Storage of Quinolizidine Alkaloids in Epidermal Tissues

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Epidermal cells of lupine leaves, petioles and stems are the main storage site for quinolizidine alkaloids with an alkaloid content of 5−6 mg/g fw (= 15−25 mmol/kg). Epidermal tissue also store malate and hydrogen ions and show a vacuolar pH of < 5. It is argued that the specific storage of alkaloids in the vacuoles of epidermal cells might be due to active transport via carrier proteins and cannot be explained by a simple diffusion and ion trap mechanism.

Introduction

In order to produce large quantities of a secondary product a plant not only needs the ability to synthesize these compounds but also to accumulate and store them. While we have rather extensive knowledge on the biosynthetic pathways, our information on the conditions required to accumulate and store the products is comparably scanty. We observe as a general rule that the site of synthesis is different from the site of accumulation: Many secondary compounds are probably formed in the cytosol, some in organelles such as mitochondria (*e.g.* amines [1]) or chloroplasts (*e.g.* terpenes [2]; quinolizidine alkaloids [3, 4]; Conium alkaloids [5]). The subcellular site for the accumulation of most of these compounds seems to be the vacuole [6−8]. In many instances the biosynthesis and the accumulation of a secondary product occur within the same cell although in different compartments (*e.g.* phenolics, flavonoids, anthocyanins). In other cases synthesizing cells differ from accumulating cells: It has been established that tropane alkaloids are produced in the root and are then transported *via* the xylem to stems and leaves, where the transformation from hyoscyamine to scopolamine takes place [9]. We have found in our laboratory that lupine alkaloids are formed in the leaf, and are then transported via the phloem to all other plant organs [10−13].

Cell suspension cultures of lupines produce quinolizidine alkaloids at a level which is 2−3 orders of magnitude lower than that of the intact plant [14]. We already found that lupine alkaloids are excreted into the cell culture medium where they are rapidly degraded by exocellular enzymes [11, 15−18]. Do lupine cell cultures fail to produce alkaloids because they do not have the adequate storage capacity? To answer this question we have to characterize the alkaloid storage cells of differentiated lupine plants, which are the epidermal cells according to preliminary experiments with laser desorption mass spectrometry (LAMMA 1000) [19].

Results

Localization of lupine alkaloids

Stems, petioli and leaflets of *Lupinus polyphyllus, L. albus, L. mutabilis* were stained with an KJ/J$_2$ solution, which precipitates lupine alkaloids [10, 20]. In all these tissues a very intensive brown precipitate could be observed in the epidermal tissue layer (Fig. 1A, 2A). As far as we could see, all epidermal cells were positive. Mesophyll and parenchymatic cells were weakly stained only. In stem sections also the first subepidermal cell layer and phloem cells were heavily stained. We are sure that the precipitates observed are indeed due to quinolizidine alkaloids for the following reasons: 1. When analyzing equivalent parts of "sweet" *L. albus,* which were almost free from alkaloids according to glc-analysis, these precipitates could not be observed (Fig. 2, II). 2. Using laser desorption mass spectrometry (LAMMA 1000) we could record lupine alkaloids only in epidermal cells of stem and petiole sections of *L. polyphyllus* and *Cytisus scoparius* [19]. 3. It is possible to peel off the epidermal tissue. As shown in Table I, the highest alkaloid concentration can be measured by glc in epidermal cells. 4. Our observations confirm histochemical studies, made at the turn
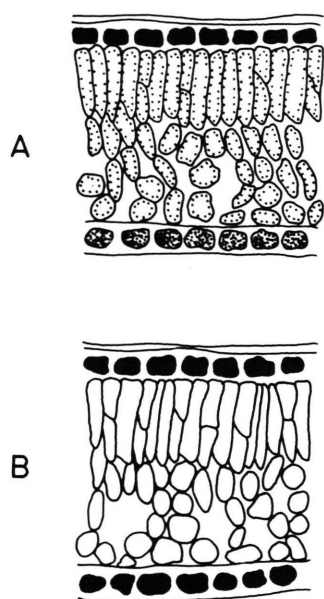
Fig. 1. Schematic illustration of the histochemical characterization of a cross section through a leaflet of *Lupinus polyphyllus*.
A. Staining with KI/I$_2$. Precipitation of alkaloids is indicated by the black or densely spotted areas. The upper epidermis seemed to contain more alkaloid than the lower epidermis.
B. Staining with methyl red. Red-stained cells (*i.e.* pH < 5) are indicated by black areas. The pH-value of the other cells was > pH 5.7.
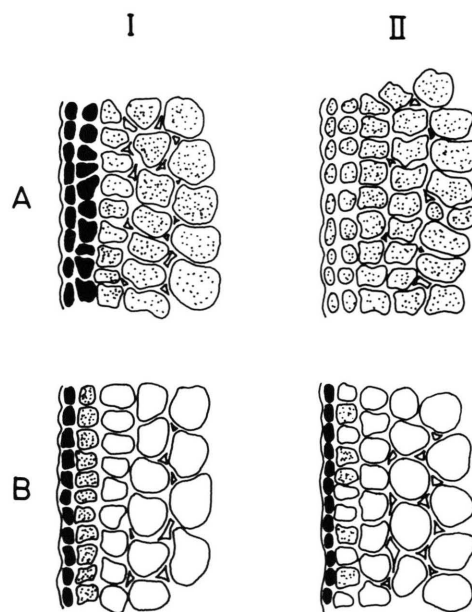
Fig. 2. Schematic illustration of cross sections through stems of *Lupinus albus*. I = bitter, alkaloid-rich ecotype; II = "sweet" variety (Llaima).
A. Staining with KI/I$_2$. The blackened areas represent alkaloid precipitates. Protein precipitates which showed a different brownish colour are illustrated by dots.
B. Staining with methyl red. Intensive red (*i.e.* pH < 5) areas are shown in black, cells with a pH between 5 and 5.5 are dotted. The pH of the remaining cells was > 5.7. Note that both, sweet and bitter lupines had epidermal tissues with acidic pH-values, whereas alkaloid precipitates only occurred in the bitter ecotype.

Table I. Alkaloid content and alkaloid pattern of isolated epidermal and nonepidermal tissues.
Alkaloid extracts were analyzed by capillary GLC according to standard procedures [14, 23, 24].
I = Stem, II = petiole, III = lower leaf surface. 1 = lupinine, 2 = sparteine, 3 = tetrahydrorhombifoline, 4 = lupanine, 5 = 4-hydroxylupanine, 6 = 13-hydroxylupanine, 7 = 13-angeloyloxylupanine, 8 = 13-tigloyloxylupanine, 9 = multiflorine.

Species	Tissue	Alkaloid content [mg/g fw]	Alkaloid composition								
			1 [mg/g fw]	2	3	4	5	6	7	8	9
L. luteus	Epid.	0.6	0.2	0.4	−	−	−	−	−	−	
	Non-E.	0.02	0.01	0.01	−	−	−	−	−	−	
L. mutabilis	Epid.	5.3	−	0.2	0.07	3.5	0.2	0.8	0.07	0.4	+
	Non-E.	0.4	−	−	0.03	0.3	0.01	+	+	0.03	−
L. albus	Epid.	6.3	−	−	−	3.8	−	1.0	+	0.5	1
	Non-E.	0.4	−	−	−	0.4	−	+	−	+	+
L. polyphyllus	I. Epid.	6.3	−	−	+	2.5	0.08	0.6	0.6	2.5	+
	Non-E.	1.3	−	−	−	0.9	0.07	0.2	0.02	0.08	−
	II. Epid.	1.7	−	−	−	0.8	−	0.1	0.1	0.7	−
	Non-E.	1.6	−	−	−	1.3	−	+	+	0.3	−
	III. Epid.	3.7	−	−	2.0	0.7	−	0.4	+	0.6	−
	Non-E.	1.3	−	−	0.7	0.3	−	0.2	−	0.05	−

Epid. = Epidermis; Non-E. = non-epidermal cells; + = traces; − = not detected.

of our century [21] on the localization of lupine alkaloids in various plant tissues.

Properties of epidermal cells

Alkaloid content and alkaloid pattern of epidermal cells

Epidermal tissue was peeled off from stems of *L. luteus*, *L. mutabilis*, *L. albus* and *L. polyphyllus*. For *L. polyphyllus* it was also possible to strip the epidermis off the petiolus and the lower leaf surfaces. Light microscopy showed that the peeling procedure left the epidermis intact, but also took away part of the first subepidermal layer. Since lupine stems often contain anthocyanin, a coloured natural product known to occur in epidermal or subepidermal cells [20, 22], the distribution of anthocyanin between the epidermal and the remaining tissues was taken to evaluate the efficiency of the peeling method. Measuring the absorbance at 520 nm showed that usually 95% of the anthocyanin could be recovered from the epidermal peel.

Table I summarizes the results of the alkaloid analysis of epidermal cells as compared to the remaining tissue. The alkaloid concentration of epidermal peels from stems of *L. polyphyllus*, *L. albus* and *L. mutabilis* accounted for 5−6 mg/g fr. wt. (equivalent to 15−25 mmol/kg) and was thus much higher than that of the remaining tissue with values between 0.3−1.4 mg/g fr. wt. For *L. luteus* a similar ratio was observed. But since this variety was a "semi-sweet" form, the overall concentration was lower than in the bitter species. The glc-analysis also confirmed that the epidermis of leaves and petioles are alkaloid-rich. Non-epidermal petiole tissue was also relative rich in alkaloids, probably because it contains a substantial amount of phloem tissue, which transports the alkaloids.

The alkaloid patterns observed in epidermal tissues were similar to those reported in the literature and from our institute for the respective plant part [10, 12, 14, 19, 23, 24] with lupanine being the major alkaloid in *L. polyphyllus, L. albus,* and *L. mutabilis.*

Hydrogen ion concentration of epidermal cells

Since epidermal cells of lupines are obviously specialized for alkaloid storage, we tried to characterize this tissue further by studying the cellular hydrogen ion concentrations of the respective cells.

Indicator dyes provide a convenient and rapid tool to measure vacuolar pH [25]. Since we expected pH-values between pH 4 and 7, we chose methyl red as indicator dye, which is red at pH 4 and 5, but colourless at pH 6−7. Stems and leaflets were incubated in the methyl red solutions, sectioned and studied by light microscopy. For all plant parts and lupines studied we observed that the epidermis was always stained dark red (Fig. 1 B, 2 B), which according to a calibration curve was equivalent to a pH-value between 4 and 5. In some stem sections about 60% of the subepidermal cells were red, but all other cells were only slightly coloured or colourless, indicating a vacuolar pH-value higher than pH 5.5 (Fig. 2 B). Using neutral red or bromphenol blue we came to similar conclusions.

Because of possible experimental errors [25], we tried to confirm these results using a different approach. We isolated about 2 g of epidermal tissue and the equivalent amount of the respective non-epidermal cells from petioles of *L. polyphyllus* and from stems of *L. mutabilis*. The tissues were disrupted by freezing at −20 °C, followed by thawing and centrifuged at 40,000 × g for 10 min to obtain a clear supernatant. The pH-value of this supernatant (*i.e.* the so-called cellsap) was in the range between 4.4 and 5.2 for epidermal and between 5.5 and 6.0 for non-epidermal cells. Since the cytoplasm and the vacuole contribute to the overall hydrogen concentration, the cell sap data are necessarily rather crude.

In another approach we employed a non-destructive method, *i.e.* ^{31}P *in vivo* NMR (V. Wray and M. Wink, unpublished results). The rationale of this method is that the signal of inorganic phosphate shows a pH-dependant shift [26]. Employing a Bruker 400 MHz − instrument we found in preliminary experiments that the pH-value of isolated epidermal tissues was at least 0.6−0.8 pH-units lower than that of the respective non-epidermal tissue. This approach was limited by the rather low concentration of inorganic phosphate in the vacuole, which prolongs each experiment considerably. Furthermore the shift of the ^{31}P signal is rather small in the relevant pH-range (< pH 5).

All these results unequivocally show, that a high hydrogen ion concentration is indeed a speciality of epidermal cells. Preliminary glc-analysis indicated that the epidermal cells are rich in malate (2 mg/g fr. wt = ca. 15 mmol/kg). Further experiments must show if malic acid contributes substantially to the low

pH-value of the vacuoles and whether it will function as a counter ion for the charged alkaloid molecules, which are present in a similar concentration (s. above).

Discussion

Our results clearly show that epidermal cells and in stems to a lesser extent subepidermal cells are the main storage sites for quinolizidine alkaloids. This result should to be discussed in an ecological context. In previous studies we could show that the biological roles of quinolizidine alkaloids lie in two areas: 1. Since the alkaloids are not inert end products but active metabolites [11, 12], they can serve as nitrogen transport and nitrogen storage compounds [27, 29]. 2. The main function, however, is that of chemical defense. Lupine alkaloids deter the feeding of herbivores, inhibit the growth of microorganisms and of other plants [27—32]. In view of this activity the epidermal storage of alkaloids fits very well. Since epidermal tissues serve as the first defense barrier, a high concentration of toxic alkaloids would be a helpful strategy. It should be recalled that the necessary inhibitory or repellent concentrations of lupine alkaloids fall in the range of 1—5 mM. Therefore, it seems probable that the 20 mM concentration of lupine alkaloids in epidermal tissues constitute a relevant chemical barrier for small invading organisms.

Are lupines extraordinary in this respect? It has been generally accepted that most of the so-called secondary products of plants are important for the fitness of a plant and either serve to attract pollinating or seed dispersing animals or to deter herbivores and inhibit the growth of microorganisms and other plants [33—35]. The localization of plant secondary products had been an active research area at the turn of the century (for summary see [20, 22]). Using histochemical methods the distribution of many products had been studied. Almost as a general scheme an epidermal localization has been reported for many compounds, e.g. colchicine, aconitine, tropane alkaloids, steroid alkaloids, nicotine, Papaver-, Veratrum-, Nuphar-, Ruta-, Conium alkaloids, anthocyanins, saponins, cyanogenic glucosides, phenolics, mustard oil glucosides, various essential oils, oxalate and silica crystals [20, 22]. These findings should be rechecked with modern methods, since the implications are very important for the understanding of the

biology of secondary products. It should be mentioned that already at the turn of the century the occurrence of many toxins in epidermal tissues was interpreted in terms of a chemical defense barrier [20, 22].

What are the biochemical characteristics of epidermal cells? From the cytological point of view they are highly specialized in that they produce a protective cuticle, which shows distinct biotoxic properties [36]. Epidermal cells with the exception of the guard cells have no chloroplasts and are therefore strictly heterotrophic. These cells are able to store also high concentrations of secondary products and in some species also of hydrogen ions [25].

Is the acidic character of epidermal cells a requirement for alkaloid storage? According to [37] alkaloid storage cells of *Catharanthus* are characterized by a high hydrogen ion concentration. In *Atropa belladonna* too, the alkaloids are concentrated in epidermal cells [20] and their cell sap is also acidic, according to methyl red staining (Wink unpublished). Judging from all these findings one could indeed conclude that the acidic nature of epidermal cells is essential for alkaloid storage and discuss an ion trap mechanism to be the driving force for alkaloid accumulation [37]. This theory also assumes that alkaloid transport through the tonoplast membrane is by diffusion of the free base. But quinolizidine alkaloids (e.g. sparteine) have a pKb value > 11.8, so that the percentage of the free base is 0.17% at pH 9 and 0.02% at pH 8. This would mean that under physiological conditions nearly 100% of all alkaloid molecules would be present as charged molecules in epidermal as well as in nonepidermal cells. The ion trap mechanism could therefore not explain the difference in alkaloid storage between epidermal and nonepidermal cells, since this mechanism would work in both systems.

Recent results of Deus-Neumann and Zenk [38] excluded an ion trap mechanism for alkaloid transport into vacuoles: The authors found that the ability of vacuoles to store a specific alkaloid was obviously due to specific carrier proteins which rules out that simple diffusion is the mechanism of alkaloid transport. There is good evidence that an ATP-driven proton pump is the driving force for active transport of metabolites into plant vacuoles [39]. This could mean that the high hydrogen ion concentration found in epidermal cells is an indication that in these cells this proton transport system is highly active. We

have experimental evidence that epidermal cells have carrier proteins for lupine alkaloids (P. Mende, M. Wink to be published) which would explain the selective accumulation of alkaloids in these cells against a concentration gradient.

In view of our failure to find lupine cell cultures with a high yield of lupine alkaloids we are inclined to assume that these storage cells are not present under *in vivo* conditions, so that when alkaloids are produced they are not stored but are rapidly degraded [11, 15−18]. It would be interesting to see whether this is relevant in other cell culture systems employed for the production of valuable secondary products but which do not come up to the researchers' expectations. On the other hand, however, the successful cell culture systems seem to have a common advantage: Shikonin, berberine and rosmarinic acid are produced and stored by the same cell respectively [40−42], which obviously made selection of high yielding strains much easier.

Experimental

Plant material

Epidermal tissue was collected from flowering or fruiting lupine plants, which were grown outside in our experimental garden.

Alkaloid analysis

Plant material was homogenized in 0.5 M HCl and left standing at room temperature for 30 min. After filtering off the solid material, the homogenate was made alkaline with NaOH. About 20 ml were applied onto a standard extrelut column (Merck, Darmstadt). Alkaloids were extracted by Cl_2CH_2. Extracts were evaporized in a rotavapor and analyzed by capillary gas-liquid chromatography according to Wink *et al.* [14, 23, 24]. Alkaloids were identified by their specific retention indices in comparison to those of authentic standards [12, 14, 23, 24].

Histochemical studies

Localization of alkaloids. Lupine alkaloids form a dark brown precipitate with KI/I_2 solution [10, 20]. Tissues were incubated in a diluted solution (1:10) of a KI/I_2 − stock, containing 14 g KI and 9 g I_2 per 100 ml water. After 5−10 min the plant material was rinsed with water, thin sections were excised and analyzed by light microscopy (Zeiss photomicroscope III).

Determination of cellular pH. Plant material was incubated in an aqueous 0.5% methyl red solution for 10 min. After rinsing the plant material with water, sections were excised and analyzed by light microscopy for red stained cells.

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Note added in proof: Recent research by Zenk and coworkers provide further evidence that the accumulation of alkaloids in plant vacuoles does not involve an ion-trap mechanism [43, 44].

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