanine. From the cell culture and plant experiments it can be assumed that the alkaloids which are formed during illumination are translocated to some degree afterwards. In cell suspension cultures the alkaloids are subjected to rapid and rhythmic turnover. Exogenous alkaloids, fed to the cultures, are taken up by the cells and disappear usually within the first 72 h. A possible mechanism for a light-mediated regulation of quinolizidine alkaloid biosynthesis which was found to be localized in leaf chloroplast, is discussed.

Introduction

Early grafting experiments with alkaloid-rich and alkaloid-free lupins revealed that the formation of quinolizidine alkaloids is restricted to the green parts of the plant [1]. Subsequent tracer experiments showed that excised shoots were able to convert lysine or cadaverine into the tetracyclic alkaloids (summarized in [2]). Recently we could isolate the first enzymes involved in the biosynthesis of quinolizidine alkaloids, lysine decarboxylase and 17-oxosparteine synthase [3–5]. Whereas lysine decarboxylase is present in all plant organs, 17-oxosparteine synthase is restricted to the leaflets [6]. Cell suspension cultures of *Lupinus polyphyllus* and *Sarothamnus scoparius* accumulate low amounts of alkaloids. But the alkaloid formation seems to be positively correlated with greening, *i.e.* chlorophyll formation of the cultures [7, 8]. This is in accordance with the finding that the subcellular site of alkaloid formation is the chloroplast [9, 10].

Localization of a biosynthesis in the chloroplast raises the question of light-mediated control of product formation. From earlier experiments with *L. albus* it is known that the alkaloid content is higher in the afternoon than in the morning [11]. This may be a first indication for a diurnal rhythm of quinolizidine alkaloid biosynthesis, similarly to hemlock alkaloid formation in *Conium maculatum* [12]. It should be mentioned that recent results of Roberts presented evidence that chloroplasts are also involved in the biosynthesis of hemlock alkaloids [13].

In this communication we report on diurnal fluctuations of quinolizidine alkaloid contents in leaves of four species of alkaloid producing legumes. A similar phenomenon is observed in photomixotrophic cell suspension cultures concerning alkaloid accumulation, turnover, uptake and excretion of quinolizidine alkaloids.

Materials and Methods

Plant cultivation

Lupinus polyphyllus, L. hartwegii, Baptisia australis, and *Sarothamnus scoparius* plants were grown in a green house at 15 °C and 16 h of daily illumination. The experiments were performed in spring (March/April) at which time the plants had commenced to flower. Four days prior to the experiments the plants were removed from the greenhouse and exposed to natural illumination of 14 h

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length daily. Other plants of these species were kept outdoors under natural conditions in the Botanical Garden of Braunschweig and experiments were performed with flowering specimens in summer (July).

Harvest of plant material

Samples of 0.5 to 1.0 g of leaflets were collected at two-hour intervals for a period of 30 to 36 h. Leaflets were harvested from the same leaf each time as far as possible. Collected material was deep-frozen immediately after harvest.

Cell suspension cultures

Photomixotrophic cell suspension cultures of *Lupinus polyphyllus* and *Sarothamnus scoparius* were established and cultured as described [8, 14]. They were kept in 50 ml or 180 ml medium in 300 ml or 1000 ml Erlenmeyer flasks on rotary shakers (120 r.p.m.), 16 h of illumination (Osram-L 40 W/77 Fluora and Philips TL 40 W/25 white), 25 °C and 70% r. H.

Cell cultures of *S. scoparius* consisted of green cell clusters (about 120 µg chlorophyll a/b g^{-1} fr.wt.) which showed the tendency of shoot bud formation. *L. polyphyllus* cell suspension cultures formed smaller cell aggregates and had a lower chlorophyll content (1 µg g^{-1} fr.wt.).

About 72 h prior to the experiments a set of 15 to 30 culture flasks with 50 or 180 ml fresh medium was inoculated with 20 g of cells. During the experimental period of 12 to 14 h one culture flask was harvested every hour. Collected cells and the remaining cell-free medium were deep-frozen immediately. Exogenous alkaloids were filter sterilized (0.45 µm Millipore) prior to application.

Alkaloid extraction

Frozen plant material was homogenized in 0.5 mol/l HCl using a Waring blendor. The filtered or centrifuged homogenate was alcalinized with 25% ammonium hydroxide and extracted with methylene chloride [6, 7, 13]. Total alkaloid content was determined photometrically [6, 7] or by capillary gas-liquid chromatography (glc), using sparteine (Roth, Karlsruhe) as an external standard.

Capillary gas-liquid chromatography

Alkaloid extracts were separated by glc on wall-coated fused silica ("quartz") columns (15 m × 0.25 mm; SE 30; J & W Scientific). A Perkin-Elmer gaschromatograph (Sigma 1 b) equipped with flame ionization and nitrogen specific detectors was employed. The chromatograms were evaluated electronically using the data system Sigma 10.

All alkaloids mentioned in this study have been previously identified by capillary glc/mass spectrometry (AEI MS 30, Data System DS 50) [8, 14–16]. The alkaloid signals in the different glc-runs were identified using reference alkaloids and their specific retention indexes.

Radioactivity

Radioactive samples were mixed with a dioxan cocktail (Packard) and measured in a Berthold-Friesecke liquid scintillation counter (Betascint BF 5000). [1,5-^{14}C]cadaverine was obtained from NEN, Boston, [U-^{14}C]L-lysine from Buchler-Amersham, Braunschweig.

Enzyme assays

Lysine decarboxylase and 17-oxosparteine synthase were assayed in crude extracts from acetone powders of *L. polyphyllus* cell suspension cultures as described in Wink *et al.* and Hartmann *et al.* [3, 6]. Lysine decarboxylase: 100 mg acetone powder was dissolved in 4 ml 0.1 mol/l phosphate buffer, pH 8.0, containing 1 mmol/l DTE, 1mmol/l $FeSO_4$, and 0.2 mmol/l [U-^{14}C]L-lysine (1 µCi). Incubation was performed at 37 °C for 5 h. The radioactivity of the cadaverine formed was determined by liquid scintillation counting [3, 6].

17-oxosparteine synthase: 30 mg acetone powder was dissolved in 1 ml 0.1 mol/l phosphate buffer, pH 8.0, containing 10 mmol/l diethyldithiocarbamate, 1 mmol/l dithioerythritol (DTE), and 0.02 mmol/l [1,5-^{14}C]cadaverine (1 µCi). Incubation under N_2 at 30 °C for 5 h. The radioactivity of the alkaloid formed was determined by liquid scintillation counting [4, 6].

Results

Diurnal fluctuations in alkaloid contents of leaflets

Fluctuations in content of total quinolizidine alkaloids in leaflets of *Lupinus polyphyllus, L. hart-*

wegii, Baptisia australis, and *Sarothamnus scoparius* were as shown in Fig. 1. The variation of alkaloid content within the 4 species studied during the 36 h period is quite similar. There is an increase of alkaloid concentrations of up to 300% during the first 6 to 8 h of day light and a steady decline in the evening. Between 2 and 4 o'clock at night the lowest alkaloid levels are reached which are about 60% lower than the amounts found at the beginning of the experiments in the morning. The increase of alkaloid content was lower the second morning, which was probably due to misty weather conditions prevailing in contrast to a sunny day the first morning.

In another set of experiments with *L. polyphyllus* and *B. australis* which are not shown in detail, we observed an increase of alkaloid content of leaflets from 400 and 600 μg g^{-1} fr.wt. in the morning to about 1900 μg g^{-1} fr.wt. in the afternoon.

In this case the plants grown under natural outdoor conditions were studied on a warm summer day.

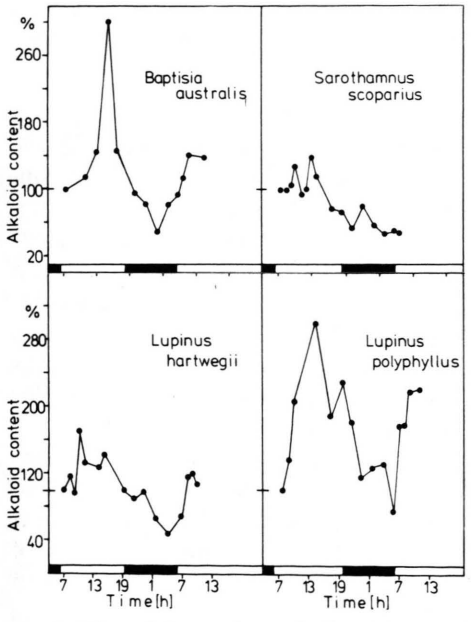

Fig. 1. Diurnal fluctuations of alkaloid contents in leaflets of flowering *Lupinus polyphyllus, L. hartwegii, Baptisia australis,* and *Sarothamnus scoparius* plants. Total alkaloid contents were estimated by glc. In *B. australis* 100% represent 900 μg alkaloid g^{-1} fr.wt., in *L. polyphyllus* 1050 μg g^{-1} fr.wt., in *L. hartwegii* 200 μg g^{-1} fr.wt., and in *S. scoparius* 170 μg g^{-1} fr.wt.

Diurnal variation in the alkaloid composition of leaflets

The variation in abundance of the individual alkaloids of the four species studied is shown in Table 1. In the *L. polyphyllus* plant angustifoline was the main alkaloid, which reached its highest abundance in the afternoon. The concentrations of the other alkaloids changed quite similarly to total alkaloids in Fig. 1. The situation was somewhat different for the lupin plant studied in July under natural conditions. In this plant lupanine and angustifoline increased 3 to 4fold, whereas N-methylangustifoline and 13-hydroxylupanine esters increased 5 to 6fold.

In *L. hartwegii* the only significant change between day and night was a decrease of lupanine and an increase of epiaphylline in the dark.

In *B. australis* lupanine, which is present in very low amounts in this species, increased during illumination but decreased and was not detectable in the night. A similar result was found in the *Baptisia* plant studied in summer, although this effect was much more pronounced then.

In *S. scoparius* sparteine reached a higher abundance in the afternoon than in the morning in contrast to 17-oxosparteine which displayed the opposite trend.

Diurnal rhythm of alkaloid accumulation and alkaloid translocation in cell suspension cultures

We have established photomixotrophic cell suspension cultures of *L. polyphyllus* and *S. scoparius* which accumulate quinolizidine alkaloids at low levels [7, 8]. The alkaloid content of *L. polyphyllus* cell cultures displays a maximum three days after medium transfer. This increase coincides with an increased activity of both enzymes of alkaloid formation, lysine decarboxylase and 17-oxosparteine synthase (Fig. 2). In general this phenomenon, although still unsufficiently understood, seems to be a common feature of primary and secondary metabolism in plant cell suspension cultures. Usually an increased RNA formation and subsequent protein biosynthesis is observed within the first three days after medium transfer [17]. Similar effects as in *Lupinus* cell cultures are reported from flavonoid biosynthesis in cell suspension cultures of *Petrosilinum hortense* [18] and from indole monoterpene alkaloid biosynthesis in *Catharanthus roseus* cell suspension cultures [19].

Table I. Diurnal fluctuations in the alkaloid patterns of leaflets of *L. polyphyllus, L. hartwegii, B. australis* and *S. scoparius*. Alkaloid extracts were separated by glc and evaluated as described previously [8, 13–15]. Early morning values (7.30 o'clock) are compared with data from early afternoon (13.30–15.00 o'clock) and night (2.00–4.00 o'clock).

Species:	Alkaloid abundance (%)											
	Lupinus polyphyllus			*L. hartwegii*			*B. australis*			*S. scoparius*		
Day time (o'clock)	7.30	15.00	4.00	7.30	15.00	4.00	7.30	15.40	2.00	7.30	13.30	4.00
Alkaloid												
Sparteine	0.4	0.3	0.4	–	–	–	0.1	0.0	0.5	55	77	62
11,12-Dehydro-sparteine	–	–	–	–	–	–	–	–	–	6.5	4.0	5.9
17-Oxosparteine	4.5	4.9	2.4	–	–	–	< 0.1	< 0.1	< 0,1	26.7	12.3	21
Lupanine	21.6	18.9	22.6	21	23	7.2	0.05	0.3	n.d.	2.2	1.5	1.9
Isolupanine	–	–	–	0.5	1.5	1.0	–	–	–	–	–	–
Aphylline (10-Oxospart.)	–	–	–	35	35	38	–	–	–	–	–	–
Epiaphylline (10-Oxospart.)	–	–	–	42	39	53.2	–	–	–	–	–	–
17-Oxolupanine	1.5	0.6	0.7	–	–	–	–	–	–	–	–	–
Angustifoline	29.3	30.4	24.2	–	–	–	–	–	–	–	–	–
N-Methylangustifoline	1.5	1.6	1.2	–	–	–	–	–	–	0.5	0.4	0.8
4-Hydroxylupanine	–	–	–	0.9	2.4	1.3	–	–	–	–	–	–
13-Hydroxylupanine	16	18.9	18.4	–	–	–	–	–	–	0.3	0.3	0.6
13-Hydroxylupanine ester*	22	20.1	23.2	–	–	–	–	–	–	–	–	–
Cystine	–	–	–	–	–	–	4.5	5.9	8.9	–	–	–
N-Methylcytisine	–	–	–	–	–	–	95	93	89	–	–	–
Anagyrine	–	–	–	–	–	–	0.4	0.3	0.6	–	–	–
13-Acetoxyangyrine	–	–	–	–	–	–	0.2	0.05	0.3	–	–	–
Tinctorine	–	–	–	–	–	–	0.2	0.05	0.05	–	–	–

* Including 13-angeloyloxylupanine, 13-tigloyloxylupanine, 13-butyryloxylupanine, 13-benzoyloxylupanine, 13-*cis*/*trans*-cinnamoyloxylupanine; n.d. = not detectable.

Cell suspension cultures of *L. polyphyllus* and *S. scoparius* were kept under 16 h of daily illumination to have conditions which are comparable to the situation of the intact plants studied above. During the first 12 h after onset of illumination cells were collected every hour. These experiments were performed about 72 h after subculturing, *i.e.* in the maximum of enzymic activity (Fig. 2).

The alkaloids were extracted and analyzed by capillary glc. In cell cultures of *S. scoparius* we observed an enhanced alkaloid accumulation after onset of illumination which reached a maximum after about 7 h (Fig. 3). Afterwards a steady decrease of the cell alkaloid content was registered which was sometimes accompanied by an increase of the alkaloid concentration in the culture medium (Fig. 3). Lupanine and sparteine were the only alkaloids detectable in the alkaloid extracts [8]. The light dependent increase in total alkaloid content was due to an enhanced accumulation of lupanine. Sparteine, which accounts for 10 to 40% of the total cell alkaloids, was predominantly formed in the night. The alkaloid excreted into the medium consisted almost exclusively of lupanine.

A similar situation was observed in cell suspension cultures of *L. polyphyllus* (Fig. 4). However, a significant difference existed in the alkaloids accumulating in the cells and the medium. Lupanine was the only alkaloid which accumulated in the cells whereas 13-cinnamoyloxylupanine accounted for the alkaloid detectable in the culture medium.

These results imply that excretion of alkaloids into the culture medium may be a regular phenomenon which follows a diurnal rhythm, too. This was supported by another set of experiments. Cultures of *L. polyphyllus* and *S. scoparius* were incubated with 10 μmol/l [1,5-^{14}C]cadaverine (1 μCi), which was taken up by the cells almost quantitatively within 24 h and was found to be converted into quinolizidine alkaloids such as lupanine. The radioactivity of the culture medium of both cultures was determined every 30 min for a period of about 30 h. In

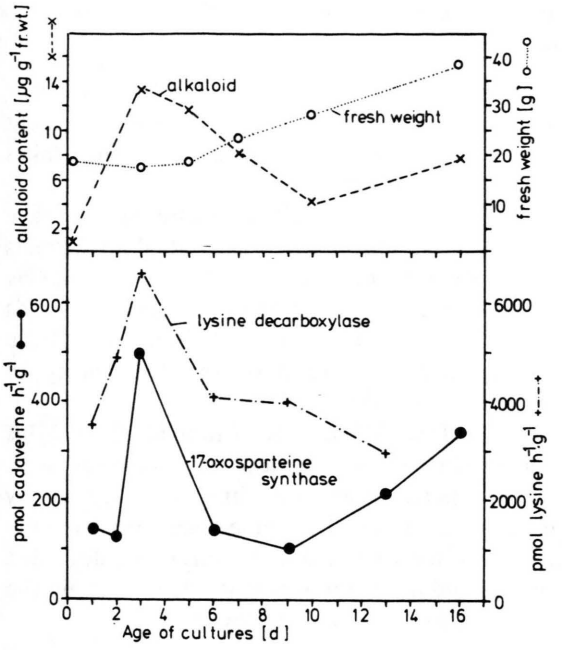

Fig. 2. Time course of alkaloid accumulation in *L. polyphyllus* cell suspension cultures in relation to the activity of lysine decarboxylase and 17-oxosparteine synthase. Data on lysine decarboxylase were produced by G. Schoofs.

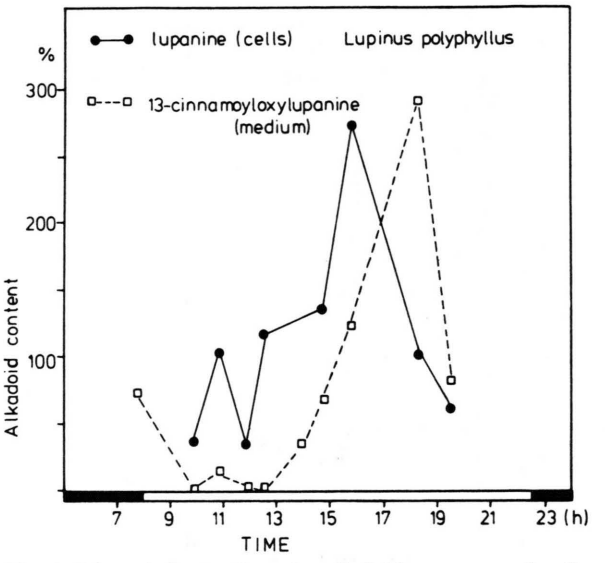

Fig. 4. Diurnal fluctuations in alkaloid contents of cell suspension cultures of *L. polyphyllus*. Alkaloid composition and alkaloid content was determined by glc. Cells contained 0.1 µg g^{-1} alkaloid (100%), respective media 0.02 µg ml^{-1} (100%). Total alkaloid content of a culture flask accounted for 3.0 µg (2 µg in cells, 1.0 µg in culture medium).

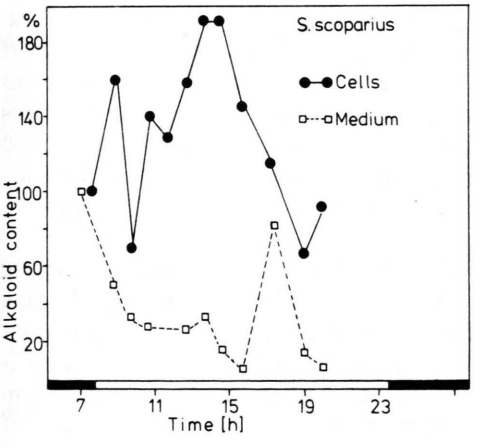

Fig. 3. Diurnal variation of alkaloid contents in photomixotrophic cell suspension cultures of *S. scoparius*. Alkaloid contents (determination by glc) accounted for 0.9 µg g^{-1} fr.wt. (100%). The respective media contained 0.1 µg ml^{-1} alkaloid. In general a culture flask contained about 35 µg total alkaloid (20 µg in cells, 15 µg in medium).

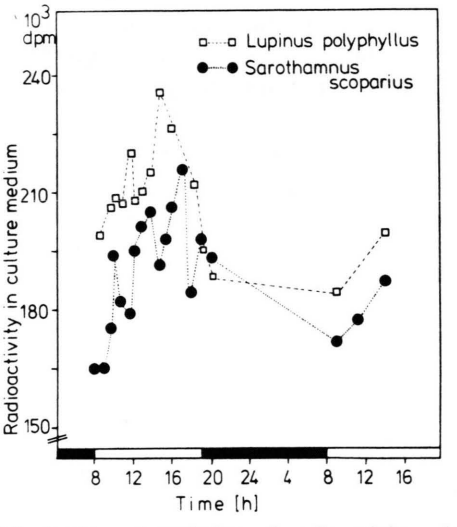

Fig. 5. Diurnal variation of radioactivity released by *L. polyphyllus* and *S. scoparius* cells into the culture medium. Cell suspension cultures (after 15 d of growth) were fed with 1 µCi [2,5-^{14}C]cadaverine 72 h prior to the experiments and were cultured under day-light regime. Aliquots of 100 µl medium were taken from the culture medium every 30 min and its radioactivity was determined by liquid scintillation counting.

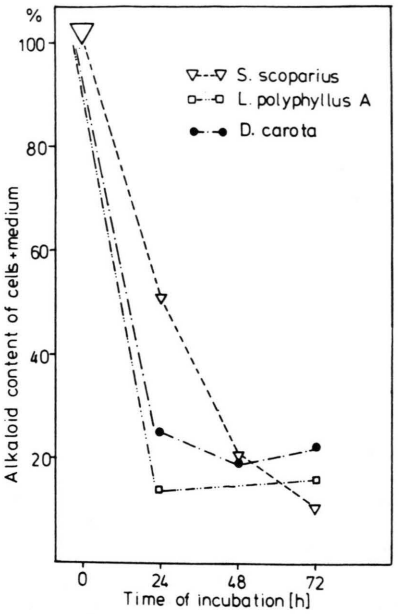

Fig. 6. Turnover of exogenous quinolizidine alkaloids by plant cell suspension cultures. Alkaloid solutions containing 0.5 to 1 mg alkaloid were added to cell suspension cultures directly after subculturing (10 g cells in 50 ml medium). Cultures were harvested as indicated and its alkaloid contents and alkaloid composition was determined by capillary glc.

both cases we observed a rhythmic variation of soluble radioactivity in the culture medium. A maximum was reached 6 to 8 h after onset of illumination which was followed by a decrease in the dark.

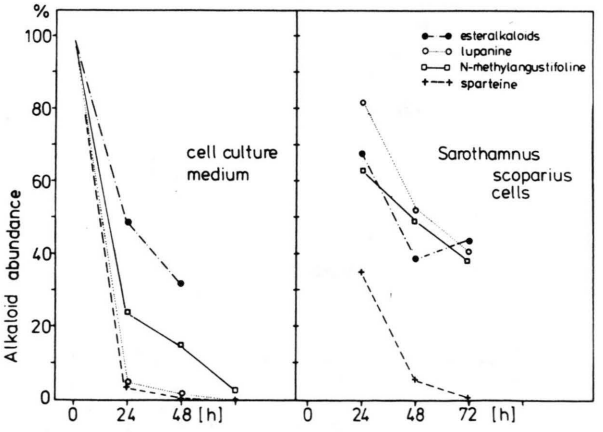

Fig. 7. Uptake and turnover of individual alkaloids by cell cultures of *S. scoparius*. The alkaloid mixture added to the cell cultures contained about 50% sparteine, 30% lupanine, 10% N-methylangustifoline and 10% 13-tigloyloxylupanine.

Patterns of alkaloid turnover in cell suspension cultures

Considering the alkaloid contents of both cells and corresponding medium (Fig. 3, 4) it is evident that a rapid turnover of the alkaloids formed takes place within a daily cycle.

To test the degree of alkaloid turnover we have fed cell suspension cultures with alkaloid extracts (85 µmol/l) from *L. polyphyllus* leaflets. As can be seen from Fig. 6 most of the exogenous alkaloids disappeared from the cultures within 48 h. Even cells of *Daucus carota* possessed the ability to metabolize exogenous alkaloids.

We have followed the fate of individual alkaloids in detail (Fig. 7). In cell suspension cultures of *S. scoparius* sparteine and lupanine are taken up by the cells most rapidly. Sparteine disappears from the cells within the first 72 h and is probably degraded to non-alkaloidal compounds not detectable by the non-radioactive methods employed.

Discussion

The accumulation of quinolizidine alkaloids in leaflets of *Lupinus, Baptisia,* and *Sarothamnus* and in photomixotrophic cell suspension cultures grown under day-night regime shows a marked diurnal fluctuation. There is an increase of the alkaloid contents in the light and a decrease during the dark. The diurnal patterns of accumulation and transport of mineral salts, carbohydrates, and nitrogenous compounds (other than alkaloids) in lupin plants [20, 21] are very similar: The accumulation and subsequent export of photosynthates from the leaflets is very active during the day and less active in the night.

Since we know that at least the principal enzymic steps of quinolizidine alkaloid biosynthesis take place within the chloroplast [9, 10] it seems reasonable to relate the observed diurnal rhythm of alkaloid accumulation to the light-mediated metabolism of the chloroplast. Although primary products of photosynthesis such as ATP and NADPH are not involved in alkaloid biosynthesis, there are several factors which could favour quinolizidine alkaloid biosynthesis in the light:

1. The shift of stromal pH from pH 7 to 8 upon illumination [22]. Both enzymes of alkaloid bio-

synthesis, lysine decarboxylase and 17-oxosparteine synthase, show optimal activity at pH 8 and are considered less active at pH 7 [23].

2. Activation by reduced thioredoxin, which is known to activate many light-dependent chloroplast enzymes [24]. Reduced bacterial thioredoxin has been shown to activate crude preparations of the alkaloid specific enzymes [23].

3. Lysine, the ultimate precursor of lupin alkaloids, is also synthesized in the chloroplast [25, 26]. Its formation from aspartate is active during the day and inhibited at night. Thus the observed diurnal rhythm may be influenced also by precursor availability.

Since quinolizidine alkaloids accumulate in all plant parts, especially roots and fruits, which are not capable of alkaloid biosynthesis [6], we must assume that alkaloids are exported from the leaflets into these organs. This is supported by our observation that phloem-feeding aphids contain quinolizidine alkaloids (Wink and Hartmann unpublished) and that phloem sap of lupins contains a significant load of alkaloids (Wink, Hartmann, and Pate unpublished). Therefore, the observed decrease of alkaloid content of the leaves during the afternoon and evening may be due to alkaloid translocation. It is remarkable that this process is also observed in cell suspension cultures which excrete alkaloids time-dependently into the medium. However, the cell culture experiments show that rapid alkaloid turnover and degradation have to be considered, too. This process was found to be very important for nicotine metabolism in *Nicotiana tabaccum* [27]. At the present state we are not able to discriminate between alkaloid translocation and turnover and to evaluate these processes quantitatively.

It should be recalled in this context that alkaloid accumulation in lupin cell cultures is up to 2 orders of magnitude lower than in the differentiated plant [7], although active enzymes of alkaloid biosynthesis are present. Maybe alkaloid formation is active too, but the alkaloids produced are not accumulated as in the plant but are rapidly degraded. In cell cultures of *Lupinus* and *Sarothamnus* alkaloid production seems to be significantly influenced by biological rhythms, alkaloid turnover, and alkaloid excretion into the medium. Maybe these factors are of similar importance in other cell culture systems employed for biotechnological purposes.

Acknowledgements

This work was supported by research grants of the Land Niedersachsen and the Deutsche Forschungsgemeinschaft. We would like to thank Prof. Dr. T. Damroth (FAL, Braunschweig) for providing green house facilities and K. Baeske (Botanischer Garten Braunschweig) for plant material. Technical assistance of C. Theuring is gratefully acknowledged.

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