Chemical Defense of Lupins. Mollusc-Repellent Properties of Quinolizidine Alkaloids

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Polyphagous molluscs such as *Helix pomatia* and *Arion rufus* generally do not feed on plants containing alkaloids. Of 19 species tested 10 species were totally avoided, the other 9 species were less attacked than *Lactuca sativa*, which was readily taken. Plants containing quinolizidine alkaloids were studied in detail. Those species with the α-pyridone alkaloids cytisine and N-methylcytisine were avoided to a higher extent than plants with lupanine as the major alkaloid. Since the repellency observed could be due to other natural products present in the plants besides the alkaloids, the feeding response of *Helix pomatia* was tested on artificial diets containing quinolizidine alkaloids in various concentrations as the only variable. If the snails had the choice they clearly preferred alkaloid-free food or a diet with only low alkaloid concentrations. Half-maximal repellency of cytisine is less than 2 mM, of sparteine 1–5 mM, and of lupanine 1–8 mM. Since the *in vivo* concentrations of sparteine, cytisine, and lupanine are equal to or higher than the inhibitory concentrations required it is concluded that quinolizidine alkaloids constitute a potential antimolluscan principle of legumes.

Introduction

QA are common secondary compounds of many Leguminosae, with a few isolated occurrences in other families [1]. The biochemistry and physiology of QA biosynthesis in plants and cell suspension cultures of *Lupinus polyphyllus* has been studied in detail in our laboratory: The biosynthesis using lysine as the only precursor [1], takes place in the aerial green parts of the lupin and could be localized in the leaf chloroplast [2, 3]. QA are formed only in the light following a diurnal rhythm [4, 5]. The QA are then translocated from the leaf via the phloem [6] to all the other plant organs [2], in case of *Cytisus (Sarothamnus) scoparius* even to its root parasite *Orobanche rapum-genistae* [7]. Especially the peripheral tissues and the reproductive organs such as the seeds accumulate high amounts of QA.

We came to the conclusion that QA constitute a means of a general chemical defense system of lupins active in plant-plant, plant-microbe, and plant-herbivore interrelationships [6, 8, 9, 10]: It could be shown experimentally that QA inhibit the germination of *Lactuca sativa* and of grass seeds, whereas seeds of *Lupinus albus* were unaffected [8]. Since seedlings of *L. albus* exudate QA via their roots [8], QA potentially constitute an active principle of plant-plant, *i.e.* allelopathic interrelations.

Abbreviations: QA, quinolizidine alkaloids.

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QA seem to be active also against microorganisms and inhibit growth of bacteria at concentrations which are clinically unsuitable due to high toxicity but match the concentrations present in the intact leaf [9]. QA seem to be less effective against fungi [9, 10]. It should be remembered, that lupins and other Leguminosae contain effective antifungal compounds, the isoflavones or pterocarpanes [11, 12].

QA seem to play an important role as an antiherbivoral principle in lupins: Mammals such as sheep and hares can discriminate between alkaloid-containing and alkaloid-poor (the "sweet") varieties [10, 13] and preferentially feed on the latter. QA are toxic to mammals and teratogenic effects have been observed in the offspring of mammals which had been fed α-pyridone-alkaloids, especially anagyrine-containing plants [14]. Since lupin seeds are rich in proteins and lipids, comparable to those of the soy bean [15], they could constitute an important crop for Homo sapiens. On account of their high alkaloid load – the seeds accumulate up to 5% d.w. QA – lupin seeds are unpalatable (bitter) and toxic to man. However, man has found a way to circumvent the antiherbivoral principle of lupins, not by evolving a QA-orientated physiological detoxification system (as some specialized insects did) but by using physical means: Inhabitants of mediterranean countries and of Southern America have traditionally used lupin seeds as food. Seeds were boiled and the QA leached out in running water

[16]. Considering their biological activity it is not surprising that a few QA have a medical application in cardiology and obstetrics [17]. Furthermore the α-pyridone-type QA cytisine and N-methylcytisine have halucinogenic properties ([18], Wink unpublished observation).

A few examples indicate that QA are also antifeedants for insects, including thrips [19], locusts [20], bruchid beetles [21], and aphids [6]. Aphids feed on many Leguminosae but usually avoid feeding on QA containing plants. We could show that this holds true for the bitter lupins, but not for the alkaloid-poor "sweet" varieties. With increasing "sweetness" *L. luteus* plants were progressively infested by aphids [6, 10]. Since QA are translocated via the phloem [6], the QA constitute a "systemic pesticide". Whereas QA seem to deter most insects, a few species have evolved which are specialized on QA producing plants, such as *Aphis cytisorum*, *Acyrthosiphon spartii* [6, 21], and a few Lepidoptera [10]. The aphids accumulate QA and might be thus rendered toxic themselves to their predators.

These data although incomplete suggest that QA deter most herbivores from feeding on QA containing plants, a fact which has been reported also for many other alkaloids and secondary products [10, 12, 23–27].

One important group of herbivores, the snails and slugs, have been somewhat neglected in studies concerning the ecological role of alkaloids and other natural products, although exciting experimental data have been provided by the pioneering studies of E. Stahl [28] nearly 100 years ago, and later by Jones [29] and Cates and Orians [30]. Stahl could unequivocally show that most molluscs are opportunistic feeders which generally avoid feeding on plants accumulating secondary compounds or plants with physical weapons such as trichomes, hairs or raphides. Stahl did not include QA containing plants in his studies. Since QA are likely to have a general biotoxicity it seemed interesting to explore if they play a role as an antimolluscan principle in legumes.

Materials and Methods

Plants

Plants of *Lupinus polyphyllus*, *L. mutabilis*, *L. albus*, *Baptisia australis*, and *Laburnum anagyroides* were grown in our experimental garden. Other plants were from the Botanical Garden of the University. Experiments were performed in June and July with leaves of flowering plants.

Molluscs

Helix pomatia and *Arion rufus* were kept in small cages (30 × 45 × 20 cm) under laboratory conditions (60% relative humidity, 20 °C, 16 h light). About 8–12 molluscs were kept in a cage and received lettuce leaves ad libitum between experiments. In the wild the molluscs fast on dry days and come out to feed on wet days or nights usually being rather hungry. To simulate these conditions and to have animals which immediately started to feed, the molluscs did not receive food 2–3 d prior to an experiment. The animals were chosen at random for an experiment and were usually employed in feeding experiments only 2 or 3 times, with an intervall of at least 4 days between experiments.

Feeding experiments. To test feeding preferences about 6–10 g portions (Table I, II) of plant material were fed to the snails. In other experiments (Fig. 1) 10 petri dishes were placed into the cages containing 200 or 300 mg dried food material suspended in 5 ml water or alkaloid solutions, which were all adjusted to pH 6.5. After 16 h the weight of the plant material was determined and compared to the respective values prior to feeding; untreated leaves lost about 1–5% of their weight during this interval. The remains of the petri dish experiments were dried at 60 °C for 24 h and their dry weight was determined.

Artificial diet

Leaves of *Lupinus polyphyllus* were suspended in ethanol until most of their chlorophyll and the other natural products were extracted. These leaves were dried for 10 h at 60 °C. The dried leaf material was soaked with water and employed in the feeding test; it was readily consumed by the molluscs.

Alkaloid extraction and determination

QA were extracted and determined according to standard methods as outlined in previous papers [2, 4, 6, 7].

Chemicals

Sparteine sulfate, cytisine were obtained from Roth, Karlsruhe and lupanine was isolated from lupins in our laboratory.

Results

Helix pomatia and *Arion rufus* are polyphagous molluscs and vigorous feeders. One slug may eat up to 4.5 g of leaf material in 24 h [28]. Since these molluscs are abundant at sites where we grow our lupins (about 60 *H. pomatia* and 50 *A. rufus* were counted on 500 square meters on a rainy evening) we have chosen both species to test whether they can discriminate between alkaloidal and non-alkaloidal plants.

In a first set of experiments the molluscs were allowed to feed on a selection of alkaloidal plants, known to contain alkaloids of various types (Table I). *Lactuca sativa* was chosen as a control since lettuce has been selected to be palatable by the herbivoric *Homo sapiens* and as all gardeners know lettuce is often eaten by snails and slugs.

Whereas lettuce was consumed to 60–90%, most alkaloidal plants were not or only slightly eaten. It could be observed that the snails tried to feed on all these plants but usually gave up after a few "bites". However, these results do not necessarily indicate that alkaloids constitute the repellent factor, since other natural products of known mollusc repellency such as phenolics [28] or cyanogenic glucosides [29] are also present in these plants.

In the following results are reported which indicate that QA are indeed potentially mollusc repellent. Species containing QA of the α-pyridone-type, such as cytisine and N-methylcytisine as major alkaloids, were generally not consumed by both species of molluscs. With lupins the situation differed to some degree since their deterrent effect was more gradual: If leaves of *L. polyphyllus* were fed as the only food item to hungry snails and slugs, the leaves were devoured by 80–90% within 20 h (Table II). But this situation does not reflect the

Table I. Feeding preferences of *Helix pomatia* and *Arion rufus*. In each test 8–12 snails were employed which had the choice to feed on about 4 different plant species (6–12 g leaves and stems) containing alkaloids and on *Lactuca sativa* (20 g). After 16–20 h the amount of plant material devoured by the snails was determined. n.d. = not determined.

Species	Alkaloidal type	Alkaloid content	% of plant material devoured after 18 h	
		[µg/g fw]	*H. pomatia*	*A. rufus*
A.				
Atropa belladonna	tropane	n.d.	11	n.d.
Datura stramonium	tropane	n.d.	23	n.d.
Solanum dulcamara	steroid	n.d.	0	n.d.
S. tuberosum	steroid	n.d.	0	n.d.
Papaver somniferum	benzylisochinoline	n.d.	50	n.d.
Eschscholzia californica	benzylisoquinoline	n.d.	0	n.d.
Macleaya microcarpa	benzylisoquinoline	n.d.	0	n.d.
Erythrina crista-galli	benzylisoquinoline	n.d.	0	n.d.
Aconitum napellus	diterpenoid	n.d.	6	n.d.
Conium maculatum	piperidine	n.d.	0	n.d.
Sedum reflexum	piperidine	n.d.	0	n.d.
Catharanthus roseus	indole	n.d.	15	n.d.
B.	QA			
Cytisus purpureus	cytisine	200	0	n.d.
Chamaespartium sagittale	cytisine	800	0	n.d.
Laburnum anagyroides [a]	cytisine	150	30	30
Baptisia australis	cytisine	800	0–5	10
Lupinus polyphyllus	lupanine	1600	31	10
Lupinus albus	lupanine	900	36	41
C.				
Lactuca sativa	–	–	60–90	70–90

[a] Green pods of *L. anagyroides* with > 400 µg/g fw cytisine were totally avoided.

Table II. Feeding of molluscs on lupin leaves. A. molluscs were fed with leaves of one species only. B. snails had the choice between lupin leaves and lettuce leaves. H.p. = *Helix pomatia*).

Species	Alkaloid content [μg · g⁻¹ f.w.]	% of leaf material eaten H.p. $\bar{x}$ (n)	H.p. $\bar{x}$ (n)
		A	B
L. polyphyllus	1600	85 (4)	25 (2)
L. mutabilis	140	85 (5)	41 (2)
Lactuca sativa	–	100	60–75

conditions in the wild, where the molluscs always have a chance to choose between several food items. Providing the choice between lettuce leaves and lupin leaves the snails clearly preferred lettuce (Table II). Leaves of *L. mutabilis* with a lower QA content than *L. polyphyllus* were obviously more palatable than those of the latter species. These experiments indicate that QA containing plants are not the food of choice of molluscs. This is in accordance with field observations, in which I could not detect snails or slugs feeding on intact lupins. Decaying lupins, however, were readily consumed. All these experiments with intact plants had the disadvantage that the alkaloid content of lupins is variable within plants and within time [4] and that other mollusc repellent compounds might also be present which could obscure the influence of alkaloids.

Therefore we have devised experiments employing "artificial" diets, to which specified amounts of QA were added, so QA content was the only variable of the system. The artificial diet which had no mollusc repellent properties consisted of leaves of *L. polyphyllus* from which the natural products were leached out with ethanol. The dry diet was soaked in alkaloid solutions of increasing concentrations thus simulating the spectrum from "sweet" to "bitter" lupins. Generally the alkaloid-free food was readily and nearly completely devoured whereas the samples with higher alkaloid concentrations deterred the snails from feeding (Fig. 1 A). Given the choice of more alkaloid-free material, such as lettuce in relation to the alkaloid-containing diet, the snails stopped feeding on alkaloid-containing samples at even lower QA concentrations (Fig. 1 B):

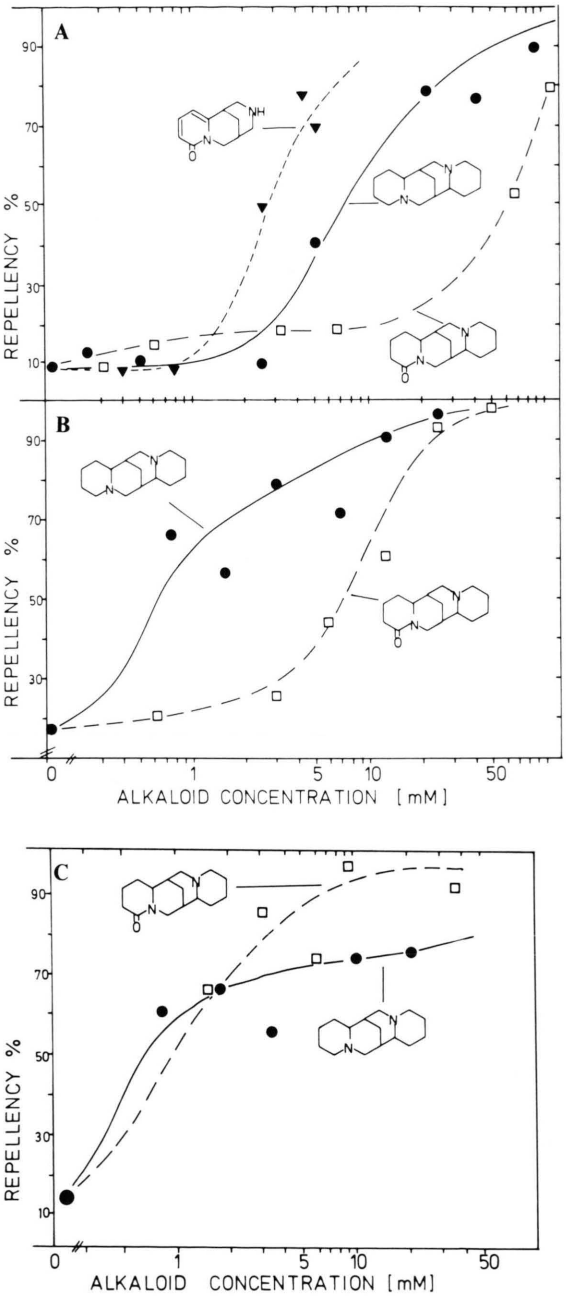

Fig. 1. Feeding response of *Helix pomatia* on artificial food containing QA. Alkaloid-free food (leached leaves of *L. polyphyllus*) was reconstituted with QA of different concentrations. A. Only food containing alkaloids was offered to the snails. B. In addition to alkaloid containing food, 15 g lettuce leaves, were given. C. Instead of reconstituted lupin leaf material lettuce leaves were soaked in respective alkaloid solutions (as above). The snails had the choice between the alkaloidal (2 g fw. each) and normal lettuce (10 g fw). Repellency was calculated from the amount of leaf material not devoured within a 16 h feeding experiment.

Table III. Comparison between half-maximal *in vitro* repellency (ED_{50}) of QA and their *in vivo* concentrations (leaves, stems).

Alkaloid	Half-maximal deterrency		*in vivo* concentrations	
	[mM]	[µg/ml]	[mM]	[µg/g fw]
Sparteine [a,d]	0.6–0.7	140–163	1.3–175	300–41 000
Lupanine [b,d]	1.0–7.0	248–1800	0.8–20	200–5000
Cytisine [c,e]	2.5	475	0.26–58	50–11 000

[a] *Cytisus scoparius.*
[b] *Lupinus polyphyllus.*
[c] *Laburnum anagyroides.*
[d] from Fig. 1 B, C.
[e] from Fig. 1 A. This value is probably lower under *in vivo* conditions. On account of a limited cytisine supply, this compound could not be tested in experiments of Fig. 1 B and C.

alkaloid-free samples and lettuce were clearly preferred. A similar result was obtained in experiments with lettuce imbibed in alkaloid solutions of increasing concentrations (Fig. 1 C). As can be seen from Fig. 1 cytisine displayed the strongest deterrency, followed by sparteine and lupanine. Since the half-maximal inhibitory concentrations of all 3 QA are equal to or lower than the concentrations of the respective alkaloids found in the living plant (Table III), we assume that the QA are potentially mollusc repellent *in vivo.*

Discussion

E. Stahl [28] was one of the first scientists who studied chemical aspects of plant-animal relationships in detail. He was aware that during coevolution of plant and animals very important adaptations had occurred: The scents and colours of flowers evolved to attract pollinating animals, whereas the thorns, spines, stinging hairs and plant poisons (the so-called "secondary compounds") were preferentially aimed to protect the plants against predating herbivores (mammals, insects). He studied how plants are protected against predation by molluscs and could show that molluscs feed less on plants with hairs (trichomes), raphides, or secondary plant substances, such as phenolics, oxalic acid, essential oils, or alkaloids.

Stahl had studied a few alkaloid-containing plants, and could show that these plants are usually not eaten by molluscs. We obtained nearly the same results studying the feeding preferences of *Helix pomatia* to 19 plant species containing alkaloids of 7 different types. Plants with QA were analyzed in more detail. If the snails had the choice between lettuce leaves and those of QA containing plants, they clearly preferred the former. However, lupin leaves from which the alkaloids were leached out, were readily accepted. That QA are really mollusc-repellent could be shown in experiments with artificial diets which contained QA solutions of increasing concentrations as the only variable. The results show that the half-maximal inhibitory concentrations of sparteine, lupanine, and cytisine are equal to or much lower than the respective QA concentrations in the intact plant. QA of the α-pyridone type, such as cytisine, were most repellent. This corresponds to the higher toxicity of cytisine in mammalian systems as compared to sparteine or lupanine [30, 31]. It is likely that the mollusc repellent properties of QA are even stronger expressed under natural field conditions because the molluscs have the choice of a wider variety of food plants there, a trend indicated in Fig. 1 B. Furthermore synergistic effects between different QA and other natural products present in a plant have also to be taken into account. The concentration of QA can increase 4-fold within 2–4 h after wounding of a lupin leaf. Thus a leaf being wounded by a herbivore becomes more and more repellent in a short time as compared to the feeding speed of a larva or mollusc [34]. Therefore we conclude that QA constitute a potential defense system of legumes against molluscs and other herbivores. Since QA are also effective against bacteria, other plants, insects and mammals, these compounds have evolved as molecules of general biotoxicity. This is true for a number of other secondary compounds such as phenolics, gossypol, cardiac glucosides and saponins [12, 24] only to mention a few.

In addition to the ecological role of QA, they may play a role also in the primary metabolism of a lupin, as has been postulated for other compounds [35]. Possible functions could include nitrogen storage in seeds and nitrogen transport in phloem.

Thus QA constitute a good example for the raison d'etre of secondary plant products, as conceived in the work of Fraenkel [36, 37], Ehrlich and Raven [38], Zenk [39], Whittacker and Feeny [40], Harborne [12, 25], Levin [24], Rosenthal and Janzen [27].

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