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Review

Lathyrus sativus (grass pea) and its neurotoxin ODAP

Ze-Yi Yan [a], Peter S. Spencer [b], Zhi-Xiao Li [a,*], Yong-Min Liang [a], Ya-Fu Wang [c], Chun-Ying Wang [c], Fen-Min Li [c]

[a] *State Key Laboratory of Applied Organic Chemistry, Lanzhou University, Lanzhou 730000, PR China*
[b] *Center for Research on Occupational and Environmental Toxicology, Oregon Health and Science University, Portland, OR 97239, USA*
[c] *Key Laboratory of Arid and Grassland Ecology of Ministry of Education, School of Life Science, Lanzhou University, Lanzhou 730000, PR China*

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Abstract

Lathyrus sativus (grass pea) is a high-yielding, drought-resistant legume consumed as a food in Northern India and neighboring countries as well as in Ethiopia. Its development into an important food legume, however, has been hindered by the presence of the neurotoxin – β-*N*-oxalyl-L-α,β-diaminopropionic acid (β-ODAP) in seeds which, if consumed in large quantities for prolonged periods, can cause irreversible paralysis. Recently, some low-toxin lines have been developed that may prove safe for both animal and human foods. Cultivation of *L. sativus* should thus be considered in suitable regions because the demand for legume animal feed protein products is expected to increase. This paper addresses advances in understanding *L. sativus* from the perspective of its taxonomy, genetics, ecology, chemistry, nutrition, medicine, biology and for animal nutrition.

Keywords: *Lathyrus sativus*; Leguminosae; ODAP; Neurotoxin; Protein; Nutrition

Contents

* Corresponding author. Tel.: +86 931 8911181; fax: +86 931 8912582.
E-mail address: lizhixiao@lzu.edu.cn (Z.-X. Li).

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1. Introduction

Grass pea (*Lathyrus sativus* L., Leguminosae) is an annual leguminous crop cultivated in Eurasia, North America, temperate parts of South America, and East Africa for animal or human consumption (Smartt, 1990). Common names include *shan li dou* in China; grass pea, chickling pea or India vetch in the UK and North America; *almorta*, *muela*, *tito* and *guijo* in Spain; *khesari* or *batura* in India; *alverjas* in Venezuela; *gilban* in Sudan; *guaya* in Ethiopia; *matri* in Pakistan; *gesette* in France and *pisello bretonne* in Italy.

The origin of *L. sativus* is unknown. Carbonized remains indicate grass pea was domesticated in the Near East and cultivated with cereals since the Neolithic period in the Balkans (Kislev, 1985, 1989; Smartt, 1990). Today, grass pea is produced throughout the arid regions of the Near East, North Africa, west Asia, and Indian subcontinent, and grown on a small scale in South America, Canada and China (DCLU, 1975; Kay, 1979; Muehlbauer, 1992).

L. sativus has a number of advantageous biological and agronomic characters, namely extensive tolerance of drought, water-logging and poor semiarid soils; resistance to insects and pests; nitrogen fixation; high grain-yielding capacity and high protein content of its seed (Kaul et al., 1986; Spencer, 1989; Campbell et al., 1994; Croft et al., 1999). The legume can thus provide an economic yield under adverse environmental conditions and offers great potential for use in marginal low-rainfall areas. Indeed, this has made it a popular crop in subsistence farming in certain developing countries that have extreme weather conditions (Rao et al., 1964; Ludolph et al., 1987; Tekele-Haimanot et al., 1990; Praveen et al., 1994).

Heavy and prolonged consumption of grass pea triggers a characteristic motor system disease (a form of spastic paraparesis known as neurolathyrism) in both animals and humans. The harmful potential of grass pea dependency was known to ancient Hindus and to Hippocrates (460–377 BC) (Spencer, 1995; Paleacu et al., 1999). Physicians from ancient Greece also knew of the disease and warned against the danger of eating grass pea (Spencer, 1995). Centuries later, in 1671, Duke George of Wurttemberg banned consumption of *Lathyrus* flour in his principality because of its ability to "paralyze" the legs, an edict that was subsequently twice enforced by his successor Leopold in 1705 and 1714 (Lambein et al., 2001). The disease was also described by Ramazzini in 1690, and another Italian, Cantani (1873), who coined the name *latirismo* ("lathyrismus") to describe the disease. Human consumption of grass pea is currently largely confined to Ethiopia, India, Nepal and Bangladesh but, over the past 200 years, it has also been eaten by other populations in Europe (France, Spain and Italy), Russia, Afghanistan and northwest China (DCLU, 1975; Jackson and Yunus, 1984; Bell, 1989; Spencer, 1995). Outbreaks of neurolathyrism occurred throughout Europe, northern Africa, the Middle East, Afganisthan, and India during the 18th, 19th and 20th centuries (Hanbury et al., 2000).

Consumption of seed of *L. sativus* for more than three months as a staple diet precipitates neurolathyrism (Selye, 1957; Ludolph et al., 1987; Spencer et al., 1993). The agent responsible for this cortical motor neuron disease appears to be β-*N*-oxalyl-L-α,β-diaminopropionic acid (β-ODAP) β-**1**, also known as β-*N*-oxalyl-amino-L-alanine, BOAA, which is present in seed as the free amino acid (Murti et al., 1964; Rao et al., 1964; Ross et al., 1985; Spencer et al., 1986; Nunn et al., 1987; Kuo et al., 1998). When grass pea is a minor component of a varied diet, **1** is tolerated without any known adverse effect. However, when food shortages occur and nutrition is marginal, grass pea intake may increase to form a major part of the diet. This occurs at times of persistent drought or flood, when most other crops fail thereby causing food scarcity. The more resistant *L. sativus* becomes the only affordable survival food for the poor and relative consumption of grass pea increases. The higher intake of the neurotoxin by poorly nourished people is the characteristic setting for neurolathyrism epidemics.

This review brings together knowledge about *L. sativus* and **1** from various disciplines. It builds on the earlier initiative of the U.S.-based Third World Medical Research Foundation known as INILSEL – the International Network for the Improvement of *L. sativus* and the Eradication of Lathyrism (Kaul et al., 1986) – to promote research cooperation and information sharing among scientists of disparate disciplines.

2. Botany

2.1. General

Grass pea is a food, feed and fodder crop belonging to the family Leguminosae (=Fabaceae), Subfamily Papilionoideae, tribe Vicieae. The genus *Lathyrus* consists of about 160 annual and perennial species (Plitmann et al., 1995; Chtourou-Ghorbel et al., 2001), also reported as

187 species and subspecies by Allkin and coauthors (1986). The species are separated into 15 sections based on their morphological traits (Smartt et al., 1994).

Inter-specific hybridization with other *Lathyrus* species has been attempted by many researchers; most attempts have failed. While many thousand cross-combinations are theoretically possible, only 16 have been reported to be successful. Interspecific hybridization involving *L. sativus* has only succeeded in two species: *L. sativus* crossed with *L. ciliolatus* or *L. cicera* gave viable hybrids. Six other *Lathyrus* species crossed with the cultivated species produced pods with shriveled or aborted seed (Khawaja, 1988; Smartt, 1990; Smartt et al., 1994). Taken together, these findings suggested a close association between *L. sativus* and *L. ciliolatus*, and *between L. sativus* and *L. cicera*. Indeed, *L. sativus* is probably a derivative of the genetically nearest wild species, *L. cicera* (Hopf, 1986).

Germplasm collections of grass pea exist in several locations, including India (Raipur, 2659 genotypes), Syria (ICARDA, 1560 genotypes), and France (University of Pau, 1807 genotypes) (Benková and Žáková, 2001; Abd El Moneim et al., 2001). The European Cooperative Programme for Crop Genetic Resources also has approximately 4000 grass pea accessions. ICARDA is collaborating with national partners to develop new grass pea lines with the objectives of improving yield potential, adaptability, and nutritional quality through reduction of **1** for human consumption and animal feed (Dahiya, 1976). Proof of safety would require animal (primate) feeding studies (see below).

2.2. Genetics

Grass pea has $2n = 14$ chromosomes (Smartt et al., 1994), but the location of genes responsible for the plant's remarkable environmental tolerance and **1** production are unknown. The biotechnological potential of grass pea as a source of multiple agro-environmental tolerance genes for general crop improvement remains to be exploited. Several attempts have been made to reduce **1** content through mutation and conventional breeding (Briggs et al., 1983; Campbell and Briggs, 1987; Roy et al., 1993; Addis and Narayan, 1994). Strains with low concentrations of **1** have been obtained but were unstable (Addis and Narayan, 1994; Santha and Mehta, 2001).

However, plant regeneration in tissue culture has proved to be a bottleneck. Shoot regeneration from shoot apices and stem calli were reported by various laboratories (Mukhopadhyay and Bhojwani, 1978; Gharyal and Maheshwari, 1980, 1983; Sinha et al., 1983), but little practical progress was made in these early experiments. However, in the 1990s, Roy and colleagues reported the in vitro production of plants from cultured leaf discs (1991), root explants (1992) and internode explants (1993). The seed and leaves of primary and secondary generations of these plants reportedly had low and stable **1** contents. In a similar timeframe, Malik and colleagues (1992, 1993) reported in-vitro shoot bud differentiation or plant regeneration via callus derived from direct shoot regeneration from epicotyl explants and seed cultures. Subsequently, Mehta and coworkers (1994) obtained somaclones of *L. sativus* from leaf, internode and root-derived callus cultures, which in several phenotypic characters, including **1** concentration, were largely stable over three generations. In addition, relative to the parent plant, the somaclone had a significantly reduced **1** content (down to 0.03%, 10-fold lower) and higher seed yield. Ochatt and colleagues (2000a,b,2001) obtained some regenerants with a normal DNA content and phenotype, and rooted and fertile plants were consistently and reproducibly obtained on hormone-free medium. More interestingly, they produced the somatic hybrid calli by fusing with leaf protoplasts of grass pea and pea protoplasts. The availability of these reliable regeneration techniques in the production of fertile plants may help in the breeding of *L. sativus* itself, and regenerating somatic hybrids might also yield genotypes with the disease resistance from grass pea coupled with the grain quality from other leguminous crops.

These advances underline the interest of flow cytometry as an early screening strategy to avoid hyperhydricity in cultured tissues, and for the optimization of plant regeneration in grain legumes (Ochatt et al., 2001). The regeneration protocol proposed has cleared the decks for genetic manipulation of this crop for both basic and applied work as well as for exploitation of somaclonal variation for low-**1** strains of *L. sativus*. Somaclones with low β-**1** contents (less than 0.1%) have been developed using these recently developed protocols, and these somaclones are being tested in different environments to assess stability of the neurotoxin content in the ripe seeds (Abd El Moneim et al., 2001).

Barna and Mehta (1995) attempted to transform *L. sativus* genetically via particle bombardment or agrobacterium, but without evidence for inheritance of transgenes. Very recently, a new in vitro protocol was developed for prolific shoot regeneration from two elite genotypes of *L. sativus*: Vegetative apical and axillary bud explants from greenhouse-grown plants were used to induce regeneration-competent green nodular callus (Zambre et al., 2002). Because regenerated shoots were readily converted with high efficiency into sexually mature plants either by planting shoots that were induced for rooting on hormone-free medium or by grafting of non-rooted shoots – the regeneration method based on green nodular callus is considered the most efficient amongst the available protocols for obtaining fertile plants of *L. sativus.* Thus, there is no doubt that it will prove possible to exploit grass pea lines with the objective of improving yield potential, adaptability and nutritional quality through reduction of β-**1** for human consumption and animal feed. Animal studies are needed to establish the threshold concentration for **1** neurotoxicity in mammalian species. Very recently, Getahun et al. (2003, 2005) demonstrated that consumption of grass pea mixed with cereals rich in sulfur amino acids

was a protective factor in the epidemiology of neurolathyrism, and breeding programmes, alongside traditional attempts to reduce the toxin content, should enhance the content of sulfur amino acids and antioxidants in grass pea.

2.3. Ecology

The plant has a very hardy and penetrating root system that supports growth on a wide range of soil types, including very poor soil and heavy clays. As a legume, it contributes to soil quality through the action of nitrogen-fixing symbiotic bacteria associated with the root system. Grass pea is extremely tolerant to environmental extremes, grows with as little as 250 mm of rain per year, and is typically the last plant standing in times of drought (Tekele-Haimanot et al., 1990; White et al., 2002). Our own experiments demonstrate that *L. sativus* tolerates the extremely low rainfall (200–400 mm p.a.) in areas of northwestern China, where the plant has an acceptable yield under conditions of limited humidity (10% moisture) (Lu et al., 1990). In Ethiopia, the legume is grown in the off season on residual moisture in vertisols at altitudes ranging from 1600 to 2700 m, across the semi-arid regions of the country, where the mean temperature fluctuations during the growing season range from 10 to 30 °C with annual rainfall ranging from 600 to 1200 mm (Duke, 1981; Campbell et al., 1994; Tadesse and Bekele, 2001b). In short, *L. sativus* is a hardy plant suited to dry climates, producing good seed crops on poor soils in some of the world's most difficult farming areas. It is thus a good model for investigating the mechanism of drought resistance and seeking genes associated with drought resistance.

The crop is not only tolerant to the extremely dry conditions found in Ethiopia but also withstands prolonged water logging as in Bangladesh (Smartt et al., 1994). The ability of grass pea to tolerate both drought and flood, the low cost required for its production, its high protein content, and its contribution to soil fertility through fixation of atmospheric nitrogen, have made this throughout history a highly desirable subsistence and insurance crop (Tadesse and Bekele, 2001a).

Furthermore, grass pea has commercially important traits in the form of resistance to extreme environmental conditions (flooding, high salinity, low soil fertility), infectious disease, and ability to flourish without expensive inputs (Campbell et al., 1994; Croft et al., 1999). Although the concentration of β-**1** in ripe seeds is variable and influenced by genetic and environmental factors, such as water stress, salinity, and drought, the biological role of β-**1** in *L. sativus* is unknown. According to Lambein et al. (1994) report, β-**1** is hypothesized to function as a carrier molecule for zinc ions, soils depleted in micronutrients or poor in available zinc and with high iron content may be responsible for the high level of neurotoxin in ripe seeds.

The possible function of β-**1** in conferring drought tolerance and resistance to oxidative stress has occupied our research attention. We have studied the relationship of **1** with H_2O_2 (Xing et al., 2001a; Xing et al., 2001b), hydroxyl radicals (Zhou et al., 2001; Xing et al., 2001b), polyamines (Xing et al., 2000a), abscisic acid (Xing et al., 2000b) under conditions of water stress and polyethylene glycol stress, and oxalic acid (Yan et al., 2004). These studies have shown that the contents of **1** increased continuously in relation to the duration of water stress, and there is a relationship between **1** contents and extent of lipid peroxidation, the enzyme activities of superoxide dismutase, catalase, peroxidase, glutathione reductase and polyamine, abscisic acid and oxalic acid concentration. These preliminary results suggest that drought resistance and oxidative stress are correlated phenomena that may be cause-effect related. Very recently, we also found that **1** play a protective role, and protect the activity of glycolate oxidase (GO) by scavenging the hydroxyl radicals (Zhang et al., 2003).

3. Chemistry

3.1. Nutritional composition

The seed of *L. sativus* provide a source of protein and carbohydrate that are able to sustain life during periods of famine when other food is unavailable (Spencer, 1989). The nutritive composition of *L. sativus* compares favorably with that of *Cicer arietinum*, field pea, faba bean and lupin (Table 1). On the whole, protein values for grass pea are higher than contents for *C. arietinum* (18.0%), field pea (25.7%) and faba bean (26.9%), but lower than lupin (35.1%) (Table 1). The amino acid composition of *L. sati-*

Table 1
Comparison of important composition in *L. sativus*, chickpea (*Cicer arietinum*), field pea, faba bean and lupin seeds[a]

Source	*Lathyrus sativus*						Chickpea	Field pea	Faba bean	Lupin
	I[b]	II	III	IV	V	VI	I[b]	VII		
Composition (% dry matter)										
Protein	27.3	26.4	25.6	26.3	26.9	35.9	18.0	25.7	26.9	35.1
Ash	3.5	2.8	–	3.2	2.9	2.7	5.7	2.8	3.0	3.0
Fat	1.6	1.7	1.3	0.7	0.8	1.2	2.8	1.2	1.4	6.5
Crude fiber	5.5	6.0	–	5.5	5.9	5.3	3.7	6.6	9.4	16.8

[a] Sources from: (I) Roy and Spencer. (1989); (II) Infascelli et al. (1995); (III) DCLU (1975); (IV) Dhiman et al. (1983); (V) Low et al. (1990); (VI) Kuo et al. (1995); (VII) Hanbury et al. (2000).
[b] Based on g/100 g seed (Moisture: in *Lathyrus sativus* 4.60%; in chickpea 7.40%.).

vus (Table 2) is similar to those of many grain legumes (Duke, 1981; Cai et al., 1984; Ravindran and Blair, 1992; Williams et al., 1994) – rich in lysine but deficient in methionine, cysteine and tryptophan (Ravindran and Blair, 1992; Gatel, 1994). *L. sativus* has a low content of polyunsaturated fatty acids and a high starch content, similar to field pea and faba bean; conversely lupin has high fat and low starch content (Chavan et al., 1999; Grela et al., 1999; White et al., 2002). The mineral content of grass pea is also similar to that of other agriculturally important grain legumes (White et al., 2002), although this will likely vary with soil mineral content. More detailed reports in chemical components, amino acid profiles, fatty acid contents and antinutritional factors of *Lathyrus sativus L. sativus* can be found in the review (Hanbury et al., 2000).

3.2. Lathyrogens

3.2.1. Lathyrism

Several substances that are toxic to laboratory animals have been isolated from the seed of *Lathyrus* species, including β-**1** in *L. sativus* and β-(*N*-γ-glutamyl)-aminopropionitrile – the γ-glutamyl derivative of β-aminopropionitrile (BAPN) **2** in *L. odoratus* (sweet pea), *L. hirsutus*, *L. pusillus* and *L. roseus* and L-2,4-diaminobutyric acid (DABA) **3** in *L. sylvestris*, *L. latifolius* (Barrow et al., 1974; Foster, 1990; Murti and Seshadri, 1964; Roy & Spencer, 1989). However, *L. sativus* is not the only plant containing toxic β-**1**, besides being present in the seeds of 21 *Lathyrus* species (mainly *L. sativus*, *L. cicera* and *L. clymenum*), β-**1** was also found in some other genera of leguminous plants including 17 species of Acacia and 13 species of Crotalaria (Quereshi et al., 1977) as well as in older ginseng roots of different species and of different origins including *P. ginseng*, *P. notoginseng* and *P. quinquefolius* – the only non-legume plant in which β-**1** is known to be present (Long et al., 1996).

Three experimental toxic syndromes associated with the ingestion of *Lathyrus* seeds by laboratory animals have been recognized by Selye (1957). Experimental neurolathyrism, an acute neurotoxic disorder of laboratory animals (chicks, rodents, and other species) mostly represented by convulsions (which are not features of human neurolathyrism), is induced by **1** (Roy & Spencer, 1989). Second, primates fed *L. sativus* or **1** for prolonged periods develop central motor pathway and hindlimb deficits similar to those seen in the human disease (Spencer et al., 1986). Third, experimental rodent osteolathyrism and angiolathyrism are associated with ingestion of *L. odoratus* (sweet pea) and its principal toxic constituent **2**. Compound **2** inhibits the activity of lysyl oxidase, an enzyme of importance in collagen formation. The resulting connective tissue abnormalities may give rise to collapse of the vertebral column, leading to secondary paralysis unassociated with neurolathyrism, and abnormalities of blood vessel walls (angiolathyrism) (Selye, 1957; Barrow et al., 1974; Roy, 1988) that are not reported in human neurolathyrism.

3.2.2. Isomerization of α- and β-*1*

Bell and O'Donovan (1966) reported that β-**1** slowly equilibrates with its isomer α-**1**, and the inter-conversion is facilitated when heated. The natural abundance of the alpha isomer is approximately 5% of the total **1** content in seed of *L. sativus* (Roy and Rao, 1968). Animal studies

Table 2
Comparison of common amino acid in *L. sativus*, chickpea (*Cicer arietinum*), field pea, faba bean and lupin seeds [a]

Source	*Lathyrus sativus*						Chickpea	Field pea	Faba bean	Lupin
	I	II	III[b]	IV	V	VI	III[b]	VII		
Amino acid (g/16 g N)										
Alanine	4.53	4.53	9.82	3.20	2.19	3.92	7.66	4.00	4.17	3.19
Arginine	8.04	6.35	21.4	6.13	6.11	3.29	14.1	10.04	9.46	12.03
Aspartic acid	11.8	11.61	27.6	8.53	14.6	9.97	20.0	10.16	10.53	9.29
Cystine	1.39	Trace	4.50	–	–	–	1.80	1.49	1.37	1.48
Glutamic acid	17.43	14.08	39.5	13.40	17.47	13.99	29.2	15.88	16.03	20.77
Glycine	4.20	3.82	9.70	3.45	3.91	3.91	7.51	4.13	4.20	4.12
Histidine	2.61	2.22	6.61	2.82	3.47	2.70	4.54	2.37	2.54	2.41
Isoleucine	4.41	4.34	9.77	3.41	4.82	3.89	6.42	3.89	3.80	3.97
Leucine	6.90	5.69	15.9	5.93	8.60	6.42	12.6	6.54	7.27	6.61
Lysine	6.73	5.65	16.7	4.08	6.27	9.65	12.8	6.81	6.29	4.66
Methionine	0.82	0.37	0.94	0.24	0.61	0.59	1.22	0.85	0.78	0.72
Phenylalanine	4.49	4.36	10.6	3.26	3.89	2.95	8.92	4.17	4.12	3.65
Proline	4.00	3.75	9.50	3.07	4.42	3.50	7.51	4.24	3.82	4.28
Serine	4.73	4.66	10.9	–	5.08	4.40	9.33	4.13	5.04	4.85
Threonine	4.08	3.22	8.43	2.59	5.15	3.82	6.62	3.35	3.54	3.36
Tyrosine	2.45	2.52	6.07	2.39	2.92	1.44	3.26	2.87	3.39	3.46
Valine	4.90	4.14	12.2	3.91	5.08	5.88	7.53	4.29	4.30	3.91
Protein (%)	24.5	25.6	28.6	27.4	32.3[c]	25.6	18.0	23.0	24.1	32.2

[a] Sources from: (I) Low et al. (1990); (II) Cai et al. (1984); (III) Roy and Spencer (1989); (IV) Latif et al. (1975); (V) Kuo et al. (1995); (VI) Ronda Lain et al. (1963); (VII) Hanbury et al. (2000).

[b] Based on mg/g seed.

[c] Based on estimated as 0.90% dry matter.

show the alpha isomer to be less neurotoxic than β-**1** (Wu et al., 1976) or non-neurotoxic when introduced into cerebrospinal fluid (Chase et al., 1985). It is also documented that β-**1** in solution undergoes a transformation to the alpha isomer via an unstable intermediate until an equilibrium mixture 3:2 ratio (Bell and O'Donovan, 1966; De Bruyn et al., 1994; Padmajaprasad et al., 1997). However, dry heating grass pea seed is reported variously to lower, raise, or effect no change on β-**1** content (Tekele-Haimanot et al., 1993). Abegaz and associates (1993) as well as De Bruyn and co-workers (1994) showed the thermal isomerization of β-**1** by NMR spectroscopy. Although it requires longer time, the same equilibrium can be established by starting with α-**1** (Abegaz et al., 1993). The stereoisomer of **1** lacks neurotoxic properties when tested in tissue culture (Nunn et al., 1987).

3.2.3. Grass pea detoxification

Compound **1** is a water-soluble amino acid that can be leached from seed by soaking in water (Mohan et al., 1966; Tekele-Haimanot et al., 1993; Akalu et al., 1998). Steeping grass pea in a large volume of cold water for 3 min leached out approximately 30% of **1**, with greater losers when hot water was employed (Tekele-Haimanot et al., 1993). Similarly, steeping dehusked seed in hot water for several hours and boiling the seed in water removed 70–80% of the neurotoxin (Mohan et al., 1966). Moslehuddin and colleagues (1987) also found that washing seed partially removed **1**. Padmajaprasad and associates (1997) reported that boiling grain and discarding the water reduced **1** levels up to 90%. Boiling has widely been used in the preparation of *L. sativus* seed as *dahl*, in bread-making and in vegetable preparations (Kay, 1979).

3.2.4. Assay of *1*

The most commonly used method based on *o*-phthalaldehyde (OPA) was introduced by Rao (1978) and later modified (Briggs et al., 1983; Li et al., 1992; Hussain et al., 1994) for spectrophotometric determination of total **1**. Attempts to separate α- and β-isomers by copper chelation fail to yield the α-isomer with sufficient purity (O'Brien and Nunn, 1982).

High-performance liquid chromatography (HPLC) methods for determination of **1** have been developed, which include methods with *o*-phthalaldehyde chiral thiols (Euerby et al., 1989), 9-fluorenyl methylchloroformate (Kisby et al., 1988; Geda et al., 1993) and phenyl isothiocyanate (Khan et al., 1993, 1994). We have also published a few HPLC methods that successfully separate α- and β-isomers; these are based on pre-column derivatization with 1-fluoro-2,4-dinitrobenzene (FDNB) (Wang et al., 2000), 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) (Chen et al., 2000), 5-dimethyaminonaphthalene-1-sulfonylchloride (DnsCl) (Xing et al., 2001) and *para*-nitro-benzyloxycarbonyl chloride (PNZ-Cl) (Yan et al., 2005a). Sequentially, we developed an assay for the simultaneous determiation of polyamines, β-**1**, α -**1** and homoarginine in *L. sativus* extracts (Yan et al., 2005b). Some other methods to determine **1** including capillary zone electrophoresis (CZE) and flow injection assay were also reported (Moges and Johansson, 1994; Arentoft and Greirson, 1995; Belay et al., 1997; Zhao et al., 1999; Yigzaw et al., 2001).

4. Biomedical aspects

4.1. Human disease

Neurolathyrism is characterized by spastic paraparesis involving the lower limbs almost exclusively. This results from degenerative changes in the Betz cells of the motor cortex and their axons in corticospinal tracts; together these constitute the principal central nervous system pathway for the regulation of muscle function (Ludolph and Spencer, 1996). Several weeks or months of heavy grass pea consumption leads to prodromal symptoms of muscle cramping associated with a sensation of heaviness and weakness of the legs. Initial manifestations of the disease appear to be reversible but, with continued grass pea reliance, individuals develop increasingly severe and irreversible spastic paraparesis arising from the selective involvement of the central motor pathway.

Clinical (Cohn and Streifler, 1981) and neuropathological studies (Streifler et al., 1977) have shown the involvement of upper motor neurons in the cerebral cortex, preservation of anterior horn cells in the spinal cord and loss of axons in the pyramidal tracts in the lumbar spinal cord in humans affected by neurolathyrism. Central motor conduction deficits are observed upon electrophysiological examination following stimulation of the motor cortex (Hugon et al., 1988, 1993). In the absence of further grass pea intake, the disease is largely static throughout the lifetime of the individual although there may be some progression in central motor system deficits.

The key etiological factor for neurolathyrism is prolonged and heavy dietary dependency on grass pea or other neurotoxic *Lathyrus* spp. Other suggested risk factors include heavy physical labour, febrile illnesses and diarrhoeal episodes (Tekele-Haimanot et al., 1990; Haque et al., 1996), and zinc deficiency in the soil (Lambein et al., 1994). In regions of grass pea dependence, neurolathyrism may be a highly prevalent neurotoxic disorder (Tekele-Haimanot et al., 1990); the condition predominantly affects males, especially young men (Shourie, 1945; Attal et al., 1978; Roy, 1988), although the reasons for this gender preference are not understood (Hugon et al., 2000).

4.2. Animal studies

Of the many species in which neurolathyrism has been reported, the horse appears to be the most susceptible and thus a useful species with which to test the safety of low-**1** strains of grasspea. Animals fed only 1–2 quarts per day for 2–3 months develop neurological signs resem-

bling stringhalt, and a pure grasspea diet is said to precipitate equine neurolathyrism in only 10 days (Stockman, 1917, 1929).

Experimental studies have examined the acute and chronic neurotoxic properties of grass pea and **1** in birds and mammals. Young animals are more susceptible to **1**. Seizures and opisthotonus develop in newborn and young mice given large doses of **1** by intraperitoneal injection (225–1350 mg/k) or by gavage (3900 mg/kg), respectively (Mehta et al., 1979). The LD_{50} by intraperitoneal injection of **1** is ~750 mg/kg in mice and ~700 mg/kg for 3-day-old chicks (Liu et al., 1989; Chen et al., 1992). Neuropathological changes in rodents treated with convulsive doses of **1** are largely confined to the circumventricular organs of the brain, where the blood–brain regulatory interface is normally absent (Olney et al., 1976). Although neurolathyrism reportedly occurs in many species that consume grass pea, experimental reproduction has proved challenging. In our laboratory, mice, rats, chicks, sheep, pigs and donkeys were fed up to 70–85% grass pea (**1**, 0.55%) for long periods (6–12 months). Pathological studies of animals (mice, donkey and sheep) showed nerve cell necrosis and degeneration in the cerebral cortex. Changes were also found in the liver and kidney. While neuronal degeneration was observed, tumors were not (Liu et al., 1989; Chen et al., 1992).

There have been several attempts to model neurolathyrism in primates, with limited success. Pyramidal dysfunction resembling the early reversible phase of human neurolathyrism was demonstrated in a carefully controlled study of well-nourished cynomolgus monkeys (*Macaca fascicularis*) fed a fortified diet of pure *L. sativus*. Animals additionally received by oral intubation a daily infusion of an alcoholic extract of grass pea, for a total daily intake of 1.1–1.4 g of ODAP/kg body weight. Other animals were given pure **1** with and without grasspea extract and fed fortified chickpea (*Cicer arietinium*) matched for protein, carbohydrate, fat, mineral and vitamin content. Animals developed comparable neurological signs after 2–4 weeks (300 mg/kg/day, increasing by 300 mg/kd day after 15 days of synthetic **1** alone), 2–6 weeks (alcoholic extract plus synthetic **1**), and 3–10 months (alcoholic extract plus grasspea diet). Affected monkeys showed a variable combination of fine tremor, periodic myoclonic-like jerks, mild-to-moderate increase in muscle tone of leg muscles, hindlimb extensor posturing, and a skater-like gait. Electrical stimulation of the motor cortex showed prolonged central motor conduction but neuropathological studies showed no convincing evidence of neuronal degeneration (Spencer et al., 1986, 1988). This suggests that the early reversible phase of primate and human neurolathyrism results from changes associated with the neuropharmacologic (neuroexcitatory) properties of **1**.

4.3. Mechanisms of neurotoxicity

The mechanism of **1**-induced neurotoxicity is still not completely understood. First and foremost, while the primate studies noted above suggest that **1** can traverse the blood–brain barrier in healthy subjects, the underlying mechanisms are not known. It is entirely possible that malnutrition increases the amount of **1** entering the brain but studies have not been performed to address this question. Second, the critical cellular actions of **1** that culminate in neuronal degeneration have yet to be established. Previous in vitro studies show that β-**1** acts as a potent agonist at α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors (Nunn et al., 1987; Ross et al., 1989; Sawutz et al., 1995; Ikegami et al., 1995), and inhibits glutamate uptake in a synaptosomal preparation (Ross et al., 1985). When administered to rats at appropriate doses, β-**1** increases cerebellar cyclic guanosine monophosphate (GMP), and induces a downregulation of glutamate receptors in the primary motor cortex (La Bella et al., 1993a,b). These results suggested that β-**1** might induce neuronal degeneration through excessive stimulation of nerve cells via plasmalemmal glutamate receptors. Electrophysiological studies also reveal that **1** is a potent agonist at non-NMDA glutamate receptor sites (Pearson and Nunn, 1981). Recently, a report proposed that **1** can be metabolized by man, which may explain the low incidence of neurolathyrism in humans and also as to why the disease has not surfaced in many regions despite consumption of *L. sativus* by large populations (Rudra et al., 2004).

However, the above studies do not explain some phenomena, such as remarkable species differences in susceptibility to the neurotoxin and the very low specific binding of ^{3}H **1** to synaptic membranes of the chick or rat (Jain et al., 1998). Compound **1** is also reported to alter the ability of astrocytes to regulate levels of glutamate, the natural central nervous system excitatory neurotransmitter (Miller et al., 1993). Increased glutamate levels arising possibly as a result of inhibition of glutamate transport were also suggested as the mechanism of neurotoxicity from a study of freely moving rats (La Bella and Piccoli, 2000). Other studies have proposed that **1** disrupts energy transformation through inhibition of the mitochondrial enzyme NADH-dehydrogenase (Pai and Ravindranath, 1993), but these findings could not be confirmed (Sabri et al., 1995). Kalivendi et al. (1997) found that C57BL6J black mice are susceptible to **1** neurotoxicity while BALB/c white mice are resistant but become susceptible if pre-treated with tyrosine. They established that **1** produces a stereospecific inhibition of tyrosine aminotransferase and predicted an increase in catecholamine synthesis. While these studies raise the possibility that tyrosine metabolism is involved in the acute behavioral toxicity of **1** in mice, it is unclear whether they have relevance to the pathogenesis of human neurolathyrism.

5. Biosynthesis of (1)

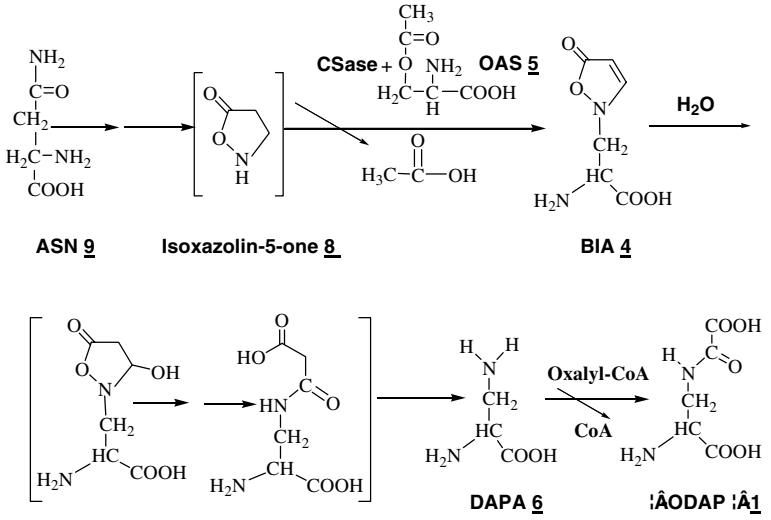
The first hypothetical pathway was put forward by Murti and Seshadri (1964) who suggested that **1** can arise

by oxalylation of diaminopropionic acid (DAPA) **6** and the latter may be formed from serine or asparagine. Malathi and colleagues (1967) investigated the possible involvement of the oxalyl moiety in the synthesis of **1** by germinating seeds of *L. sativus* in the presence of [$^{14}C_2$]-oxalic acid. Further investigation showed that **1** is derived from free **6** and oxalyl-coenzyme A (Malathi et al., 1970).

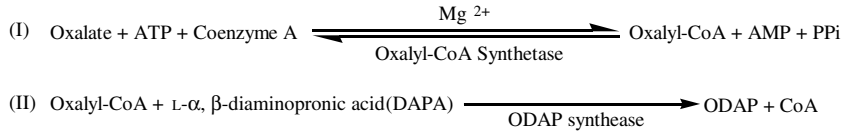
Leguminous plants, including *L. sativus* in particular, synthesize a large number of secondary products such as β-(isoxazolin-5-one-2-yl)-L-alanine (BIA) **4**, which has been studied in relation to **1** synthesis (Murakoshi et al., 1973, 1975; Ikegami et al., 1991; Kuo et al., 1998). When *O*-acetyl-[3-^{14}C] serine (OAS) **5** was fed to *L. sativus* seeds, the radioactive label was incorporated into **4** and with lower concentration into (**1**); further studies with [^{14}C] labeled L-serine or L-**5** revealed that the cotyledons are presumably the site for this biosynthetic step (Lambein et al., 1990). When ^{14}C-labelled **4** was supplemented with free **6**, or with oxalate in freshly cut callus tissue, the kinetics of the incorporation of [^{14}C] into **1** confirmed that **4** is the precursor for **1** and indicated that **6** may indeed be the short-lived intermediate (Kuo and Lambein, 1991); other isotope-labeling experiments showed that [^{15}N]-β-*N*-hydroxyl-α,β-diaminopropionic acid (HDAP) **7** may also be a short-lived intermediate in the biosynthesis of **1**, and confirmed that **6** is the essential precursor for the formation of **1** (Kuo et al., 1994a). When **4** was labeled with ^{13}C at carbon atoms C-3, C-4 (C═C) and C-5 (C═O) on the ring as well as with ^{15}N-2 fed to the callus tissues (Kuo et al., 1994a), observations confirmed that the breakdown of the isoxazolinone ring is absolutely in agreement with the proposed chemical mechanism (De Bruyn et al., 1992), and that the carbons of **1** molecule derive from the alanine side chain of **4**. Therefore, in accordance with the above experimental facts, a systematic hypothesis for the biosynthesis of **1** was proposed, namely that **4** formed from the free ring and *O*-acetyl-serine by cysteine synthase of *L. sativus* (Ikegami et al., 1993) is the biosynthetic precursor of β-**1** in different stages of *L. sativus* (Lambein et al., 1990; Kuo and Lambein, 1991; Kuo et al., 1994a; Kuo et al., 1994b, 1998), and the ring of **4** opens to give rise to the short-lived **6** which is further oxalylated by oxalyl-CoA to form **1** (Scheme 1).

Cysteine synthase (CSase, *O*-acetyl-serinelyase), which can catalyse the formation of some heterocyclic β-substituted alanines in plants, is normally present in two forms (Murakoshi et al., 1985; Murakoshi et al., 1986; Ikegami et al., 1987; Ikegami et al., 1988). Two different forms of cysteine synthase (CSase A and CSase B) were also purified from grasspea (Ikegami et al., 1993). Data on the substrate specificity revealed that both isoenzymes catalyse the formation of **4** and some other heterocyclic β-substituted alanines from **5** (Scheme 1). Although the CSase isoenzymes display somewhat different relative activities, their responses to **5** are essentially the same (Murakoshi et al., 1985; Ikegami et al., 1990; Ikegami et al., 1992). Further studies suggested that the biosynthetic site of **1** is located in mitochondria and chloroplasts (Hock Ng and Anderson, 1978; Ikegami et al., 1992, 1993). Although the above hypothesis proposed that **7** is a potential intermediate in the breakdown of **4**, and **6** is the metabolic intermediate between **4** and β -**1** in the callus tissues of *L. sativus*, neither **7** nor **6** has ever been identified as natural constituents in *L. sativus* seeds or plants even after germination. It was noteworthy, however, that even in the absence of added **6**, there is a small but significant amount of **1** formation in cell-free extracts, which suggests the possible presence of an endogenous precursor for **6** (Malathi et al., 1967) (Scheme 1).

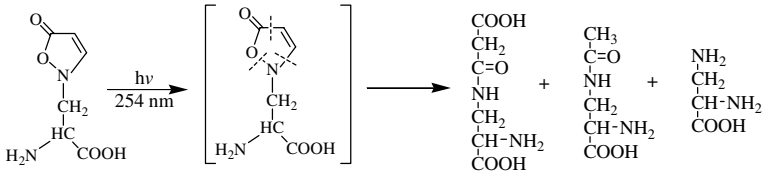
The proposed hypothesis based on **6** as the intermediate in **1** genesis is recognized as the most systematic, detailed theory regarding the biosynthesis of **1** (Scheme 1). There



Scheme 1. Proposed biosynthetic pathway for β-**1** in *Lathyrus sativus*: formation of **8** from**9;** formation of **4** from **8** and **5**; ring-opening from **4** by the occurrence of H_2O addition with formation of free**6** as short-lived intermediate; formation of β-**1** from **6** and Oxalyl-Coenzyme A. The products in brackets have not been detected in *L. sativus* (Ikegami et al., 1990; Lambein et al., 1990; Kuo and Lambein, 1991; Ikegami et al., 1992, 1993; Ikegami et al., 1993; Kuo et al., 1994a; Kuo et al., 1994b; Kuo et al., 1998; Ikegami et al., 1999).



Scheme 2. The proposed oxalylating steps: the oxalylating steps including two reactions catalysed by two different enzymes have been proved by Malathi et al. (1970).



Scheme 3. The photodegradation scheme of **4** by UV-light at 254 nm. The products in brackets which was hypothesized as the water addition product have not been detected (Lambein et al., 1976; De Bruyn et al., 1992).

are two proposed steps: The first reaction is catalyzed by oxalyl-CoA synthetase, which has properties similar to that of the enzyme in peas. The second reaction is catalyzed by another enzyme, which is specific to *L. sativus* and is designated as oxalyl-CoA-α,β-diaminopropionic acid oxalyl transferase (Scheme 2) (Malathi et al., 1970). Oxalyl-CoA, which was first found in *Pisum sativum* seedlings, is the donor for the oxalyl moiety in the in vitro enzymatic synthesis of **1** (Kuo and Lambein, 1991). Oxalyl-CoA from *L. sativus* showed almost the same substrate specificities as those from *P. sativum* and other sources. However, the ODAP synthetase from *L. sativus* appears to be unique (Ikegami et al., 1993).

The mechanism for the biological breakdown of the isoxazolin-5-one ring is not yet known. Potentially, it may follow the same scheme as the breakdown after activation with UV-light at 254nm (Scheme 3), where an addition of H_2O to the ring is involved (De Bruyn et al., 1992). Ikegami and associates (1999) proposed that the enzymatic breakdown of **4** with formation of **6** – the direct precursor of the neurotoxin β-**1** in *Lathyrus sativus* – was confirmed in vitro and some properties of the enzyme responsible for the biosynthesis of **6** are described. However, additional information on this subject has yet to be made available. According to Misra and Barat (1981), the second oxalylating step may not need any enzymatic catalysis and be a purely chemical reaction. If so, the enzyme opening the heterocyclic ring of **4** should be the factor determining the toxicity of the species.

6. Animal nutrition

It is reported that seed yields of grass pea crops range from 900 to 1500 kg per hectare in general, while crops sown from Rhizobium inoculated seeds produced up to 2000 kg per hectare in the United States (Kay, 1979; Duke, 1981). In India, the seed yields of *L. sativus* are proportional to seeding rates, which vary from 45 to 90 kg per hectare depending on the method of cultivation (Duke, 1981). At the seeding rate of about 14 kg per hectare in mixed cultivation, yield per hectare is about 300 kg of pulse and 0.5 metric tons of straw; an average crop at a seeding rate of 40 kg per hectare yields about 925 kg per hectare of pulse and 3.2 metric tons per hectare of forage (Duke, 1981). Our previous study showed that the optimum seeding rate (for purpose of seeds) is 150–187.5 kg per hectare, seed yields of grass pea range from 3187.5 to 3225 kg per hectare; indeed, the optimum seeding rate for forage is 225–300 kg per hectare, whereas the average yield per hectare for forage (fresh weight) is 26.4 metric tons (Lu et al., 1990; Chen et al., 1992). A report from Hungary (Lazányi, 2000) showed that the mean grain yield could increase to 3.546 ton per hectare for five grass pea varieties by selection within ecotypes and plant breeding. In Canada, grass pea seed yields up to the equivalent of 5232 kg per hectare have been obtained by a selection and breeding program (Briggs et al., 1983).

The studies at the Center for Legumes in Mediterranean Agriculture (CLIMA) suggested that two *Lathyrus* species, *Lathyrus cicera* and *Lathyrus sativus*, have potential in low-to-medium rainfall (250–500 mm p.a.) areas of southern Australia (Siddique et al., 1996; Hanbury et al., 2000). They are well adapted to southern Australian dry land conditions and envisioned to have a role as multi-purpose crops providing feed grain, fodder, hay and green manure with a potential growing area of 100,000–300,000 ha (Hanbury et al., 2000; White et al., 2002). Most of the reported work on grass pea improvement has been on the reduction of **1** content in the seed. In India, Roy and colleagues (1993) reported the finding of reduced **1** content in somaclones derived from internode explants of *L. sativus*; In Canada, a breeding and improvement program was established in the release of the germplasm LS8246, which has a seed **1** content of 0.03% ((Campbell and Briggs, 1987). In 1989–90, an extensive screening program was initiated for five years to explore the possibility of identifying toxin-free lines from germplasm of different origins. The results

indicated that no accession of any *Lathyrus* species was β-**1** free, although in several lines the β-**1** content was low. Four IFLLS lines of *L. sativus* – 522, 588, 516, and 563 — were found to have low β-**1** content in the seeds, ranging from 0.02% to 0.07% (Abd El Moneim et al., 2001). In conclusion, analysis of a large number of germplasm accessions of *L. sativus* revealed that samples originating from Bangladesh, Ethiopia, India, Nepal, and Pakistan were high in **1** content in the dry seeds, in a range from 0.7% to 2.4%, whereas samples from North Africa, Syria, Turkey, and Cyprus had significantly lower **1**, ranging from 0.02% to 1.2% (Abd El Moneim et al., 2001).While low-**1** strains have been developed, the stability of the ODAP level in various soils and under various environmental conditions has yet to be established. In addition, it cannot be assumed that low-**1** levels are safe for human consumption until the bioavailability of **1** has been studied in an appropriately susceptible monogastric species, such as the horse. If it can be shown that stable low-**1** strains generate correspondingly low serum levels of **1** in the absence of any acute and chronic illness, the prospect of using these strains for human food could be considered. It should be noted, however, that while grass pea seed has a high protein content, it is low in sulfur amino acids.

The effects of feeding *L. sativus* are species-specific. Horses are very susceptible to neurolathyrism and sometimes die after seed consumption (Hanbury et al., 2000; López Bellido, 1994). Dhiman et al. (1983) fed calves on *L. sativus* up to 30% of daily diet for prolonged periods without any signs of toxicity, ill health, or changes in behavior or growth. There is some evidence that rumen micro-organisms may be able to degrade **1**, which might account for the absence of neurotoxicitty in non-monogastric species (Kuo et al., 1995, 2000). Very recently, eighty individually penned Merino wethers (35 ± 0.3 kg) were fed on controlled *Lathyrus* diets up to 13 weeks. There were no visible or biochemical signs of ill health in any sheep fed *Lathyrus cicera* (low **1**). Quality testing of meat showed equal or better results for *L. cicera* than for lupins, and wool growth was similar for all diets. Compared with lupin grain, low-**1** *Lathyrus* grain appears to be of high nutritional value for sheep, with no evidence of adverse effects on animal health (White et al., 2002). In our previous experiments, three groups of sheep (4 months old) were treated with *L. sativus* (0.55% **1**) up to 30%, 50% and 70% for 6–9 months, respectively; six piglets (50 days old) were fed on *L. sativus* (0.25% **1**) up to 80% for 6 months; two groups of six young donkeys were treated with *L. sativus* (0.25% **1**) up to 50% and 80% for 6 months, respectively. No signs or symptoms of neurolathyrism or malnutrition were observed, body weights increased normally, and there were no differences from controls. However, pathological examination showed that liver and kidney cells had undergone pathological changes (Liu et al., 1989; Chen et al., 1992). Liu and associates (1989) also found that intake of 0.55% **1** *L. sativus* grain at 85% of daily diet for 12 months did not induce any symptoms of neurolathyrism in young rats (18–24 g weight), but growth rates were markedly lower than those of a control group. La Bella and colleagues (1997) reported that male albino Wistar rats treated with *L. sativus* (6–12 g seeds/day) showed no signs or symptoms of hypovitaminosis or malnutrition: their body weights increased normally, and there were no differences from a control group treated with *C. arietinum* (CA) seeds.

According to the review of Hanbury and colleagues (2000), pig feeding studies indicated that grain of *Lathyrus sativus* with a low **1** concentration could be fed at 30% of the diet without any effect on animal performance. Sheep, and presumably other ruminants, can tolerate high levels of inclusion (70% grass pea with 0.09% **1**) with no reduction in performance, and **1** is probably broken down in the rumen (Hanbury et al., 2000). Since a 70% inclusion rate is unlikely to be used in practical ruminant feeding, *Lathyrus* spp. containing higher concentrations of **1** could certainly be tolerated at inclusion rates below 70%. Poultry seem able to tolerate up to 40% of their diet of *L. sativus* of moderate **1** concentrations (0.27%) with minor reductions in growth. However, the susceptibility of animal species to neurolathyrism is poorly understood and further feeding studies to estimate a relatively safe dose are necessary. Moreover, reports also showed that young animals are more susceptible to **1** than older animals when animals are treated with *L. sativus* (Rao, 1967; Mani, 1971; Liu et al., 1989; Chen et al., 1992). Therefore, caution should be taken when using *L. sativus* in the diet of young animals.

7. Future directions

Four decades has elapsed since the discovery of **1** as the neurotoxic constituent of *L. sativus* seed. Several questions relating to the relationship between **1** and human neurolathyrism remain. While a complete understanding of molecular mechanisms is desirable, this may not be required for the purposes of developing a safe strain of grass pea for animal feed and even human consumption. Among the information that is required to develop a safe strain of grass pea are the following: (i) A convincing animal model of neurolathyrism is needed to assess the bioavailability of **1** from wild-type and low-**1** strains of grass pea: the primate shows promise but the horse offers the advantage of disease susceptibility. (ii) Mechanisms underlying the zinc dependency, drought tolerance and nitrogen-fixation of grass pea are needed. (iii) The precise enzymatic pathways for the biosynthesis and degradation of **1** are needed. (iv) Screening and breeding of new grass pea lines with increased forage production and higher-yielding ability are needed. This information will be generated through continued multidisciplinary research that has been established under the International Network for the Improvement of *Lathyrus sativus* and the Eradication of Lathyrism (INILSEL).

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Additional on-line information of *Lathyrus* and lathyrism

Lathyrus Lathyrism Newsletter - (http://go.to/lathyrus OR http://www.clima.uwa.edu.au/lathyrus). An online newsletter with many articles on lathyrism.

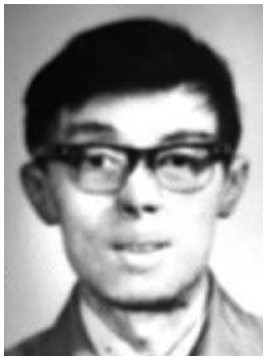
An on-line bibliographic database of *Lathyrus*-(http://mansfeld.ipk-gatersleben.de/ris/risweb.isa). This bibliography provides access to the complete *Lathyrus* literature until 1998.

The grass pea monograph - (http://www.ipgri.cgiar.org/publications/pdf/430). An online monograph by Clayton Campbell introduce the outline on grass pea.

Toxic Food Materials Consumed -(http://allafrica.com/stories/200007100005.html). This article at allAfrica.com contains information about lathyrism in Ethiopia.

Scientist to fight disease-(http://www.expressindia.com/ie/daily/19990317/ige17010.html). Genetic engineering being used to neutralize the toxin in *Lathyrus sativus*.

Ze-Yi Yan became his chemical research and received his bachelor's degree at Shaanxi University of Science & Technology, China. He completed his diploma majoring in biosynthesis of toxic ODAP of *Lathyrus sativus* at Lanzhou University in 2004. Since then he has been working for his Ph.D. at the same University (Prof. Yong-Min Liang). The main topic of his doctor research moves to develop innovative and fundamentally new organic reactions and multi-component reactions with high "atom efficiency".



Zhi-Xiao Li is a professor at Lanzhou University, China. His research interests include to develop general assays of various toxins in plants, screen and cultivate low- or non-toxic *Lathyrus sativus* lines, and isolate and identify natural products.