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Variability for the presence of pyrrolizidine alkaloids in *Crotolaria juncea* L.

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Received September 14, 2004, accepted October 20, 2004

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Pharmazie 60: 620–622 (2005)

Sunn hemp (*Crotalaria juncea* L.) is the most widely grown legume used as green manure in the tropics where it is also grown as a fiber and animal fodder crop. It has been reported that sunn hemp seeds contain several pyrrolizidine alkaloids that when ingested in sufficient amount can be toxic to animals and birds. No information is available regarding variability for the presences of the different types of pyrrolizidine alkaloids in the seeds. The objective of this research was to analyze sunn hemp seeds of nine populations that originated in different parts of the world for several pyrrolizidine alkaloids to determine their level of variability for the presence of these compounds and to quantify the total amount of pyrrolizidine alkaloids in the seeds. Of the nine pyrrolizidine alkaloids tested, the sunn hemp populations only had junceine and trichodesmine. PI 207657 had very low levels of both alkaloids and PI 314239, PI 322377, PI 346297, and the US cultivar Tropic Sun had very low levels of trichodesmine. Although juncein was present in higher amounts than trichodesmine in the seeds of most accessions, its value was deemed to be small. The amount of pyrrolizidine alkaloids present in the sunn hemp populations studied was low.

1. Introduction

Sunn hemp (*Crotalaria juncea* L.) is the most widely grown green manure in the tropics where it is also grown as a fiber and animal fodder crop (Purseglove 1981). It is a summer legume adapted to a wide range of environmental conditions and soil types. Thus, it can be used as a summer cover crop to protect and conserve soil and water resources.

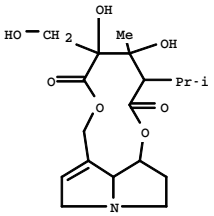
Plants are tolerant-resistant to several nematodes. Sunn hemp can be effective in management of reniform nematode, *Rotylenchulus reniformis* Linford and Oliveira, which does not reproduce on the plants (Wang and Sipes 2000). In fact, it was reported that sunn hemp reduced reniform nematode populations at least as well as fallow (Caswell et al. 1991). Also, it was found to be a very poor host or non-host of the root-knot nematodes *Meloidogyne arenaria* race 1, *M. incognita* race 1, and *M. javanica* (McSorley 1999).

It has been reported that sunn hemp seeds contain various pyrrolizidine alkaloids such as junceine, riddelliine, senecionine, seneciphylline and trichodesmine (Smith and Culvenor 1981). Pyrrolidizidine alkaloids ingested in sufficient amount can be toxic to animals and birds. Although pyrrolizidine alkaloids are not directly toxic, metabolic derivatives formed in the liver cause toxic by interacting with cell DNA (Mattocks 1978). Most commonly, they cause liver damage (hepatoxic effect) and can affect other organs such as lungs (Hooper 1978).

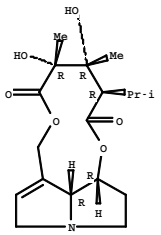
Trichodesmine, that has hepatotoxic and neurotoxic effects (Yan and Huxtable 1995) on some farm animals such as pigs (*Sus scrofa* L.), was reported to be the main toxic compound present in the seeds of sunn hemp (Zhang 1985). However, there seems to be a variability for the presence and quantity of toxic alkaloids among sunn hemp populations. Purseglove (1981) reported that seeds are fed to farm animals in some countries with no toxic effects and Rotar and Joy (1983) determined that seeds of the cultivar Tropic Sun were not toxic to animals. Furthermore, sunn hemp seeds were found to be non toxic to chicks when fed at 10 mg/g of body weight (Williams and Molyneux 1987). The content of pyrrolizidine alkaloids in sunn hemp seeds was reported to be low, thus Adams and Gianturco (1956) measured 0.003% and Williams and Molyneux (1987) measured 0.02%. No information is available regarding variability for the presences of the different types of pyrrolizidine alkaloids in the seeds. Thus, the objective of this research was to analyze sunn hemp seeds of populations originated in different parts of the world for several pyrrolizidine alkaloids to determine their level of variability for the presence of these compounds and to quantify the total amount of pyrrolizidine alkaloids in the seeds.

2. Investigations, results and discussion

Of the pyrrolizidine alkaloids junceine, riddelliine, senecionine, seneciphylline and trichodesmine reported to be present in sunn hemp, only junceine and trichodesmine were detected in the accessions studied (Table 1). The additional pyrrolizidine alkaloids heliotrine, retrorsine, inte-



Junceine



Trichodesmine

gerrimine and lasiocarpine were not detected either (Fig). Junceine was detected in all accessions but one, PI 207657, whereas trichodesmine was detected in four accessions. Four of the nine accessions were from the Indian subcontinent from where sunn hemp is thought to have originated (Wiersema et al. 1990). Among those four accessions was PI 207657, the only accession for which junceine or trichodemine were not detected. Accessions PI 219717, PI 250486, PI 391567 and PI 426626 had both junceine and trichodemine. The chromatograms indicated that the amounts of junceine were higher than trichodemine in those accessions except for PI 391567 which had a higher amount of trichodemine.

The limit of detection for HPLC/ELSD is about 40 μg compared to about 1 μg for mass spectrometric detection. Consequently, the samples were run again using an HPLC equipped with mass spectrometric detection. The pyrrolizidine alkaloids junceine and trichodesmine were detected in all the accessions studied whereas riddelliine, senecionine and seneciphylline or any of the other pyrrolizidine alkaloids were not present in any of the sunn hemp accessions.

Content of pyrrolizidine alkaloids in sunn hemp ranged between 0.82 and 3.8 μmol/g whereas seeds from *Crotolaria spectabilis* from the USA, a species known for its toxicity to birds and animals, had 60 μmol/g, whereas PI 1849043, a *C. spectabilis* that originated in Nigeria, had 96 μmol/g. The pyrrolizidine alkaloids content of sunn hemp accessions was only 1.4 to 6.3% of the content in *C. spectabilis* from the USA. Consequently, the amount of pyrrolizidine alkaloids present in the sunn hemp populations studied was deemed to be low what agrees with the reports of Adams and Gianturco (1956) and Williams and Molyneux (1987). Although there was variability for pyrrolizidine alkaloids content in sunn hemp, the results indicate that the values are so small that it is unlikely that ingestion of sunn hemp seed may be toxic to birds or animals. Further research is need to determine if long term ingestion by chicks, the most susceptible animals, will have a negative economic effects such as reduced weight gains.

In summary, seed of sunn hemp populations that originated in several continents only had the pyrrolizidine alkaloids junceine and trichodesmine. PI 207657 had very low levels of both alkaloids and PI 314239, PI 322377, PI 346297, and the US cultivar Tropic Sun had very low levels of trichodesmine. Although juncein was present in higher amounts than trichodesmine in the seeds of most accessions, its value was deemed to be small. The amount of pyrrolizidine alkaloids present in the sunn hemp populations studied was low.

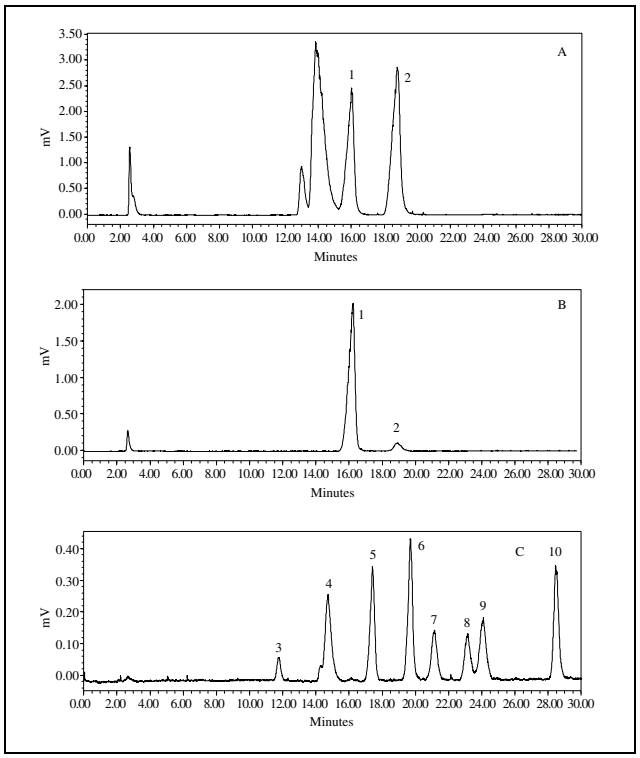


Fig. 1: Typical HPLC-ELSD chromatograms of sunn hemp seed (A), pyrrolizidine alkaloids (B) and (C) (1. junceine; 2. trichodesmine; 3. riddelliine N-oxide; 4. heliotrine; 5. riddelliine; 6. retrorsine; 7. seneciphylline; 8. integerrimine; 9. senecionine; 10. lasiocarpine)

Table 1: Pyrrolizidine alkaloids detected by HPLC using evaporative light scattering detector (ELSD) in sunn hemp seeds of populations from several countries

Accession	Origin	Junceine	Trichodesmine
PI 207657	Sri Lanka	−	−
PI 219717	Myanmar	+	+
PI 250486	India	+	+
PI 314239	Former Soviet Union	+	−
PI 322377	Brazil	+	−
PI 346297	India	+	−
PI 391567	South Africa	+	+
PI 426626	Pakistan	+	+
Tropic Sun	USA	+	−

+ detected − not detected

Table 2: Pyrrolizidine alkaloids content as measured by NMR spectroscopy in *C. juncea* and *C. spectabilis*

Accession	Origin	Moles of alkaloid (μmol/g)	% based on *C. spectabilis* from the USA
PI 207657	Sri Lanka	2.0	3.3
PI 219717	Myanmar	1.7	2.8
PI 250486	India	2.3	3.8
PI 314239	Former Soviet Union	1.7	2.8
PI 322377	Brazil	1.5	2.5
PI 346297	India	1.4	2.3
PI 391567	South Africa	0.8	1.4
PI 426626	Pakistan	3.8	6.3
Tropic Sun	USA	1.6	2.7
C. spectabilis	USA	60	100
PI 189043	Nigeria	96	160

3. Experimental

3.1. Plant material

Sunn hemp populations that originated in Southeast Asia, South America, Africa, and Europe were obtained from the US National Pant Germplasm System. Seed of eight sunn hemp populations from seven countries (Ta-

ble 1) were planted in the greenhouse on March 17, 2003, and transplanted to the field in Tallassee, Alabama, on April 14. Plants were grown until seed maturity. Seed of the cultivar Tropic Sun had to be purchased from Hawaii because the plants cannot produce seed in the continental US.

Showy crotalaria (*C. spectabilis* Roth), which has become naturalized in the Southeast of the USA, is known for its toxicity and high content of pyrrolizidine alkaloids (Williams and Molyneux 1987). It has a high content of monocrotaline. Seeds of *Crotalaria spectabilis* were collected from the wild and used as a point of reference when measuring total pyrrolizidine alkaloids. Furthermore, PI 189043 that originated in Nigeria and tentatively classified as a showy crotalaria was grown in the greenhouse in 2003 until it produced seeds. Those seeds were also included in the analysis when measuring total pyrrolizidine alkaloids.

Seeds from each sunn hemp population were analyzed for the pyrrolizidine alkaloids junceine, riddelliine, senecionine, seneciphylline and trichodesmine which have been reported to be present in sunn hemp (Smith and Culvenor 1981). Furthermore, the samples were evaluated for the presence of pyrrolizidine alkaloids heliotrine, retrorsine, integerrimine and lasiocarpine.

3.2. Extraction

A weighed powdered seeds (about 0.5 g) was extracted with methanol by sonication. After filtration, the filtrate was concentrated. The extract was then partitioned between chloroform and 2 N HCl. Once the two phases were separated the aqueous phase was stirred for 1 h with zinc dust, then basified to pH 9–10 with ammonium hydroxide. The basic aqueous fraction was then extracted with chloroform, and the chloroform removed to give the pyrrolizidine alkaloid extract for analysis. The alkaloid extracts were dissolved in 1 ml methanol and filtered through 45 μm nylon filters (Phenomenex, Torrance, CA) prior to injection.

3.3. HPLC-UV-ELSD analysis

It was used a Waters Liquid Chromatograph Module I with UV detector (Waters, Milford, MA), utilizing an XTerra RP_{18} column (150×4.6 mm; 5 μm particle size) from Waters (Milford, MA) at ambient temperature and a Sedex 55 evaporative light scattering detector (ELSD) by S. E. D. E. R. E. of France and was run at 40 °C and 2.3 bar nitrogen. The mobile phase consisted of water (0.015 M ammonium acetate) (A) and acetonitrile (B). At a flow rate of 0.75 mL/min, the gradient elution was as follows: 95A/5B to 50A/50B in 40 min. After the injection of 10 μL, the data was collected and analyzed by Waters Millennium^{32} software (Milford, MA). Injection volume 50 μL. Identification was made by comparing the sample retention times and the abundant with UV spectra at 210 nm and 254 nm. This method was chosen because of its low cost and high sensitivity to detect pyrrolizidine alkaloids when present above 40 μg (Schaneberg et al. 2004).

3.4. HPLC-UV-MS analysis

The samples were further analyzed using HPLC-UV-MS. Finnigan AQA Mass Spectrometer and Finnigan HPLC with AS3000 autosampler, P4000 pump, and a UV6000LP detector (San Jose, CA) were used. The HPLC conditions were the same as HPLC-UV-ELSD. Nitrogen (60 psi) was used as a nebulizing and drying gas. The conditions of the MS detector was positive ESI mode, with capillary voltage (probe (kV)) 3.0 kV, source voltage (AQAmax) 30 V, and probe temperature 450 °C.

3.5. NMR spectroscopy

Ground seeds from each population were placed in a Soxhlet thimble (45×123 mm) where were extracted with methanol for 20–30 h, and the resulting solution was evaporated to dryness at reduced pressure. The residue was taken up in 2 M HCl, washed with ether to remove non-basic material, and basified with NH3 gas. The aqueous phase was extracted with $CHCl_3$ (3×50 mL), and the combined extracts were dried over $MgSO_4$, filtered, and evaporated to dryness. A weighed quantity of the residue, and a weighed quantity of *p*-dinitrobenzene was dissolved in $CDCl_3$. NMR spectroscopy was performed on a Bruker AV-400 instrument, operating at a proton frequency of 400.18 MHz.

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