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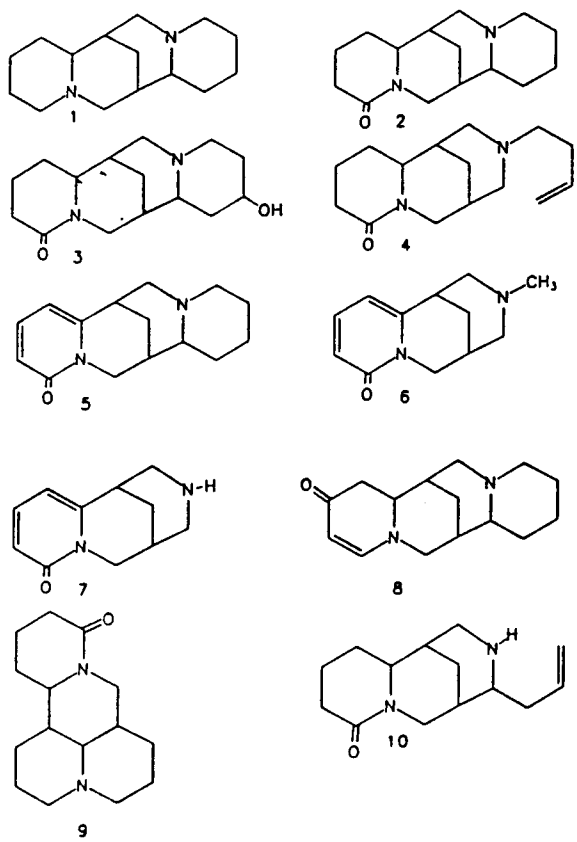
PLANT BREEDING: LOW OR HIGH ALKALOID CONTENT?

Michael Wink, Universität Heidelberg, Institut für Pharmazeutische Biologie, Im Neuenheimer Feld 364, D-6900 Heidelberg.

1. Introduction

Quinolizidine alkaloids constitute a characteristic class of natural products in legumes, specially of the General Lupinus, Baptisia, Thermopsis, Genista, Cytisus, Chamaecytisus, Laburnum and Sophora. Over 150 individual quinolizidine alkaloids are known which can be grouped in the classes illustrated in Fig. 1.

Fig. 1 Structures of quinolizidine alkaloids
1 = sparteine, 2 = lupanine, 3 = 13-hydroxylupanine,
4 = tetrahydrorhombifoline, 5 = anagryne, 6 = N-methylcytisine,
7 = cytisine, 8 = multiflorine, 9 = matrine, 10 = angustifoline.



Since the quinolizidine alkaloids occur in complex mixtures often involving more than 15-25 individual structures only chromatographic methods with a high resolution power are adequate for their complete analysis. As a method of choice we have applied capillary GLC, which allows the separation of nearly all the alkaloids present, even of most of the enantiomers. Furthermore, since this method can be combined with mass spectrometry (GLC-MS; EI and CI-mode) it is relatively easy to identify the individual compounds present (Kingholm and Balandrin, 1984; Wink et al. 1980, 1981, 1982, 1983; Wink and Witte, 1984, 1985, 1991). If a nitrogen-specific detector is used, the sensitivity of this method is in the nanomole range and therefore adequate even for those studies where minute amounts of alkaloids have to be analyzed. TLC, HPLC and colorimetric tests work for some studies, but have less resolution power and are less sensitive (Table 1). Within the last few years immunological assays have been developed: an ELISA for lupanine in Australia (Allen et al., 1990), and RIA, ELISA and SPA-assay for lupanine in my laboratory (Wink unpublished). These assays are highly specific and precise and allow the determination of picomole amounts of lupanine-type alkaloids.

Tab.1. Sensitivity of analytical methods for the determination of quinolizidine alkaloids.

Technique	Sensitivity 1 mg	1 µg	1 ng	1 pg
Photometry	---------->			
Thinlayer chromatography (TLC)	---------->			
HPLC	---------->			
Capillary GLC	---------->			
GLC-MS	---------->			
^{1}H-NMR	------>			
^{13}C-NMR	--->			
ELISA	---------->			
RIA	---------->			

In general, all parts of a lupin plant contain alkaloids, although their biosynthesis is restricted to the green tissues, specially the leaves (review: Wink, 1987). Rich in alkaloids are the seeds (Tab. 2), which are also a good source for proteins and lipids. Some general trends can be observed for total alkaloid contents: seed alkaloid levels of wild specimens are positively correlated with seed size but negatively with seed number. I.e. they are relatively low in species with many small seeds, but high in those species which form few and large seeds (Wink, 1985). The alkaloid levels in the green parts of scrubby legumes, such as brooms seem to be negatively correlated with the formation of spines: in spiny species, such as Ulex chemical defence seems to be substituted by mechanical defence.

Tab.2. Composition and abundance of quinolizidine alkaloids in lupin seeds of different ecotypes.

Alkaloid	Alkaloid composition (total alkaloid = 100%) L. albus	L. angustifolius	L. mutabilis	L luteus
Sparteine	+	+	5-20	30-50
Lupanine	50-80	50-80	50-70	+
17-Oxolupanine	+	+	-	-
4-Hydroxylupanine	-	-	7	-
13-Hydroxylupanine	5-15	10-20	10-20	-
Albine	5-15	-	-	-
Multiflorine	3-10	-	+	-
Angustifoline	+	5-20	+	-
Tetrahydrorhombifoline	+	+	4	+
Lupinine	-	-	-	40-70
Esteralkaloids	1-5	1-3	1-5	+

+ = traces

We have studied the physiology and biochemistry of quinolizidine alkaloids (biosynthesis, transport, storage, degradation, etc.) in some detail (for review: Wink, 1985, 1987, 1988; Wink and Hartmann, 1985).

2. What is the function of quinolizidine alkaloids in lupins?

For many years it has been argued that alkaloids and other secondary compounds are waste products, a view which is not supported by experimental evidence, however; in case of lupin alkaloids we could observe that the alkaloid content of old leaves decreases substantially (Wink and Hartmann, 1981). Furthermore, alkaloid contents of lupin show diurnal and seasonal fluctions (Wink and Witte, 1984; Wink, 1985), but a positive correlation with time should have been expected for a waste product. Also from a theoretically point of view this waste product argument is not plausible; nitrogen constitutes a limiting growth factor for plants and its elimination as waste product would be highly uneconomical. Plants are essentially autotrophic whereas animals are heterotrophic organisms which have to break down organic substrates for energy provision. And only for the heterotrophic organism a need exists to eliminate waste products by faeces or urine.

Another argument is that alkaloids might be important for primary metabolism or the development of a plant acting as a growth regulator or hormone. However, no such function has been discovered so far. A minor function of lupin alkaloids could be nitrogen transport or nitrogen storage, since the alkaloids are translocated in the phloem together with amino acids and are stored in the seeds together with the storage proteins (Wink and Witte 1984, 1985).

Thirdly, it was postulated that alkaloids have no function at all and only exist as a play of nature (Mothes, 1955). However, it has been shown with several other features that functionless traits are usually not maintained during evolution. Since the production and storage of alkaloids is energetically costly, it is highly unlikely that such a trait would have been conserved when having absolutely no function.

Already in 1888 it was postulated by Stahl (1888) that secondary metabolites are important for the fitness of a plant in that they protect against predation by herbivores. Plant physiologists at the beginning of this century turned down Stahl's concept as being teleologic, since they were anti-Darwinian and not willing to accept evolutionary arguments. Starting with Fraenkel in 1959, the old concept was resurrected and by now it is generally accepted in the fields of "Chemical ecology" that secondary metabolites or allelochemicals have evolved in Nature as means of protection against microorganisms, herbivores and competing plants. In addition, some compounds serve to attract pollinating or seed dispersing animals, others are important in interspecific communications (e.g. pheromones) (Swain, 1977; Levin, 1976; Rosenthal and Janzen, 1979; Harborne, 1982; Nahrstedt, 1989; Wink, 1988).

We have tested whether the defence concept can be supported in case of lupin alkaloids by experimental evidence.

In a first set of experiments with purified quinolizidine alkaloids, such as sparteine, lupanine and 13-tigloyloxylupanine, we set up bioassays for antiviral, antibacterial and antifungal properties, allelopathy, mollusc deterrence and insecticidal or insect deterrent activities:

Sparteine inhibits the multiplication of potato-X virus (Wink 1987), the growth of gram-negative and gram-positive bacteria (Wink, 1984a), and of some phytopathogenic fungi (Wink, 1984a). Lupanine and 13-tigloyloxylupanine inhibited the germination of spores and the formation of appressoria of Erysiphe graminis (Wippich and Wink, 1985).

Lupin alkaloid, specially lupanine and 13-tigloyloxylupanine are secreted into the surrounding soil by the growing plantlet (Wink, 1983). Both alkaloids substantially inhibit the germination of lettuce and grass seeds and reduce the growth of lettuce and cress seedlings (radicle and hypocotyl), (Wink, 1983; Wink and Twardowski, 1991). Preliminary experiments show that lupin plants can reduce competing weeds that grow in the immediate neighbourhood, at least under experimental conditions (Wink, 1985).

Snails, like Helix pomatia are deterred from feeding a diet if it contains elevated concentrations of lupin alkaloids (Wink, 1984b). Also some insects are deterred by quinolizidine alkaloids, such as bees (Detzel and Wink, in preparation), Syntomis mogadorensis (Lepidopt.) (Wink and Schneider, 1990), Manduca sexta (Heidrich and Wink, in preparation), and ants (Wink, in preparation). For other insects, such as Plutella, Phaedon, Dysdercus and Ceratitis LD_{100} values have been obtained for sparteine, lupanine and 13-tigloyloxylupanine (Wink, 1985).

Sparteine, lupanine and cystine are known to be toxic for vertebrates in that they influence the acetylcholine receptor, Na^+, and K^+ channels in biomembranes and probably also protein biosynthesis (Wink and Twardowski, 1991, and in preparation). Some quinolizidine alkaloids (such as anagyrine) are mutagenic and cause fetal malformations (so-called "crooked calf disease"), if ingested by pregnant animals (Keeler, 1976).

We can thus conclude that quinolizidine alkaloids have a broad biological activity with defines dose response curves. However, ED_{50} values are usually in the range of 1-5 mM (equivalent to 0.01-0.1%) which is rather high, as compared to many synthetic growth regulators and pesticides. These values have to be compared to the alkaloid concentrations present in the intact plant: alkaloid levels range from 5-10 mM in leaves and stems to over 200 mM in seeds. In shoots, petioles and stems lupin alkaloids are localized in the epidermal and subepidermal cell layers (Wink et al., 1984) and reach local concentrations between 20 and 100 mM (Wink, 1986). Specially these tissues have to ward off an attack in the first place. Therefore, we can conclude that the alkaloid levels in the plant are sufficiently high to ward off pathogens and herbivores. In addtion, lupins can react to experimental wounding by increasing their alkaloid levels within hours (Wink, 1983b).

We could obtained good experimental evidence that our defence hypothesis is correct: we compared the fitness of alkaloid-rich wild lupins with so-called sweet lupins (varieties of L.albus low in alkaloid content). Sweet lupins were preferentially eaten by rabbits, infested by leaf miners and aphids (Wink, 1987), whereas, the bitter alkaloid-rich varieties remained almost unmolested. The sweet varieties still contained some other natural products, such as flavonoids, isoflavones, phenolics, etc., which are certainly responsible for some residual defence against microorganisms and to some degree against herbivores.

However, no defence is absolute, which is also true for defence by quinolizidine alkaloids. A few herbivores have evolved which found a way to either tolerate QA or even to exploit them for their own defence. In the latter class we have studied some aphids, such as Aphis cytisorum, Aphis genistae and Macrosiphum albifrons, which all accumulate and store the alkaloids which they acquire from the phloem of their respective host plants (Wink et al., 1982; Wink and Witte, 1985, 1991; Wink and Römer, 1986; Scentesi and Wink, 1991). The adaptation and storage of QA is obvioulsy of some advantage to these aphids, since they are less attacked by predators, such as ladybirds and other beetles (Wink and Römer, 1986; Gruppe and Römer, 1988). As compared to the number of potential enemies the number of those adapted ones is relatively small. We should compare this situation with our immune system: it works in almost all instances against microorganisms (and we hardly notice its existance), but a few pathogens have overcome the defence barrier, such as influenca virus, and cause disease. Nobody would call the immunsystem to be inexistent or functionless. We should adopt a similar attitude when considering the chemical defence system.

An interesting case are those lupin species, such as L. polyphyllus and L.arboreous which were brought to Europe from America some 300 years ago. Both species have a number of adapted herbivores in their native country, but were almost without them in Europe, since they had left their enemies behind. Only recently, the lupin aphid (M. albifrons) made its way to the Old World (probably hidden in a Jumbo Jet) and is now on its way to infest and molest lupanine-rich lupins in Europe (Eppler and Hinz, 1987; Wink and Römer, 1986).

3. High or low alkaloid levels?

From the point of view of chemical ecology it is clear that quinolizidine alkaloids are important for the fitness of a lupin plant and are therefore a desirable trait, that confers resistance against pathogens and herbivores. On the other hand, lupin alkaloids are bitter and toxic and are threfore undesirable if animal or human consumption is concerned.

The traditional approach was and is to breed for sweet lupins, devoid of alkaloids, which was rather successful: at present low-alkaloid varieties are available from L. albus, L. angustifolius, L. luteus, L. mutabilis and L. polyphyllus. These sweet varieties are useful for animal consumption (and will therefore be exploited in the future), but have the ecological disadvantage that they can only be cultivated successfully if predators are kept away by fences and pesticides. A similar fate can be recorded for many of our agricultural crops (Wink, 1988). However, since pollution of our soils and water by pesticides causes severe environmental problems, we must think about ecologically acceptable alternatives. This holds also true for lupins, even if this species is young and still relatively unimportant as a major crop.

Are there feasible alternatives? At present, we can outline at least two different strategies: 1) The cultivation of bitter lupin varieties in combination with a technological post-harvest processing of the seeds. 2) The selection of a naturally protected lupin ("NP-lupin") which still produces alkaloids in its green parts but which does not translocate the alkaloids to the seeds any longer, which are thus alkaloid-free and ready for consumption.

3.1 Technological processing of lupin seeds

Mittex Anlagenbaucompany in Ravensburg/West Germany has developed the technology to process and to refine alkaloid-rich lupin seeds: in principle lupin seeds are dehulled, milled and then processed by water extraction. This aequous extract contains proteins and peptides, lipids and alkaloids, which are subsequently separated, so that the process yields fractions containing 1) pure protein, 2) dietary fibers, 3. lipids, 4. alkaloids, and 5. residues. Fractions 1, 2, 3 and 5 can be marketed without much further development. The alkaloid fraction could be used as plant growth regulators and as natural pesticides (because of their deterrent or insecticidal properties). This naturalproduct is biodegradable and does not cumulate in the soil or the food chain (Gross and Wink, 1986).

3.2 Development of NP-lupin

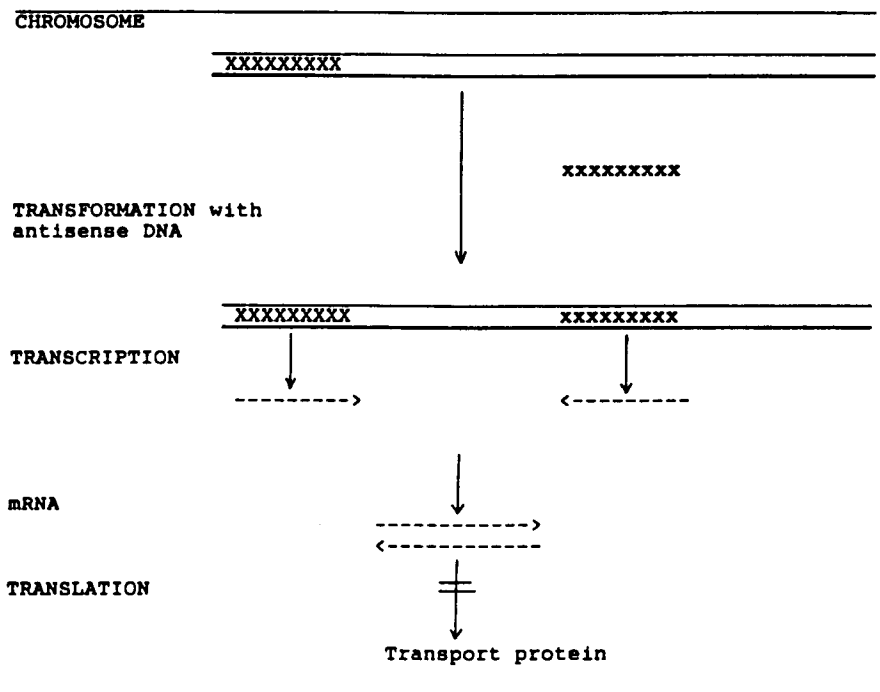
A lupin which still produces QA in its green parts would be protected against herbivores and also probably some microbial pathogens during most of its development. But are there chances to find a lupin which does not translocate the alkaloids to the seeds? We have shown that the site of lupin alkaloid synthesis is the chloroplast (Wink and Hartmann, 1982). After synthesis the alkaloids are exported from leaf mesophyll cells to all other plant parts, but specially to the fruits and seeds via the phloem (Wink and Witte, 1985). Since alkaloids are charged molecules at

physiological pH they can not pass biomembranes by simple diffusion (Mende and Wink, 1987; Wink and Mende, 1987; Hauser and Wink, 1990; Wink, 1990). Instead we could provide experimental evidence for carrier proteins in biomembranes (Mende and Wink, 1987; Wink and Mende, 1987). Since these carriers are proteins, carrier genes must also exist. Because genes are susceptible to mutation we assume that it should be possible to find and to select lupin plants which no longer export alkaloids to their seeds. This goal can be achieved by traditional lupin breeding.

It is tempting to employ the new possibilities of molecular biology in this context: we have set out to isolate the genes which are responsible for alkaloid biosynthesis and alkaloid transport. At a first step we have established cDNA libraries of L. polyphyllus, from which we have isolated and characterized a number of interesting genes (although still unrelated to alkaloid metabolism), such as a gene for a basic peroxidase (Perrey et al., 1990), a HSP70 heat shock protein (Perrey et al., 1991), a ribosomal protein rps 16 (Warskulat et al., 1991), a polyubiquitin gene (Perrey and Wink, 1990) and several cell culture-specific genes (Perrey et al., 1990a,b,; Wink, 1991). If we are successful in isolating an alkaloid carrier gene we intend to transform lupins tissue-specifically by the corresponding "anti sense" gene. We can expect that these plants then produce both the mRNA for the carrier and the respective complementary mRNA. Both mRNA species hybridize and are degraded by RNAses and no longer translated into the transport protein (Fig. 2). There are several examples already in the literature that the anti-sense strategy is very useful to down-regulate traits or pathways in higher plants. We are aware that many intermediates in this scheme are missing and that it will cost a lot of effort to achieve our goal. But we are sure that it will be worth it.

Fig. 2. Theoretical approach to inhibit alkaloid transport by antisense RNA.

XXXXXXXX= transport gene; xxxxxxxxx= antisense transport gene



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