

Towards the elimination of anti-nutritional factors in grain legumes¹

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Abstract

Anti-nutritional factors (ANFs) in grain legumes can be divided into several groups based on their chemical and physical properties such as non protein amino acids, quinolizidine alkaloids, cyanogenic glycosides, pyrimidine glycosides, isoflavones, tannins, oligosaccharides, saponins, phytates, lectins or protease inhibitors. Their elimination can be achieved either by selection of plant genotypes with low levels of such factors, or through post-harvest processing (germination, boiling, leaching, fermentation, extraction etc.). The development of new food crops from *Lupinus*, *Vicia* and *Lathyrus* species is used to illustrate the problems associated with heat stable low molecular weight ANFs. The relative merits of the various strategies for the elimination of ANFs are going to be discussed in relation to ecological function (ANFs can be important for the plants producing them, since they can function as defence compounds against herbivores and microorganisms) and economic feasibility.

1. Introduction

Plants which produce seeds rich in energy supplies (carbohydrates, lipids, proteins) usually accumulate potent chemical defence compounds. This also applies to grain legumes with comparably large and protein-rich seed which often contain substantial amounts of "anti-nutritive" factors (ANF), such as lectins, protease inhibitors, non-protein amino acids (NPAAs), alkaloids, cyanogenic glycosides, pyrimidine glycosides, saponins, tannins, isoflavones, oligosaccharides, erucic acid, or phytates (Bell and Charlwood, 1980; Conn, 1981; Rosenthal and Berenbaum, 1991, Rosenthal, 1982; Bardocz et al., 1996; Bardocz and Pusztai, 1996).

Since many of the ANFs are either toxic, unpalatable or undigestible, elimination strategies are probably as old as mankind since grain legumes were already an integral part of neolithic farming systems (Zohary & Hopf, 1988). These early means must have consisted mainly of thermal destruction of heat labile ANFs and leaching out the others by cooking. Today several more strategies are available to minimise the impact of ANFs in grain legumes in order to improve their utilisation:

- Reduction of ANFs through processing (germination, boiling, leaching, fermentation, extraction, etc)
- Reduction of ANFs through genetic manipulation (selection from natural or artificial diversity, genetic engineering of biosynthetic pathways or of the toxic protein itself)
- Improvement of tolerance to ANFs through supplementation of diets with protective factors e.g., methionine, threonine, feed enzymes

But before making a decision whether to breed for ANF free crops or whether to opt for any kind of processing it is important to recall and to discuss biological and economic constraints of ANFs. Because of space limitations we cannot give a comprehensive review covering all aspects or all relevant publications. Our paper was focussed on two groups of ANFs (lupin alkaloids and NPAAs) with which we have personal experience and references are often restricted to reviews instead of original papers.

2. ANF biology

In order to survive plants have developed defences against herbivorous animals, microorganisms and viruses during evolution. Furthermore, plants compete with other plants for light, water, and nutrients. The production of second-

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dary metabolites (including lectins and toxic peptides) is of ultimate importance as a defence strategy in this context (Rosenthal, 1982; Harborne, 1993; Wink, 1988, Wink, 1992; Wink, 1993a). The seedling is the most vulnerable lifestage in the plants' life cycle, therefore it is not surprising that many species are well equipped with anti-nutritional factors and other substances in their seeds. Plants are often very economic in that they are able to utilize defence compounds (especially the N containing ones) as nitrogen source during germination. These compounds can be either acutely toxic (such as some lectins, cyanogenic glycosides, NPAAAs or alkaloids), unpalatable (such as saponins, tannins, NPAAAs, or bitter alkaloids) or "anti-nutritive" reducing growth and fitness of the consumer by nutrient complexation (e.g., by phytates), metabolic inhibition (e.g., NPAAAs, cyanogenic glycosides, isoflavones, alkaloids) or reduction of digestion (e.g., through protease inhibitors, lectins, or oligosaccharides).

Considering the evolutionary background of ANF as either antiherbivore or antimicrobial compounds, ANFs have been selected during evolution as biologically active components. If isolated and processed they could be even useful in medicine or in agriculture (increasing resistance against pests and pathogens; as natural plant protectants "biorational pesticide" or phytomedicines). The biological background and properties of legume ANFs is briefly summarized in the following. Our results on quinolizidine alkaloids, which constitute the main anti-nutrients of lupins (Wink, 1985; Wink, 1988, Wink, 1992; Wink, 1993a, Wink 1993b) and recent work with non-protein amino acids in *Vicia* and *Lathyrus* (Lambein et al. 1990, 1992; Enneking et al. 1993, 1997; Enneking, 1994, 1995a) should exemplify this view and is therefore covered in more detail.

2.1. Lectins and protease inhibitors

Those legumes which do not accumulate low molecular weight toxins in their seeds, often store toxic peptides (e.g. protease inhibitors), or lectins (Bisby et al., 1994). Whereas protease inhibitors block the function of digestive enzymes (proteases) in animals (leading to malnutrition and other disturbances), lectins either bind to receptors in the intestinal tract and related organs (mimicking the activity of other signal compounds) or are taken up by cells and inhibit protein biosynthesis (e.g., the lectin abrine from the legume *Abrus precatorius* is one of the most potent toxins known). These compounds also serve a double purpose for the plants producing them: they are both N storage compounds (which are broken down during germination and seedling development) and defence chemicals against animals. Since lectins are gene encoded proteins, they are direct targets for genetic engineering. For example, the genetic approach will allow the downregulation of lectin synthesis (by "antisense strategies"), eventually leading to lectin-free crops. On the other hand, since lectins mediate insect toxicity, a transfer and expression of lectin genes into other crop plants would certainly be a means to enhance resistance of plants against insects.

2.2. Saponins

Triterpene saponins have been detected in soybean, lupins and several other legumes (Price et al., 1987; Bisby et al., 1994). Saponins are amphiphilous compounds which interact with biomembranes of animals, fungi and even bacteria. The hydrophobic part of the molecule complex cholesterol inside the membrane and their hydrophilic sugar sidechain bind to external membrane proteins. Thus fluidity of biomembranes is disturbed leading to holes and pores. As a consequence, cells become leaky and die. This rather unspecific membrane disturbance is responsible for the wide effects of saponins against animals, fungi, bacteria and competing plants. Thus saponins can be considered as a resistance factor in legumes against microbial infection and herbivory. Legume saponins have only moderate toxicity and present a problem only when present in the diet at higher concentrations.

2.3. Phytate

Inositols with 4, 5 or 6 phosphate groups are common in the seeds of many of our grain legumes and can reach concentrations higher than 10% of dry matter (Bisby et al., 1994). They can be regarded as stores for phosphate and mineral nutrients that are important for plant nutrition and especially valuable during germination. Since phytates complex iron, zinc, magnesium and calcium ions in the digestive tract, they can cause mineral ion deficiency in animals and humans. Again, these compounds seem to serve a double purpose, i.e. defence and phosphate & mineral store. Phytate content of food can be lowered by the addition of enzymes which hydrolyse them, i.e. of phytases (Bardocz et al., 1996; Bardocz and Pusztai 1996)

2.4. Oligosaccharides and isoflavonoids

Legume seeds are generally rich in oligosaccharides (up to 20%), such as stachyose and raffinose (Bisby et al., 1994). In animals, they produce flatulence and other disturbances. Since these symptoms are not pleasant, these compounds will contribute to the general deterrence of legume seeds to herbivores. These compounds serve as carbon sources during germination; therefore their contents can be reduced in legumes through germination which is a common practice, e.g. in soybeans. Isoflavonoids have been detected in soybean, lupins and several other legumes (Bisby et al., 1994). They are involved in plant defence against fungi, bacteria, viruses and nematodes (phytoalexins, phytoanticipins), act as signals in legume-Rhizobium interactions and exhibit estrogenic activities (Dakora and Phillips, 1996).

These hormonal effects are not necessarily welcomed in humans or life stock. Recently it was found that the isoflavone genistein inhibits tyrosine kinases. Since these enzymes are often stimulated in cancer cells, the lower incidence of some kinds of cancers in people which ingest isoflavone rich food, such as soybean products, has stimulated the hypothesis, that some legumes rich in isoflavones can prevent cancer.

2.5. Cyanogenic glycosides

Cyanogens are glycosides of 2-hydroxynitriles and widely distributed among plants, e.g. the Rosaceae, Leguminosae (e.g., in *Phaseolus lunatus* and *Vicia sativa*), Gramineae, and Araceae (Bisby et al., 1994; Bell and Charlwood, 1980; Conn, 1981; Rosenthal and Berenbaum, 1991). In case of emergency, ie. when plants are wounded by herbivores or other organisms, the cellular compartmentation breaks down and cyanogenic glycosides come into contact with an active β -glucosidase, which hydrolyses them to yield 2-hydroxynitrile. This is further cleaved into the corresponding aldehyde or ketone and HCN by hydroxynitrile lyase. HCN is highly toxic for animals or microorganisms because it inhibits enzymes of the mitochondrial respiratory chain (ie. cytochrome oxidases). In addition to the toxic effects cyanogens can serve as mobile nitrogen storage compounds in seeds important during germination.

Cassava is a crop plant rich in cyanogenic glycosides. Since farmers in marginal areas rely on "bitter" varieties for food production it has been suggested that the prevention of cyanate poisonings through improved detoxification procedures may be more effective than the development of "low cyanide" cultivars (Tylleskär et al., 1992).

Hanelt and Tschiersch (1967) found that the majority of *Vicia sativa sensu lato* accessions from the Balkan and Turkey contained the cyanogenic glycoside vicianine, whereas the material from Iran was predominantly HCN-free. In *Trifolium repens* a similar polymorphism had been observed. The distribution of cyanogenic *Trifolium repens* genotypes in Europe correlated with the occurrence of snails which feed on this legume and low winter temperatures which can disrupt cyanogenic plant tissue function (Jones, 1972).

2.6. Pyrimidine glycosides

Vicine and convicine are β -glycosides of the pyrimidines divicine and isouramil. High levels of vicine are present in the seeds of *V. sativa* and *V. faba* (which also contains significant levels of convicine). The ingestion of meals prepared from the seeds of *V. faba* can trigger the onset of Favism, an acute haemolytic disease which affects individuals lacking sufficient activity of the NADPH producing enzyme glucose-6-P-dehydrogenase (G6PD) in their red blood cells (Mager et al., 1980; Marquardt, 1989). Several genetic variants of this enzyme deficiency, which is thought to confer an adaptive advantage against malaria, are known to occur worldwide, with Favism being an extreme manifestation of this trait (Vulliamy et al., 1992). It is particularly prevalent in some Mediterranean and South-West Asian populations (Belsey, 1973). Vicine and convicine have been implicated in favism because their hydrolysis products are unstable and form radicals which can cause a depletion of reduced glutathione (GSH) in G6PD deficient red blood cells. A lack of sufficient NADPH due to G6PD deficiency impedes GSH replenishment and predisposes the red blood cells to oxidative damage which can, ultimately, result in a haemolytic crisis. Antioxidants have been successfully employed to reduce the effects of vicine in animal diets. (Mager et al., 1980; Marquardt, 1989; and references therein).

Efforts are under way to develop cultivars of *V. faba* with zero levels of vicine and in their seeds and some promising material has already been identified (Griffiths and Ramsay, 1992). The work of Bjerg *et al.* (1984) suggests that these compounds play a role in the resistance of pods and leaves to fungal pathogens, so seed specific deletion of these factors would be desirable. However their physiological and biological role during seedling establishment is still unresolved (Griffiths and Ramsay, 1992) and seed specific deletion may affect useful levels of these compounds in young seedlings.

2.7. Lupin alkaloids

The genus *Lupinus* contains several hundred species, 12 in the Old and the rest in the New world. Their evolution has recently been described by Käss & Wink (1997) using nucleotide sequences of marker genes. Since some species have big protein rich seeds (up to 50% protein), these lupins are of considerable agricultural interest. Cultivated species include *L. albus*, *L. luteus*, *L. angustifolius* and *L. mutabilis*. Reviews concerning the biochemistry, utilisation and agronomy of lupins can be found in the proceedings of the International lupin conferences which take place every 2 or 3 years (Gross and Bunting, 1982; ILA 1983; 1984;1986;1990; Neves-Martin & Beirao da Costa, 1994).

The main secondary metabolites of lupins are quinolizidine alkaloids which are sometimes accompanied by piperidine alkaloids, such as ammodendrine or indole alkaloids such as gramine (Bisby et al., 1994). After synthesis in lupin leaf chloroplasts alkaloids are exported via the phloem all over the plant and are accumulated in the epidermis of leaves and stems. Reproductive organs, such as flowers and seeds are especially rich in alkaloids (2-6% dry weight). In germinating lupins alkaloids serve as a N source (review in Wink 1987, 1992, 1993). Lupin alkaloids were shown to be feeding deterrents and lethal for a number of insects, especially aphids (Berlandier, 1996), but also moth- and butterfly larvae, beetles, grasshoppers, flies, bees and ants, other invertebrates and vertebrates, ie. alkaloids are active over a wide range of animal orders (Wink, 1985; Wink, 1988, Wink, 1992; Wink, 1993a, Wink 1993b). Furthermore, these alkaloids are inhibitory for competing plants, viruses, bacteria and fungi (Wink, 1985; Wink, 1988, Wink, 1992; Wink, 1993a). Consequently,

alkaloids appear to be important for the resistance of lupins against insects and other pests, an assumption which could be tested experimentally. Since alkaloids are toxic for humans and animals, breeders have selected lupin varieties with very low alkaloid contents, the so called "sweet" lupins. When planted in the field sweet lupins suffer substantially from herbivores, such as rabbits and hares. A similar picture was observed for a number of insects, such as aphids, beetles, thrips and leaf mining flies, ie. sweet lupins were attacked, whereas the alkaloid-rich ones were largely protected (Wink, 1985; Wink, 1988, Wink, 1992; Wink, 1993a, Wink 1993b). Plant breeders have observed that also bacterial, fungal and viral diseases are more abundant in the sweet forms.

The toxic effects of quinolizidine alkaloids for insects and vertebrates can be explained through the interaction of these alkaloids with acetylcholine receptors (sparteine activates the nAChR, lupanine the nAChR), with Na⁺, K⁺-channels and protein biosynthesis (Wink, 1992; Wink, 1993a; 1993b; Schmeller et al., 1994). Since synaptic signal transduction and protein biosynthesis are important and vulnerable processes in most animals, it is not surprising that alkaloids have toxic properties over a wide range of animals. These data clearly support the importance of alkaloids for chemical protection.

With our present knowledge on the ecological importance of alkaloids for the fitness of lupins, it seems doubtful whether the selection of alkaloid free lupins with a decreased resistance is the only possible solution. Similar strategies, ie. to eliminate unwanted chemical traits, appear to have been chosen with other agricultural crops (such as cabbage, turnip, rape seed, tomato, potato, cassava or barley) with the consequence, that the overall fitness was much reduced. In these cases the loss of natural protection had to be substituted by man made synthetic agrochemicals with their known problems (Wink, 1993b). For lupin breeding we have proposed two alternatives (Wink, 1993b):

- To select for lupin mutants which do not translocate the alkaloids to the seeds, since seeds do not produce alkaloids but store them. In this case, the plant would retain its chemical resistance in its vegetative parts but would also provide the valuable alkaloid free seeds.
- To grow alkaloid rich plants but to process the seeds and to simultaneously produce pure protein, lipids, amino acids, and dietary fibres from bitter seeds. A spin-off product would be alkaloids, which could be either used in medicine or in agriculture (Wink, 1993b). This strategy is presently developed with aid of the European Commission by Mittex company.

Considering the insecticidal and fungicidal properties, it seems most promising to exploit the alkaloids as natural plant protectants. Alkaloids do not accumulate in the soil, and plant cell culture experiments indicate that alkaloids can be metabolised rapidly. Since alkaloids will be produced as a by-product of a normal crop they could be a comparably cheap natural („biorational“) pesticide. Since proteins, insecticides and fungicides are in shortage in many countries, especially in the developing world, lupin cultivation and processing might be a valuable alternative (Wink, 1993b).

In the long run, the genetic engineering of the pathways leading to secondary metabolites should provide another option. For example, if we could transfer the genes which encode the biosynthesis, transport and storage of lupin alkaloids to other crop plants, such as cotton, it is quite likely that we would thus transfer a novel resistance towards insects. When successful, the genetic approach will be even more environmentally friendly than using "biopesticides".

A successful example for seed specific deletion (as suggested for lupin breeding) is provided from rape cultivation: Some low seed glucosinolate (00) cultivars have been shown to possess similar levels of leaf glucosinolates as high seed glucosinolate (0) cultivars and were not more susceptible to pests and diseases than the more toxic genotypes (Mithen, 1992). Thus, selection for low seed toxin levels can be achieved while the beneficial protective effects are maintained in other tissues of the plant. However, it has also been demonstrated that the susceptibility of oilseed rape seedlings to slugs is related to the seed glucosinolate content and increased use of molluscicides, and thus costs, were predicted with the more widespread 00 oilseed rape cultivation (Moens et al., 1992).

2.7. Non-protein amino acids

More than 900 non-protein amino acids (NPAAs) which are especially abundant in certain legumes (Viciae, Phaseoleae, Mimosoideae, and Caesalpinioideae) have been detected which resemble protein amino acids (structural analogues) (Bell and Charlwood, 1980; Bisby et al., 1994; Conn, 1981, Rosenthal, 1982). Concentrations in seeds can exceed 10% of dry weight and since non-protein amino acids are remobilised during germination they certainly function as N-storage compounds (Rosenthal, 1982).

If non-protein amino acids are taken up by herbivores, microorganisms or other plants, they may interfere with several targets (Bell, 1977; Rosenthal, 1982, 1991): They can be accepted in ribosomal protein biosynthesis in place of the normal amino acid leading to defective proteins (example: canavanine, azetidine-2-carboxylic acid). They can inhibit the charging of aminoacyl-tRNA synthetases or other steps of protein biosynthesis or they may competitively inhibit uptake systems for amino acids. In vertebrates, effects may be among others: foetal malformations, neurotoxic disturbances, hallucinogenic effects, hair loss, diarrhoea, paralysis, liver cirrhosis, hypoglycemia, and arrhythmia. In plants, microorganisms and insects non-protein amino acids cause reduced growth or even death. Since non-protein amino acids affect a basic target present in all organisms, they are important in plant- plant, plant -microbe and plant- herbivore interactions.

Holt and Birch (1984) correlated the presence of NPAAs in *Vicia* to aphid (*Aphis fabae*, *Acyrtosiphon pisum* (Harr.), *Megoura viciae* (Buckt.)) resistance and found that the most domesticated species were also the least resistant. β -Cyanoalanine has been shown to be active against *Locusta migratoria* where it exhibited diuretic effects and led to an

inhibition of moulting (Schlesinger et al., 1976); it has also been documented as an effective feeding inhibitor for three species locust (Navon and Bernays, 1978) and one bruchid species (Janzen et al., 1977).

The biology of canavanine is probably one of the best documented examples for a protective role of non-protein amino acids in plants, although its benefits have not yet been demonstrated in isogenic lines differing only in the canavanine biosynthesis trait (Rosenthal, 1991). It has been shown that canavanine can have toxic effects against a wide range of organisms (Rosenthal, 1977; 1986; 1991; Miersch et al., 1992; Enneking et al., 1993, Enneking, 1995).

The insecticidal properties of these compounds alone, without considering their edaphic, allelopathic (Wright and Srb, 1950; Weeks and Hunt, 1973; Weeks, 1974; Miersch et al., 1992) and possible drought tolerance functions, suggest that it may be prudent to aim for seed specific deletion of these factors whilst preserving and enhancing their beneficial role in other parts and phenological growth stages of the plant.

The work of Lambein *et al.* (1990, 1992; and references therein) has shown that seed NPAA in *Lathyrus* species act as precursors for compounds synthesised during germination. Depletion of seed NPAA toxins would also deplete the reserves from which root exudates (Kuo *et al.*, 1982) and protective chemicals are formed during early seedling establishment. Increased levels of γ -glutamyl- β -CN-alanine in young seedlings of *V. sativa* (Ressler *et al.*, 1969) and for canavanine in seedlings of *Canavalia ensiformis* (L.) (D.C.) (Rosenthal, 1972b), *Medicago sativa* L. (Gorski *et al.*, 1991; Miersch *et al.*, 1992) have been documented, suggesting a similar function for these compounds as for those isolated from *Lathyrus*.

2.7.1. Development of new grain legume crops from the genera *Vicia* and *Lathyrus* in Australia

Easily domesticated grain legumes with respect to ANFs such as peas, lentils and chickpeas have already been developed. The more difficult ones with heat stable low molecular weight ANFs in their seeds remain including *Vicia*, *Lathyrus* and *Lupinus* spp.. Several species from these genera have received increased attention by agronomists, plant breeders and hunters because of their ability to grow under adverse environmental conditions and on marginal soils.

Prior to elimination of ANFs they need to be identified. Recent studies in Australia used a porcine feed intake bioassay to identify unpalatability principles in *Vicia* species (Enneking, 1994).

V. villosa seeds are extremely unpalatable to pigs and were found to be a serious problem as contaminants of cereals used in diet formulations. Through a series of fractionations and bioassaying, the NPAA canavanine, a structural analogue of arginine was identified as the major unpalatability principle in the seed. The same study also found that under alkaline conditions canavanine degrades to the inactive deamino-canavanine (Enneking et al., 1993). *V. ervilia* and *V. articulata* seeds also contain canavanine but at much lower concentrations (0.05-0.2, 0.1-0.4% DW, respectively) (Enneking, unpublished).

The unpalatability of *V. narbonensis* seed was found to be due to the considerably less inhibitory dipeptide γ -glutamyl-S-ethenyl-cysteine (Enneking, 1994; Enneking et al., 1997) which can be inactivated by mild acid hydrolysis. This compound renders the seeds unpalatable to pigs, poultry and humans. The limited information about the toxicity of *V. narbonensis* in pigs suggests that it may cause haemolysis, and kidney damage through the formation of crystalline precipitates (The late R. L. Davies, pers. comm.). Further studies of its chemistry, pharmacology and toxicology are clearly needed.

The safe use of *V. sativa* cv. Blanchefleur for human consumption has been the subject of controversy. It is well known, that *V. sativa* contains the Favism toxin, vicine (Pitz el al., 1980) and the neurotoxic peptide γ -glutamyl- β -cyanoalanine. This compound inhibits the metabolic conversion of methionine to cysteine, leading to raised urinary levels of cystathionine, thus ultimately reducing the supply of vital glutathione. In addition, γ -glutamyl- β -cyanoalanine-glycine, a structural analogue of glutathione is formed (for a review see Ressler, 1975). Cysteine malnutrition and oxidative stress are likely to increase sensitivity to this toxin. *V. sativa* cv. Blanchefleur is still being clandestinely exported for human consumption from Australia to developing countries despite our warnings (Tate and Enneking 1992) about its toxicity.

In a feeding experiment (Enneking, 1994) with laying hens the effects on feed intake, weight gain and laying performance of cooked, hydrolysed (4% acetic acid, 30 mins, 121° C) and raw Blanchefleur vetch were compared with raw or cooked red lentils (all treatments fed at 10% inclusion level). Cooking of Blanchefleur vetch did not lead to a reduction of its anti-nutritional activity while acid hydrolysis was effective. The chemical data were in agreement with feed intake and growth performance while no significant differences on laying performance could be detected between treatments.

This assessment of three model *Vicia* species which are representative for the genus demonstrated that in addition to the pyrimidine glycosides, the major ANFs in the seeds of *Vicia* spp. are NPAAAs which, in principle, can be inactivated by either acidic or alkaline hydrolysis.

Lathyrus species which are presently of agricultural interest as grain legumes all contain the phagostimulant and neurotoxic glutamate analogue beta-oxalyl-2,4-diaminopropionic acid (beta-ODAP). One major problem associated with the development of *Lathyrus sativus* for human consumption is the stigma of lathyrism. No acceptable experimental protocol is available to provoke neurolathyrism in experimental animals. In the absence of a suitable bioassay to test the safety of the newly developed low-toxin cultivars and lack of understanding about the pre-disposing factors e.g. the malnutrition-poverty complex or genetic susceptibility, which lead to the onset of this nutritional disease, informed decisions regarding safe intake levels cannot be made at present. In contrast to these concerns, millions of people

consume diets containing *L. sativus* on a regular basis with no apparent ill effect, except during periods of famine and excessive *L. sativus* consumption.

Caution is needed with the marketing of the recently released low beta-ODAP *L. cicera* cultivar from South Australia for human consumption in developing countries, since very little information is available about the suitability of this species for monogastric consumption.

The following species are being selected in Australia for reduced grain toxicity (major institutions involved in brackets): *Lathyrus sativus* (Ag WA-CLIMA), *L. cicera* (SARDI, Ag WA-CLIMA), *V. sativa* (Waite Institute, Univ. Adelaide-SARDI), *V. ervilia* (CLIMA), *V. narbonensis* (VIDA, CLIMA). Some other species with a history of human consumption would be of interest but no specific programs are in place for them: *L. ochrus*, *L. clymenum*, and *V. articulata*. For further detail on *Vicia* spp. grain legumes see (Francis et al., this conference).

V. ervilia genotypes with low levels of canavanine (< 0.05% DW) and good seed yields have recently been identified in Australia. Interestingly, no genotype with canavanine levels approaching those found in *V. villosa* or *V. benghalensis* seeds has so far been found after screening 200 accessions of this species, include wild ecotypes from the ICARDA gene bank. This finding suggests that other, yet unidentified ANFs are present in the seeds of this species. The bitter principle in the seed remains to be isolated and chemically characterised. Sufficient variation for CN-containing compounds exists in *V. sativa* to make the selection of genotypes with very low levels of these substances a realistic possibility (Delaere, Rathjen and Tate, pers. comm.). Variation in total sulfur levels of the Australian *V. narbonensis* collection suggests that a low GEC genotype can be selected from this material, although screening seems to be confounded by soil sulphur status. Low vicine and convicine genotypes of *V. faba* have already been developed. These low toxin genotypes, combined with genotypes selected for high toxin levels provide a unique opportunity for the further study of the ecological biochemistry of *Vicia* and *Lathyrus* species. Such genotypes could be included into disease, pest resistance (and other stress factors) screening trials to assess the resilience of the low toxin. Conversely, sources of disease, pest and other stress resistances already identified in the two genera could be assessed for their NPAA and related metabolites e.g. polyamines. For *Vicia*, information concerning the biosynthesis, transport, subcellular locations, and relative concentrations of the individual NPAAs and other low molecular weight toxins during the complete life cycle of representative species e.g. *V. faba* (pyrimidine glycosides and L-Dopa-glycoside), *V. sativa* (pyrimidine glycosides, cyanogenic glycosides, cyano-amino acids), *V. ervilia* (canavanine), *V. villosa* (canavanine, GEC) *V. narbonensis* (pyrimidine glycosides, GEC) would permit predictions and correlations with anti-predator activity and toxicity to grazing animals. The influence of various factors such as grazing, insect attack, disease attack, cold, heat, water stress, salt stress, edaphic stresses may also influence the levels of these compounds.

The most attractive feature of *Vicia* and *Lathyrus* spp. as a group of grain and forage legumes for marginal areas is the tolerance of individual species or genotypes to stress factors such as drought, cold, temporary waterlogging, pests, diseases and infertile soils. Therefore it would clearly be undesirable to convert such crops with minimum input requirements into ones with higher input requirements.

Based on the available knowledge about the biology of NPAAs in plants it is likely that one of the consequences of their genetic removal from the plants is a reduced fitness because the compounds in question fulfil positive roles in the plants' biology, and could be linked to resilient traits which enable them to grow under adverse conditions. The exact nature of their biological role remains to be elucidated with the selection of low seed toxin genotypes as experimental models for NPAA biology providing an opportunity to answer some of these questions.

3. Elimination strategies

3.1. Post harvest processing

Legume seeds are hardly been consumed raw; they are usually cooked and by this procedure lectins and protease inhibitors are inactivated. Low molecular weight compounds are leached out into the cooking water, to be discarded afterwards. All these simple techniques have been "invented" by man (even without a profound knowledge of the underlying toxicology) to make legume seeds more palatable and digestible. Today, a deeper knowledge of the chemical structure of the anti-nutrients involved can help to devise technological strategies to process legume seeds in order to obtain toxin free products. Since a diversification will increase the economic value of the overall crop plant, food technology and rational processing are an alternative to breeding of ANF free plants which can be more susceptible to pests and pathogens.

Followed by modern food technology (separation, filtration etc), pure nutritionally valuable dietary products, such as protein, dietary fibres, oil and other fine chemicals can be generated. The remaining fractions containing the anti-nutrients don't need to be discarded: some of them are useful for the pharmaceutical industry (see below), others might be used in agriculture as biorational pesticides. Because of higher costs these considerations are related to the use of legume seeds which will be utilized for human consumption (an example are soybeans for which a specialized industry has been developed).

The situation is different in the case of feed industry (which is a major outlet of legume seeds in Europe) where pulses are simply ground or pelleted. For this purpose we need to develop varieties which are low in heat stable nutritional factors (e.g., alkaloids, saponins, phytates, isoflavones, non-protein amino acids). If heat labile compounds (prote-

ase inhibitors, lectins) can be denatured by heat treatment during grinding and pelleting these compounds might be maintained since they confer resistance to the plants. Thus many fields remain to be explored even in a widely known group like the temperate grain legumes.

3.2. Chemical detoxification

Deaminocanavanine is a well known un toxic deamination product of canavanine (Rosenthal, 1972; Enneking et al., 1993). The degradation of canavanine to deaminocanavanine under alkaline conditions provides therefore a chemical strategy for the detoxification of this compound and has already been successfully employed for the processing of the canavanine containing seeds of *Canavalia ensiformis* (Obizoba and Obiano, 1988). γ -glutamyl-S-ethenyl-cysteine, γ -glutamyl- β -cyanoalanine the major antinutrient in the seeds of *V. sativa* (besides vicine) can be rendered inactive by mild acid hydrolysis. It is reasonable to propose that in principle post-harvest detoxification procedures can be developed for these anti-nutritional factors.

3.3. Fermentation

The use of fermentation as an integral part of food detoxification processes is widely practised (Ochse, 1931; Horsfall, 1987; Salih et al., 1991). A wide variety of fermented foods are produced and eaten around the world (Yokutsuka, 1991; Campbell-Platt, 1987; Reddy and Salunkhe, 1989). The further development of fermented foods has been advocated amongst nutritionists because of the intrinsic nutritional benefits associated with such products (Hesseltine, 1983). Fermentation is also an effective means for food preservation (Nout and Rombouts, 1992). Fermented foods can be prepared at both, an industrial and the household scale. Indeed, many fermented foods are prepared by very simple techniques and represent grass roots technology which is already widespread; a fact which facilitates their further refinement, transfer and adoption in underdeveloped countries for the detoxification of alternative food sources.

Ayyagari et al. (1989) compared various Indian household food preparation techniques for their effectiveness in detoxifying *L. sativus*, and their data show that those methods which included a fermentation step were the most effective in reducing ODAP levels, eliminating 95% of this toxin. Further improvements in detoxification are likely to be made with selection for better ODAP degradation. Such methods can, in principle, also be used for the post-harvest detoxification of *Vicia* seeds, thus providing an alternative approach to the wider utilisation of these grains without the need for genetic removal of their low molecular weight antinutritive and unpalatability factors. The incorporation of fermentation processes into other simple food technologies also offers good prospects for a detoxification of food sources while simultaneously giving flexibility in the manipulation of flavour, texture and colour of the raw material.

3.4. Germination

Pea and lentil sprouts (even lupin sprouts in Germany) have gained popularity in recent years. Traditionally, mediterranean grain legumes have not been used as sprouts. The potential toxicity of beta-isoxazolin-5-one-alanine (BIA), the biosynthetic precursor for the lathyrism toxin beta-ODAP may be a risk factor if consumption of such sprouts is excessive. The concentration of this compound increases during the germination of lentils and peas. This kind of processing, however, which reduces the contents of oligosaccharides and of other N-containing ANFs, has a long history in Asia, where it has served to improve the palatability of soybeans. Pea sprouts are a very recent addition to Chinese cuisine (since ca. 1970).

3.5. Detoxification by ruminants

Vicia and *Lathyrus* grain legumes, viz. *V. faba*, *V. articulata*, *V. ervilia*, *V. sativa* and *V. narbonensis*, *L. sativus* *L. cicera*, *L. ochrus*, *L. chymenum* can be used as supplemental feeds for ruminant production, which is another form of post-harvest detoxification. These grains have been used for millennia as a ruminant feed in the Mediterranean, Middle-East and West-Asia. Traditional practices are rapidly disappearing with the introduction of more profitable crops, while traditional crops often remain neglected by plant breeders. It is therefore important to preserve this knowledge and transfer it combined with plant introduction to regions where such crops can be grown with profit for ruminant production.

3.6. Can "anti-nutrients" be even useful?

There can be no doubt that the so called "anti-nutrients" of legumes have a biological function. They are certainly important in the physiology of seedlings as N or C storage compounds and to facilitate nutrient uptake and rhizosphere establishment. Since they are also toxic to animals and sometimes even to microorganisms, viruses and other plants, they exhibit defence functions at the same time. As shown for lupin alkaloids, ANFs can be important for the fitness of plants and constitute relevant resistance factors.

An important question arising in the context of selection for low-toxin lines is whether the genetic reduction of anti-feedant and anti-nutritional factors is going to have a negative effect on the ecological fitness of the resulting cultivars? In the words of Bell (1977) who reviewed the ecological function of non-protein amino acids, "The development of a toxin-free crop would be totally impractical if the reduction in toxicity to man or domestic animals was accompanied by an equal or greater reduction in toxicity to predatory insects which might destroy the crop before it could be harvested".

Conversely, the breeding of crops for improved resistance and cases where this has led to increased toxicity of the resulting cultivars have been discussed by Breider (1973) and Fenwick et al., (1990).

There are also examples which show that certain levels of ANFs in legumes might be even beneficial on human health. The negative dietary impact of affluence can be held at bay through inclusion of grain legumes (cholesterol lowering properties, dietary fibres, trypsin inhibitors).

The phenylalanine derivative, β -(3,4-dihydroxyphenyl)-L-alanine (L-Dopa) can be found in free form and bound as a glycoside in some *Vicia* species (incl. *V. faba* and *V. narbonensis*) and was at one time implicated as a causal agent of Favism, however, it is no longer considered to be important in this disease (Mager et al., 1980). *V. faba* pods might be a cheap dietary source of L-Dopa for the therapy of Parkinson's disease (Kempster et al., 1993).

Canavanine is an inhibitor of soluble nitric oxide synthesis and has recently been found to be useful in treating experimental endotoxaemic shock in rats. Domesticated *Vicia ervilia* and *V. articulata* seeds contain very low to moderately low levels of canavanine (0.05-0.2, 0.1-0.3%), respectively and might be even beneficial.

Lupin alkaloids, such as sparteine, have been used as antiarrhythmic and uterotonic therapeutics. Also other lupin alkaloids exhibit similar activities which could be used either as pure compounds or in mixtures (Wink, 1993b).

Isoflavonoids which inhibit tyrosine protein kinases might be interesting as anticancer agents.

3.7. Elimination of ANFs through genetic modification

The identification of alkaloids and NPAAAs as the major anti-nutritional principles present in *Lupinus* and *Vicia* seeds, respectively, now allows for the selection of genotypes with low levels of these factors, thus enabling the development of more palatable and less toxic cultivars. The selection of genetic material with contrasting levels of anti-nutritional factors is also ideally suited for the elucidation of their biological functions (as shown for the example of bitter versus sweet lupins; see above). Because of space limitations this section will be a brief outline only. The general aim is a selection of non-toxic and palatable genotypes requiring efficient screening techniques to expedite the quantitative detection of individual ANFs for the selection of improved grain legume cultivars. Such techniques should be suitable for testing large numbers of samples to facilitate the screening of the available germplasm and material generated through breeding or artificial mutagenesis. Sometimes simple colour reagents might work for an initial test, such as Reifers reagent for quinolizidine alkaloids in lupins (it produces a brown precipitate with alkaloids; Wink, 1993). Immunological methods such as ELISA to detect specific proteins can also be established for low molecular weight compounds such as alkaloids (Wink 1993). Substantial progress has recently been achieved for *Vicia* and *Lathyrus* employing DRUID-IR for the screening of γ -glutamyl- γ -cyanoalanine, and Capillary zone electrophoresis for ODAP, GEC, canavanine and vicine (Eichinger et al., this conference). Furthermore, an enzyme based method has been shown to be highly effective for ODAP screening. ICARDA routinely screens genotypes by Near-IR; TLC is used to screen *Trifolium subterraneum* for isoflavones. Living systems can also be useful for selection: Whereas alkaloid rich bitter lupins are avoided by rabbits and aphids, sweet alkaloid poor varieties are readily accepted (Wink 1998, 1992). A careful selection of plants attacked by generalist herbivores might provide a clue to plants with lower ANF levels. A snail (*Helix aspersa*) bioassay on 24 well tissue culture plates may prove useful for the detection of phenolic compounds (Enneking, unpublished).

Mass screening is still a labour and capital intensive strategy. With an increased understanding of ANF biology new opportunities, no doubt, will arise for the use of natural selection pressures in the screening for ANFs, either to improve crop resistance or to eliminate a particular factor and replace it with ANFs which are of no detrimental consequence for a particular end-use. Tolerance levels for individual ANFs and applications need to be known so that plant breeders can define target levels in their breeding programs.

3.8. Seed specific deletion of ANFs

As discussed already under the section „lupin alkaloids“, it might be possible to select strains of legumes which no longer accumulate ANFs in their seed, but still maintain their synthesis in the rest of the plant. In this case only the seeds and seedlings need additional protection but not the whole plant. This selection would work in instances in which ANFs are produced in the leaves but transported to the seeds (such as lupin alkaloids) or in which all parts of a plant produce a certain ANF but not for a seed specific synthesis. In the first instance, selection is directed towards plants in which the translocation via the phloem is blocked and the other instances towards an organ specific inhibition of biosynthesis.

3.9. Genetic engineering options

If the gene is known which encodes a toxic protein (e.g., a lectin) or the key enzyme of a biosynthetic pathway leading to alkaloids, saponins, NPAAAs etc, genetic engineering offers a set of methodologies at present to downregulate or to knock out the respective activity. Strategies include the expression of antisense mRNA, of gene targeting, and of synthetic oligonucleotides or ribozymes.

Also the introduction of new traits into a crop plant, such as new lectins or proteins rich in methionine/cysteine can be achieved by appropriate molecular techniques. Although these strategies look straightforward in theory, their utilisation and application for a specific problem is often more complicated and tedious. Obstacles are often encountered

in that relevant genes have not been detected so far which is usually the situation for biosynthetic enzymes of ANFs. If a time-, developmental and organ specific expression is required then promoter sequences need to be known for a particular plant - and again these data are usually not available for the crop of interest. Transformed plants need to be regenerated which is a severe problem in most legumes.

Several target enzymes could be envisaged for NPAAAs or alkaloids, but since the genes are still unknown, we have to leave this issue for a later conference.

4. Economic considerations

The economics of producing low toxin *Vicia* or *Lupinus* varieties (with their added-value through improved palatability, reduced toxicity and hence marketability), versus the benefits derived from the protective and adaptive functions of these compounds are going to determine whether genetic deletion of toxins or post-harvest detoxification (itself a costly process) could become viable options for the further development and utilisation of these crops. Their sustainable development, especially for resource-poor farmers in underdeveloped countries may favour post-harvest processing, which has a long tradition in many parts of the world and is of especial importance in traditional cultures that still subsist on a variety of otherwise toxic or unpalatable food stuffs.

Australian industry also has an opportunity to develop post-harvest processes for *Vicia* grain because the inclusion of a simple hydrolysis step into a splitting or compounding operation would allow the destruction of GEC in *V. narbonensis*, while preserving the high levels of sulfur in the grain. The near complete elimination of β -cyanoalanine and γ -glutamyl- β -cyanoalanine from *V. sativa* seed has been achieved by a hydrolytic process incorporated into a commercial splitting operation (Delaere and Tate, pers. comm.), however, the lack of cost-effective and pragmatic measures to test the safety of the resultant product and the prohibitive costs incurred by the Australian lupin industry in a similar exercise have stifled this development.

The development of human food from otherwise toxic or unpalatable grain may justify the added costs for detoxification. This option is not likely to be viable under the current economic conditions, but may become more attractive in the future, especially if the rise in the human population and thus the corresponding rise in demand for food continues.

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<i>Taxon</i>	<i>ANF</i>	<i>Achievement</i>
<i>V. faba</i> L.	vicine, convicine	low
<i>V. narbonensis</i> L.	GEC	med-low
<i>V. sativa</i> L.	glu- β -CN-ala	med-low
<i>V. ervilia</i> Willd.	a) CAN, b)?Bitter	a) low, b) ?
<i>V. articulata</i> Hornem.	CAN	rel. low
<i>L. sativus</i> L.	β -ODAP	low
<i>L. cicera</i> L.	β -ODAP	low
<i>L. clymenum</i> L.	β -ODAP	med-low
<i>L. ochrus</i>	β -ODAP	med

Fig 1. Known *Vicia* toxins

