Chemical Defense of Leguminosae. Are Quinolizidine Alkaloids Part of the Antimicrobial Defense System of Lupins?

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Growth of 6 bacteria (*Serratia marcescens*, *Bacillus megaterium*, *Bacillus subtilis*, *Streptococcus viridis*, *Micrococcus luteus*, and *Mycobacterium phlei*) was inhibited by 50% if the growth medium contained sparteine at concentrations between 0.5–10 mM. Total growth inhibition, which was bacteriostatic in nature, was achieved at 20 mM. The growth of 6 phytopathogenic fungi was also affected: at a sparteine concentration of 15 mM the growth of *Alternaria porri* was reduced by 40% as compared to the untreated control. Respective values were 18% inhibition for *Piricularia oryzae*, 33% for *Helminthosporium carbonum*, 15% for *Rhizoctonia solani*, 5% for *Fusarium oxysporum*, and 42% for *Asperquillus oryzae*. Since the concentrations of quinolizidine alkaloids range from 1–200 mM (roots, leaves, or stems) or 10–200 mmol/kg (seeds) in Leguminosae, it is discussed whether quinolizidine alkaloids are involved in the antimicrobial defense of lupins, in addition to their potential role as allelopathic or herbivore repellent defense compounds.

Introduction

Most organisms are threatened by infections of bacteria and fungi. Animals, especially vertebrates, possess a very effective antimicrobial defense system, *e.g.* the humoral and cellular immune response.

Higher plants, too, are subject to attack by many bacteria and fungi as they have direct contact to the soil which contains nearly all germs. Since plants survived during evolution, however, they too must possess an effective antimicrobial defense system, which may be realized by many different mechanisms [1–3]: Texture and composition of the plant surface, unsuitable pH and osmotic pressure will not favour bacterial growth. But these factors are certainly not altogether sufficient to prevent a fungal or bacterial infection in plants. There is good evidence that the so-called secondary compounds of plants, ranging from phenolics to terpenes, alkaloids, saponins, cyanogenic, and mustard oil glycosides, play a crucial role in antimicrobial defense [1, 3–6].

Quinolizidine alkaloids are common natural products of Leguminosae [7]. They are formed in the leaf chloroplast [8], but are then translocated to the other plant parts *via* the phloem [9, 10], so that alkaloids accumulate in all organs, especially those involved in reproduction [11]. We could recently show that QA seem to be important for lupins in ecological terms: Quinolizidine alkaloids are repellent to herbivores (insects, mammals, molluscs) [9, 12, 13], and inhibit the germination of other plants [14]. Since the relevant inhibitory concentrations match those found in the plants we conclude that plant-herbivore and plant-plant interactions are mediated, at least to some degree, by the quinolizidine alkaloids.

In this communication evidence is provided that the lupin alkaloids have antimicrobial properties as well and that they may constitute an important part of the antimicrobial defense system of lupins.

Abbreviations: QA, quinolizidine alkaloids.

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Material and Methods

Microorganisms

Serratia marcescens 1534, *Bacillus subtilis* 1527, *Bacillus megaterium* 1621, *Micrococcus luteus* 1557, *Streptococcus viridis* and *Mycobacterium phlei* were from the collection of Dr. B. Wolters (Institut f. Pharmazeutische Biologie, Braunschweig), and were cultured on Difco nutrient broth (pH 6.8). *Piricularia oryzae*, *Alternaria porri*, *Fusarium oxysporum f. pisi*, *Helminthosporium carbonum*, *Aspergillus oryzae*, *Rhizoctonia solani* 4246 (collection Wolters) were grown on a malt agar containing (per 1000 ml) 40 g malt extract, 5 g peptone, 0.5 g yeast extract, 20 g agar (pH 5.4).

Antimicrobial assays

a. Bacteria: Bacteria were grown in test tubes on nutrient broth medium containing different concentrations (3 replicates) of filter-sterilized sparteine (pH 6) at 30 °C. To determine the growth inhibition the optical density (800 nm) was measured after 16–36 h, and the growth of the untreated controls was set 0% inhibition. Growth of air-borne microorganisms was determined on nutrient broth agar plates, containing filter-sterilized sparteine, lupanine or 13-tigloyl-oxylupanine at different concentrations. Growth inhibition was determined from the number of colonies formed after 48 h of incubation.

b. Fungi: Malt agar plates, containing filter-sterilized sparteine were incubated with 6 small fungal colonies (about 3 mm diametre) at 30 °C. To determine the growth inhibition by alkaloids, hyphal growth was measured every 48 h.

Capillary gas-liquid chromatography

Quinolizidine alkaloids were separated on fused silica capillary columns as described in Wink *et al.* [15, 16]. The analytical and chemical properties of these compounds have been determined previously in our laboratory.

Chemicals

Sparteine was obtained from Sigma, München. The other QA were isolated from plants in our laboratory (Wink in preparation): Lupanine and 13-tigloyl-oxylupanine had a purity of > 90% or 80%, respectively.

Results

In a first set of experiments we tested if quinolizidine alkaloids possess antibacterial properties. For this purpose we exposed agar plates for 5 min in the air of our experimental garden in close vicinity to cultured lupins to obtain a sample of the air-borne microorganisms present there. The numbers of bacterial colonies and their size are markedly reduced on plates containing increasing amounts of QA (Fig. 1), thus providing first evidence that QA possess an antimicrobial potential. Since these experiments involved many different undefined microorganisms, we have tested the antibacterial properties of sparteine in more detail in 6 different bacterial species (mostly gram-positive). As can be seen from Fig. 2 a half-maximal growth inhibition (ED_{50}) was usually obtained between 0.1 and 10 mM: Respective values were 2.5 mM for *Mycobacterium phlei*, 3 mM for *Bacterium megaterium*, 0.5 mM for *B. subtilis*, 1.5 mM for *Micrococcus luteus*, < 0.5 mM for *Streptococcus viridis* and 10 mM for the gram-negative *Serratia marcescens*. Sparteine is probably bacteriostatic and not bacteriocidal since viable bacteria could be readily isolated from all culture tubes, even those which showed a 100% growth inhibition.

In another set of experiments we examined sparteine in relation to 6 fungal, mostly phytopathogenic species. As can be seen from Table I, fungal growth is reduced with increasing alkaloid concentrations. However, the inhibitory concentrations are higher than those for the bacteria tested: At a sparteine concentration of 15 mM the growth of *Alternaria porri* was reduced by 40% as compared to the untreated controls. Respective values were 18% inhibition for *Piricularia oryzae*, 33% for *Helminthosporium carbonum*, 15% for *Rhizoctonia solani*, 5% for *Fusarium oxysporum*, and 42% for *Aspergillus oryzae*. These values constitute a rough estimate of fungal growth inhibition, since growth was assessed from the diameter of fungal colonies only.

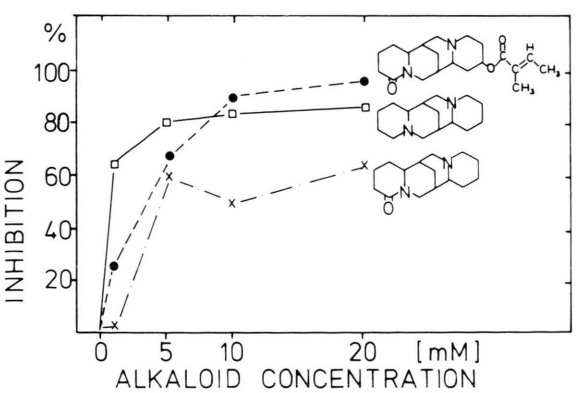

Fig. 1. Inhibition of bacterial growth by quinolizidine alkaloids. Agar plates containing increasing concentrations of sparteine, lupanine, or 13-tigloyl-oxylupanine (2 replicates each) were exposed to the air of the experimental garden for 5 min to obtain a sample of the air-borne bacteria present at the site where the lupins were cultivated. After incubation of the plates at 30 °C for 1–3 days the number of bacterial colonies were counted and compared to that of the respective control plates which were free from alkaloids. Only few fungal infections were recorded, usually on the control plates or on plates with low concentrations of alkaloids.

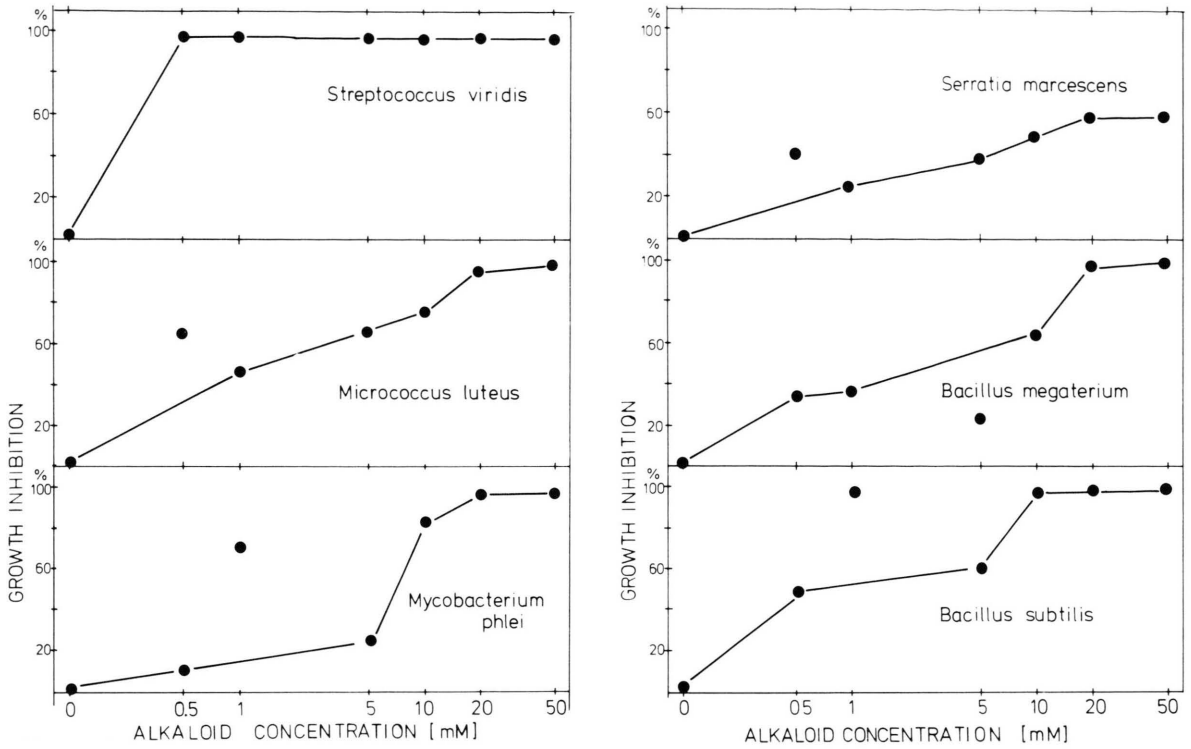

Fig. 2. Inhibition of bacterial growth by sparteine. Test tubes with 5 ml growth medium containing different concentrations of sparteine (3 replicates) were inoculated with 50 µl of densely grown bacterial suspensions. Incubation of the test tubes was performed at 25 °C on a rotary shaker. The optical density of the bacterial suspensions was determined at 800 nm after 12–36 h when the control tubes without sparteine showed an OD value of 0.4.

Table I. Inhibition of fungal growth by sparteine. Mycelial particles of about 3–5 mm diameter (3 per plate, 2 replicates) were incubated on malt agar plates containing different concentrations of sparteine. Fungal growth (diameter of the colonies; $\bar{x} \pm$ s.d. mm, $n = 6$) was assessed before the colonies came into contact with each other, usually after 5–10 days.

Fungus	Mycelial growth [mm] $\bar{x} \pm$ s.d.					
	Control	sparteine [mM]				
		1	5	10	15	50
Aspergillus oryzae	55 ± 3	25 ± 3	24 ± 3	22 ± 2	32 ± 2	28 ± 3
Fusarium oxysporum	35 ± 5	42 ± 3	31 ± 2	29 ± 3	33 ± 7	32 ± 5
Rhizoctonia solani	20 ± 4	18 ± 13	16 ± 9	18 ± 2	17 ± 2	14 ± 2
Helminthosporium carbonum	27 ± 4	29 ± 3	19 ± 1	18 ± 2	18 ± 1	12 ± 0.5
Piricularia oryzae	34 ± 8	26 ± 1	21 ± 1	27 ± 4	28 ± 5	23 ± 2
Alternaria porri	23 ± 6	18 ± 2	18 ± 2	17 ± 2	14 ± 1	7 ± 0.5

Discussion

Several conditions have to be fulfilled before we can consider a compound to be part of an antimicrobial defense system [2, 4]: 1. Inhibition of bacterial or fungal growth by a given compound must be expressed *in vitro*. 2. The concentration of the respective compound in the intact plant should be in the same order or higher as the inhibitory concentrations observed *in vitro*. 3. The compound should be present at the site of a potential infection. 4. The intact plant should be protected against microbial attack *in vivo*.

The experiments show that QA affect the growth of several bacteria and of some phytopathogenic fungi *in vitro* at concentrations between 1 and

20 mM (Table II). Although these concentrations exclude a therapeutic use of these compounds because of their high toxicity (Table II) it does not mean, however, that the antimicrobial effects observed, are without any biological significance for the lupin itself. The concentrations of lupanine, the major alkaloid of most lupins, fall in the range of 1 to 40 mM in lupin leaves, stems, and roots, and are up to 200 mmol/kg in seeds. This means that the alkaloid concentrations of the lupin plant are generally in the range where the alkaloids express effective antimicrobial activity (Table II) or even much higher.

Further, the concentrations of QA in plants are not static. They follow a diurnal rhythym with high levels in the day and low levels at night [10, 17]. Recent experiments with *Lupinus polyphyllus* show that the level of alkaloids can be triggered additionally by wounding, a situation which ressembles a herbivoral attack: Wounding of leaves increased the amount of QA 2–4-fold within 2–4 h [18]. Since wounding increases the danger of a bacterial infection [3], the spontaneous increase of QA thus amplifies the antimicrobial potential of lupins.

It is much more difficult to obtain data which unequivocally show that the condition 4 (see above) is fulfilled. Lupins and other Leguminosae (*Baptisia, Cytisus, Laburnum, Spartium, Sophora*) which accumulate QA in nearly all their tissues, are grown in our experimental garden, and only seldomly acquire a microbial infection. But this might be due to other factors than the alkaloids studied. However, alkaloid-"free" strains of lupins ("sweet lupins") often show a lower resistance against disease than the wild

Table II. Repellent or inhibitory concentrations of quinolizidine alkaloids (A) in relation to their concentrations in the plant (B). LD_{100} = lethal dose at which 100% of the animals died. ED_{50} = Dose, at which the inhibitory or repellent effect was 50% of the maximal effect possible. T = toxicity, FR = feeding repellency, GI = growth inhibition, n.d. = not determined, i.p. = intraperitoneal, p.o. = per os, s.c. = subcutan.

Organisms	Parameter		Lupanine	Sparteine	Cytisine	13-Tigloyloxy-lupanine
A.						
Mouse [a]	i.p.T LD_{100}	[mmol/kg]	n.d.	0.2–0.4	n.d.	n.d.
	i.p.T LD_{50}	[mmol/kg]	n.d.	0.2–0.3	n.d.	n.d.
	p.o.T LD_{50}	[mmol/kg]	n.d.	1.5–2.2	n.d.	n.d.
Rat [b]	i.p.T LD_{100}	[mmol/kg]	0.8	0.1–0.2	n.d.	n.d.
Cat [c]	s.c.T LD_{100}	[mmol/kg]	n.d.	n.d.	0.02	n.d.
Molluscs						
(Helix pomatia) [d]	FR ED_{50}	[mM]	1–7	0.7	2	n.d.
Aphids [e]	FR ED_{50}	[mM]	0.5	n.d.	n.d.	n.d.
Lactuca sativa [k]	GI ED_{50}	[mM]	7	50	6	2
Bacteria [f]						
air-borne bacteria	GI ED_{50}	[mM]	5	0.5–3	n.d.	3
Gram-positive bacteria	GI ED_{50}	[mM]	n.d.	0.5–7	n.d.	n.d.
Phytopathogenic fungi [f]	GI ED_{50}	[mM]	n.d.	5–50	n.d.	n.d.
B.						
Lupinus polyphyllus [g]	leaves	[mM]	2–20	0.1	–	1–10
	seeds	[mM]	100–200	0.1	–	0.5
	roots	[mM]	1–4	–	–	–
Cytisus scoparius [h]	stems	[mM]	0.5	17–200	–	0.1
	seeds	[mM]	10	–	–	–
Laburnum anagyroides [i]	stems	[mM]	–	–	0.2–5	–
	stem bark	[mM]	–	–	60	–
	seeds	[mM]	–	–	20	–

Deterrent properties of QA were also confirmed with beetles (Bruchidae) [25], Thrips [26], grasshoppers (*Melanoplus*) [27], hares [13], *Gallus domesticus* [28], and *Homo sapiens* [29].

References: [a] from [30, 31], [b] [31, 21], [c] [33], [d] [12], [e] [9], [f] (this study), [g] [11, 34], [h] [35], [i] [34], [k] [14].

"bitter" varieties [13] indicating that QA may be actually involved in the antimicrobial defense system of lupins. This defense is regularly broken by a mildew, a specialized pathogen. But since no defense is absolute some parasites have evolved during evolution which could circumvent this defense and thus found a niche free from competitors. But these pathogens do not contradict the proposal that secondary compounds constitute the means of the chemical defense system of plants but reflect a general biological principle.

All these data support the idea that QA may be involved in the antimicrobial defense system of lupins. Since QA are also effective in plant-plant and plant-herbivore interactions [9, 10–12] (Table II), the question is, whether the antimicrobial activity is a main feature or only a side effect. It is likely that phenolics and other natural products play the main role in the antimicrobial defense system of lupins, from which simple phenolics, fungitoxic isoflavones [19] and 2-*trans* hexenal [20] have been reported. However, it can be assumed that QA are synergistic to the other natural products and may be therefore also important in the antimicrobial defense. It should be mentioned, that Leguminosae which do not accumulate QA usually contain other repellent and antimicrobial compounds, such as non-proteinogenous amino acids [21, 22], *e.g.* canavanine [21], proteinase inhibitors [23], lectins [24], or cyanogenic glycosides [19].

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