

Wounding-Induced Increase of Quinolizidine Alkaloid Accumulation in Lupin Leaves

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Z. Naturforsch. **38c**, 905–909 (1983); received August 26, 1983

Quinolizidine Alkaloids, Wound-Induction, Defense Response, *Lupinus polyphyllus*

Cutting-up leaflets of *Lupinus polyphyllus* induces a rapid increase of quinolizidine alkaloid accumulation of up to 400% within 2 to 4.5 h. In contrast to the diurnal alkaloid formation, this reaction takes place both in the light and the dark and even at 4 °C. The effect is reduced in chloramphenicol-treated samples but unimpaired in cycloheximide-treated assays. Since quinolizidine alkaloids constitute probably an important means of a chemical defense system of lupins against microbes and herbivores, the wound-induction of alkaloid accumulation is discussed as a defense response.

Introduction

Quinolizidine alkaloids are widely distributed among Fabaceae [1, 2]. We came to the conclusion that these alkaloids have evolved in legumes as a means of a general defense system. Aspects of this defense include plant-bacteria [3], plant-herbivore [4] and plant-plant [5] interactions.

The question arises if this defense system is constitutive or if it can respond to environmental factors. A strong signal should be the wounding of a plant, which would be similar to a herbivorous attack.

In this communication we describe the increase of alkaloid concentration in lupin leaves after wounding. This demonstrates the dynamic state and ecological reactivity of lupin alkaloid metabolism.

Material and Methods

Plants

Plants of *Lupinus polyphyllus* Lindl. were grown outdoors under natural conditions. Experiments were performed during May and June with pre-flowering and flowering plants.

Wounding experiments

A. Outdoor experiments: leaflets of *L. polyphyllus* were clipped with scissors. After 2 and 5 h the clipped leaflets were collected and their alkaloidal contents were compared to those of unclipped leaflets from the same leaf.

B. Laboratory experiments: leaflets of about 2–3 leaves were pooled and cut into small segments of 3–5 mm width. These fragments were thoroughly mixed to obtain a homogeneous sample. About 500 mg material was taken for the non-induced control which was immediately homogenized. Samples of about 500 mg were taken for the induction experiments. These leaf fragments were left floating on bidistilled water in a petri dish. In some experiments the water contained inhibitors of translation. Samples were either incubated on the laboratory bench in the light, in the dark at 24 °, or 4 °C. After 2 and 4.5 h the samples were collected and homogenized immediately.

Alkaloid extraction

Leaf samples and the respective incubation water were adjusted to pH 1 with 2 N HCl and homogenized in a mortar or an ultraturrax. The resulting homogenate was made alkaline with 25% ammonium hydroxide and applied onto standard Extrelut (Merck, Darmstadt) columns. The alkaloids were extracted with methylene chloride and the eluate evaporated in vacuo at 30 °C [6].

Capillary gas-liquid chromatography

Alkaloid extracts were separated on fused silica capillary columns under standard glc-conditions as described in [6–8]. The quinolizidine alkaloids of *L. polyphyllus* have previously been studied in our laboratory and have been identified in this study by comparing the specific retention indexes of an alkaloid with that of an authentic sample [9, 10].

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0341-0382/83/1100-0905 \$ 01.30/0



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Assay of oxosparteine synthase

Acetone powders were prepared from cut-up leaflets after 4 h and assayed for oxosparteine synthase [11] activity according to [6].

Results

Comparing the alkaloid content of a clipped leaflet to that of an untreated neighbouring leaflet showed an increase of individual quinolizidine alkaloids up to 600% after 5 h (Table I). It was somewhat difficult to handle this system, since the alkaloid content varies from leaflet to leaflet and because wounding of one leaflet seemed to influence the alkaloid content of the neighbouring leaflet also.

The subsequent experiments were therefore performed in the laboratory using excised leaflets or leaves. In these experiments the leaflets were cut into small segments which were left floating on bidistilled water. Under these conditions the alkaloid content of the segments increased in an almost linear fashion over the 4.5 h studied (Fig. 1). No difference was observed in samples incubated in the dark or even in the refrigerator at 4 °C as

compared to those incubated at 24 °C under natural light conditions.

In another set of experiments we compared the change of alkaloidal content after wounding of intact leaves (petioles were cut), intact leaflets and cut-up leaflets (Fig. 2). Whereas intact leaves seemed to be only slightly influenced by wounding, there was a marked effect in leaflets and fragments.

To get a first idea on the nature of the wounding-induced increase of alkaloid accumulation, inhibitors of translation were added to the incubation water (Fig. 1). Whereas cycloheximide was rather inactive, a marked inhibition of the accumulation was observed when chloramphenicol, an inhibitor of prokaryotic systems, was added. In cell suspension cultures of *L. polyphyllus* quinolizidine alkaloids only accumulate in the light, thus following a diurnal rhythm. This diurnal activity can be inhibited by the application of chloramphenicol and not by cycloheximide (Wink, in preparation). These first results, obtained from the wounded leaves or the cell cultures, must be interpreted with caution, since it was not established if the inhibitors, *i.e.* cycloheximide were taken up by the cells and if other processes were also influenced. However, it is

Table I. Wound-induced increase of quinolizidine alkaloid accumulation in leaflets of different *Lupinus polyphyllus* plants. A. *In vivo* experiments: About 2–4 leaflets/leaf were clipped. After 5 h clipped and non-clipped leaflets were collected and their alkaloid contents analyzed. B. *In vitro* experiments: cut-up leaflets were incubated for 4.5 h and their alkaloid contents determined by glc.

Plant Nr.	Conditions	Alkaloid content of controls [µg/g fw]	Change of alkaloid abundance % (control = 100%)			
			Tetrahydro- rhombifoline	Lupanine	13-Tigloyl- oxylupanine	Total alkaloids
A.						
1	standard	1250	140	178	135	152
2	standard	520	153	158	160	158
3	standard	330	203	600	254	289
4	standard	500	100	216	100	216
B.						
5	standard	430	100	240	271	276
6	standard	660	177	236	141	181
7	standard	1190	356	283	337	350
8	standard	720	400	800	134	280
9	standard	720	850	1900	275	388
10	senescing leaf	110	—	110	100	105
	very young leaf	600	—	80	86	83
	mature leaf	700	—	272	168	183
11	young leaf	30	43	166	—	100
	mature leaf	150	97	134	233	154
12	senescing leaf	190	108	188	190	189
	mature leaf	1400	240	1800	666	364
13	senescing leaf	480	100	204	144	150
	mature leaf	2890	133	142	161	144

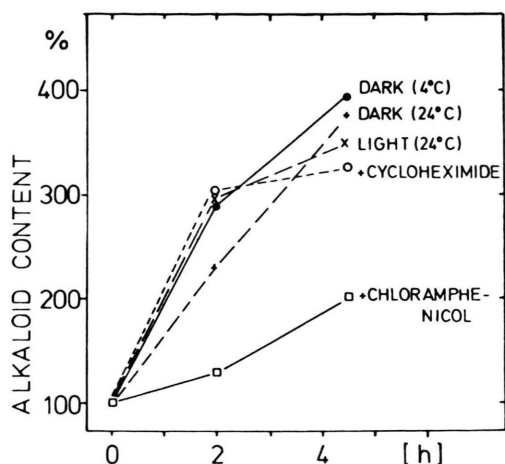


Fig. 1. Time course of wounding-induced alkaloid accumulation in cut-up leaflets of *Lupinus polyphyllus*. Cut-up leaf fragments were left floating on water in petri dishes and were incubated either under light or in the dark. Cycloheximide and chloramphenicol were added at concentrations of about 2 mM. After 2 and 4.5 h samples were collected and their alkaloids analyzed by glc. The data for cycloheximide and chloramphenicol treated assays are mean values of 3 independent experiments.

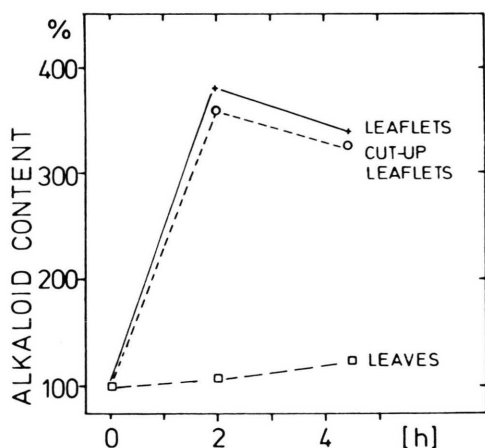


Fig. 2. Comparison of wounding-induced alkaloid accumulation between excised leaves, leaflets, and cut-up leaflets. Experiments were performed at 24 °C under natural light conditions.

tempting to assume that chloroplast genes are involved in the regulation of quinolizidine alkaloid metabolism, especially since quinolizidine alkaloid biosynthesis is localized in the chloroplast [12, 13].

As concerns oxosparteine synthase activity (the key enzyme of alkaloid formation) no difference was observed between non-induced controls,

Table II. Activity of oxosparteine synthase [11] in non-induced, induced and chloramphenicol-treated leaflets. Assay conditions [6]: 20 mg acetone powder (ap) suspended in 1 ml 0.1 M phosphate buffer (pH 8) containing 3 mM dithioerythritol, 10 mM diethyldithiocarbamate, 20 mM pyruvate, 5 μ M [1,5- 14 C]cadaverine (0.5 μ Ci); anaerobic incubation at 30 °C for 4 h.

Assay conditions	Enzymic activity	
	[pmol (h) g ap] $\bar{x} \pm$ s.d.	n
Non-induced leaf	272 \pm 102	4
Induced leaf	287 \pm 87	4
Induced leaf + chloramphenicol	303 \pm 67	4

induced leaflets or chloramphenicol-treated leaflets (Table II). This finding would indicate that it is probably not de novo enzyme synthesis which is induced as is the case in the elicitor-induced formation of phytoalexin [15, 16]. It has to be considered that an increase of alkaloid accumulation might be accomplished by a modulation of alkaloid turnover.

Studying more than 13 different plants it was evident that the wounding response differed individually (Table I). Old and very young leaves, which according to their alkaloid contents were not actively producing alkaloids, tend to be less reactive to wounding than actively producing leaves. Out of the individual alkaloids analyzed lupanine and 13-tigloyl-oxylupanine, the major alkaloids of lupin leaves, usually displayed the strongest response.

Discussion

Like other plants lupins need defense systems against infection by bacteria, fungi and against predation by herbivores. We have evidence that the lupin alkaloids are one means of the chemical defense system, since they are antimicrobial, allelopathic and herbivore-repellent [3–5]. There is evidence that structurally different secondary compounds play similar roles in other species [14, 18].

Defense systems may be either inducible or constitutive. Among the first group are the phytoalexins [14]. Fungal attack, elicitors, and other factors such as wounding and certain chemicals induce the enzymes of the phenylpropanoid pathway, especially phenylalanine ammonia lyase and conse-

quently the fungitoxic phytoalexins in some Fabaceous species [14–19]. The induction of antimicrobial acridone alkaloid accumulation in *Ruta graveolens* cell cultures by fungal elicitors seems to fall also in this group [20]. An exciting and well-studied example of an insect- or wound-induced plant defense mechanism is the induction of antimicrobial and repellent proteinase inhibitors in tomato and other plants [21]. Most of the other secondary compounds are considered to be constitutive defense measures if any role is attributed to them at all.

The wound response of lupins does not seem to fit strictly either the category of a constitutive or an induced defense, because most plants have intrinsic high levels of quinolizidine alkaloids, which can be amplified, however, by wounding. So lupin alkaloids are a qualitatively constitutive but quantitatively inducible trait. The inducibility seems to be important since the immediate increase of quinolizidine alkaloids could deter a predator from further feeding and it would decrease the risk of a bacterial infection developing in the wound. It is remarkable that the alkaloid accumulation can be induced in the dark and even at 4 °C, which is in contrast to the natural diurnal rhythm of alkaloid accumulation [6]: No alkaloid is formed in the dark under non-induced conditions. Reactivity in the dark would enable the lupin to respond to a herbivorous attack also at night.

Examination of the wound areas of lupin leaflets by fluorescence microscopy revealed white fluorescent compounds already 2–3 h after wounding. This fluorescence was very prominent after 24 h. It is likely that these compounds constitute phenolics (quinolizidine alkaloids do not fluoresce) which are considered to be antimicrobial and involved in the wound-reaction of plants [18]. This observation indicates that the chemical defense system of lupins is not composed of a single component but probably includes different groups of natural products.

Another example for a reactive defense system are silicates in grass which could be induced by clipping, *i.e.* grazing [22]. Silification in grasses has long been viewed under the aspect of coevolution of grasses and hypsodont dentition in grazing mammals. Since herbivores can discriminate between high- and low-silica plants and will preferentially feed on the latter [23], an induction of silification can amplify the deterrent effect.

The observation that wounding induces an increased alkaloid accumulation in lupins raises the question whether it is possible to induce alkaloid formation in lupin cell cultures, which usually accumulate only low amounts of quinolizidine alkaloids [7, 10]. Since these cell cultures contain relatively active enzymes of alkaloid biosynthesis [24], they thus possess the potential of alkaloid formation. Recent experiments indicate that a short-time (up to 170-fold) increase of alkaloid formation can be achieved in these cell cultures by treatment of the cells with foreign alkaloids or polyamines [25]. Since the rate of alkaloid synthesis seemed to be unimpaired in these experiments as concerned the activity of oxosparteine synthase, we suggested that the level of turnover was the part of alkaloid metabolism most influenced. We have to test whether the induction of alkaloid accumulation upon wounding is similar in nature to the induction observed in the cell cultures.

Considering these ecological interactions, another approach would be open for the modulation of secondary compound metabolism in plant cell cultures, or for the development of plant varieties resistant to plant pests.

Acknowledgement

This study was supported by grants of the Deutsche Forschungsgemeinschaft and the Land Niedersachsen. I would like to thank Prof. Dr. T. Hartmann for encouragement, helpful discussions, and C. Theuring for technical assistance.

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