

Chapter 3

Crotalaria

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3.1 Basic Botany of the Species

The genus *Crotalaria* belongs to the Fabaceae family and contains approximately 600 species that grow wild in tropical and subtropical areas (Polhill 1981). Sunn hemp (*Crotalaria juncea* L.), the main species in the genus, is grown worldwide in tropical areas and is being bred to grow and produce seeds in temperate climates (Mosjidis 2006). Sunn hemp is probably native to the Indo-Pakistan subcontinent (Wiersema et al. 1990). It belongs to the family Fabaceae, subfamily Papilionoideae, tribe Crotalariaeae, section *Calycinae* (van Wyk and Schutte 1995). *C. juncea* is a diploid species ($2n = 2x = 16$) (Purseglove 1981). The haploid nuclear content of *C. juncea* L. is approximately $1C = 1.23$ pg (Gupta 1976; Bennett and Leitch 1995).

Sunn hemp is an erect herbaceous annual that branches in the upper portion of the stem. Dense plantings reduce branching. Stems are cylindrical with ridges that end on an inflorescence. Deep yellow flowers develop acropetaly on the inflorescence, which is a terminal open raceme. Plants produce an indeterminate number of flowering stems. The number of flowering stems is much influenced by water availability, temperature and daylength. Flowers are visited by a variety of insects and humming birds. Flowers of sunn hemp are cross-pollinated by several bee species. In Brazil, it has been reported to be visited by native

stingless bees *Xylocopa* spp. (Nogueira-Couto et al. 1992; Heard 1999). Bees visit fully open flowers and prefer to collect pollen in the afternoon (Maiti 1997). Fertilization occurs only after the stigmatic surface has been damaged by bees or by mechanical means (Purseglove 1981).

Sunn hemp has as base chromosome number of 8. A karyotype characterization by chromosome banding was recently conducted by Mondin et al. (2007). They found that chromosomes can be identified during metaphase based on small differences in their morphology when Feulgen-stained. There is a secondary constriction only in the largest chromosome. This secondary restriction is the major nucleolus organizer region (NOR) in sunn hemp. However, chromosome 4 may have nucleolar activity in some stages of the cell cycle.

The genus *Crotalaria* has about 600 species distributed mostly in tropical and subtropical areas of the southern hemisphere and Africa (Polhill 1981). Chromosome number of most *Crotalaria* spp. is $2n = 16$ (Polhill 1981), but species in the section *Chrysocalycinae* subsection *Incanae* usually have $2n = 14$ and polyploids ($2n = 32$) have been reported among the American species (Mondin et al. 2007).

Sunn hemp is the fastest growing species of the genus *Crotalaria*. It has multipurpose use as green manure, fiber and animal fodder crop (Purseglove 1981) and has potential as an ornamental plant. Sunn hemp is an important source of fiber for the production of paper and ropes in the Indo-Pakistan subcontinent (White and Haun 1965). *C. spectabilis*, *C. paulina* and *C. mucronata* may have as much biomass production as *C. juncea* (Mes et al. 1957). However, *C. spectabilis* is toxic. Another species that has potential use in Africa is *Crotalaria ochroleuca* Don. known as slender leaf rattlebox (Sarwatt 1990).

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3.2 Conservation Initiatives

Genetic erosion is not a major issue for the cultivated species at this time, but germplasm stored in banks is limited. It is necessary to collect seeds of landraces and ecotypes in the Indo-Pakistan subcontinent and other countries of southeastern Asia to increase the number of accessions presently stored in germplasm banks. Seed longevity seems to be good and many species have hard seeds (Kak et al. 2007). *C. juncea* can have a small percentage (about 10% or less) of hard seed (J Mosjidis unpublished results).

3.3 Roles in Elucidation of Origin and Evolution of Sunn Hemp

The tribe Crotalariaeae has two genera, *Crotalaria* and *Lotononis*, which include species that currently have agronomic use. These two genera also form a group characterized by containing pyrrolizidine alkaloids (van Wyk and Verdoorn 1990). Pyrrolizidine alkaloids are N-containing compounds derived from ornithine, an intermediate in arginine biosynthesis.

It has been reported that sunn hemp seeds contain various pyrrolizidine alkaloids such as junceine, rid-delliine, senecionine, seneciphylline and trichodesmine (Smith and Culvenor 1981). However, Ji et al. (2005) determined that trichodesmine and junceine (Fig. 3.1) were the only pyrrolizidine alkaloids present in the seeds of nine *C. juncea* populations that originated in different parts of the world. The amounts of pyrrolizidine alkaloids reported by Ji et al. (2005) were small that agrees with other reports (Adams and

Gianturco 1956; Pilbeam and Bell 1979b; Williams and Molyneux 1987). This was confirmed again by Nurhayati and Ober (2005) who did not detect alkaloids in cotyledons, leaves, flowers or roots of *C. juncea*, which suggests that alkaloid production is limited to the seeds.

A study of free amino acids in *Crotalaria* seeds indicated a high correspondence between free amino acid distribution and taxonomic relationships in the genus (Pilbeam and Bell 1979b). *C. juncea* had an amino acid pattern different from all other *Crotalaria* species. It had low levels of pyrrolizidine alkaloids and a high content of δ -hydroxynorleucine in the seeds, up to 2% of seed weight (Pilbeam and Bell 1979a), whereas species in the section *Crotalaria* sub-section *Crotalaria* contain γ -glutamyltyrosine. Species in the section *Calcynae* also lack γ -glutamyltyrosine. Thus, *C. juncea* seems to be intermediate between sections *Calcynae* and *Crotalaria* (Pilbeam and Bell 1979b). Some *Crotalaria* species have the toxic amino acids α -amino- β -oxylaminopropionic acid, α -amino- γ -oxylaminobutyric acid and/or α,γ -diaminobutyric acid in the seeds (Pilbeam and Bell 1979b).

Another compound reported to be in sunn hemp seeds is cardenolide cardiogenin 3-*O*-beta-D-xylopyranoside (Yadav and Thakur 1994), but its significance has not been assessed. Hamana et al. (1996) determined that *C. juncea* and *C. spectabilis* seedlings had a high content of polyamides. Mangotra and Bhargava (1987) used carbohydrates present in the plants to identify taxonomic relationships, but they found them to be of limited taxonomic use in the genus *Crotalaria*. However, Anuradha et al. (1989) determined that the presence of a number of secondary metabolites in several *Crotalaria* spp. can be used to identify taxonomic relationships within the genus.

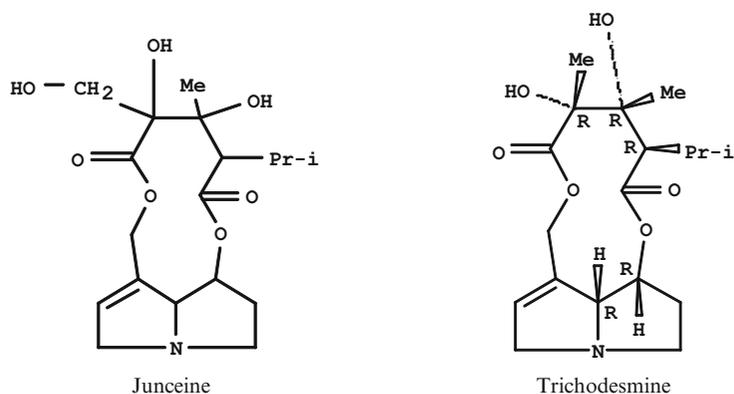


Fig. 3.1 Pyrrolizidine alkaloids junceine and trichodesmine

In most angiosperms, mitochondrial and chloroplast genomes are inherited maternally. Variation detected from mitochondrial DNA (mtDNA) and chloroplast DNA (cpDNA) can be used for population genetic studies and phylogenetic analysis at both intra- and interspecific levels (Netwon et al. 1999; Desplanque et al. 2000). Recently, Boatwright et al. (2008) used nucleotide sequences from the internal transcribed spacer (ITS) of nuclear ribosomal DNA (the plastid gene *rbcL*) and morphological data to study phylogenetic relationships in the tribe Crotalariae. They determined that this tribe is monophyletic and is sister to the tribe Genisteae. As far as we know, there are no DNA markers developed from mitochondrial genome of *Crotalaria* that can be used for population and phylogenetic studies within the genus *Crotalaria*.

3.4 Roles in Genetic Studies and Development of Cytogenetic Stocks

Lack of genomic resources such as DNA markers, large insert-size clones (e.g., bacterial artificial chromosomes) and known-function genes in *Crotalaria* has hindered advanced genetic studies, such as genetic and physical mapping, in the genus.

Most species from the genus *Crotalaria* are diploids with 16 chromosomes ($2n = 2x = 16$). However, a diploid with 14 chromosomes ($2n = 2x = 14$, for example, *C. incana*) and tetraploids with 32 chromosomes ($2n = 4x = 32$, for example, *C. paulina* and *C. stipularia*) were also found within *Crotalaria* (Oliveira and Aguiar-Perecin 1999). Karyotyping by C-banding revealed that two secondary constrictions (NOR-heterochromatin) were on the distal region (major nucleolus organizer regions) of chromosome 1 and the proximal region of chromosome 4. The secondary constriction regions on the chromosomes were also confirmed using 45S rDNA as probed by fluorescent in situ hybridization (FISH) analysis (Mondin et al. 2007). Identification of the variation of basic chromosome number among species within *Crotalaria* may help to understand and explain the process of evolution and divergence for speciation.

Classical genetic studies are almost non-existent. Miranda et al. (1989) determined that anthocyanin pig-

mentation in flowers and hypocotyl of *C. juncea* was due to a pair of alleles. The allele in the dominant form (*A*–) was required for color presence and the recessive (*aa*) for absence. However, no genetic work has been accomplished in *C. juncea* using its wild allies.

3.5 Genomics Resources

As most *Crotalaria* species are not major crops, there have been no public efforts on sequencing any species within the genus. Therefore, there is very limited sequence information available. By searching NCBI database, only 152 entries are available related to *Crotalaria* and over one-third of the sequences have a high homology either to ribosomal or to chloroplast DNA. Nucleotide sequences of cpDNA (*rbcL*) and ncDNA (ITS 1 and 2) of rDNA have been used for phylogenetic analysis within the family Leguminosae. It was found that Crotalariae shares the same ancestry with Genisteae, Thermopsidae/Podylyriace (Käss and Wink 1997).

Seeds from *Crotalaria* contain large amounts of pyrrolizidine alkaloid (PA), which are chemicals related to plant defense. Deoxyhypusine synthase (DHS) is one of the specific enzymes involved in PA biosynthesis. Two DHS genes (*DHS1* and *DHS2*) have been identified and cloned in *C. juncea*. Genes for DHS and pseudogenes with homology to the DHS-coding genes were also identified and sequenced in other *Crotalaria* species including *C. anagyroides*, *C. retusa* and *C. scassellatii* (Nurhayati and Ober 2005). There were 12 sequence entries generated through sequencing cDNA and genomic DNA of DHS genes.

From a phylogenetic point of view, some legumes are more closely related to *Crotalaria* than others. In one study (Choi et al. 2006), 274 unique gene sequences were selected from six model (or cultivated) legumes and tested on 94 legume species (including *Crotalaria cunninghamii* R. Br.). From this study, six sequences from *C. cunninghamii* have been deposited into Genbank. The successful rate of amplification across the legume family is definitely related to the phylogenetic distance with the model or crop legumes from which the primers were derived. Limited sequence resources within the *Crotalaria* genus are greatly hindering the genetic research within the genus. Although DNA markers are not available



Fig. 3.2 Selection for adaptation to humid temperate climate of the Southeast USA. In the *foreground* are the selections adapted and able to produce seed in temperate regions. In the

background is Tropic Sun, a tropical cultivar unable to produce seed in the Southeast USA

for *Crotalaria*, they can be developed by a transferable approach (i.e., testing whether DNA markers developed from other related species will work on species within the *Crotalaria* genus). Fifty-eight primer sets from *Medicago* and soybean were tested on *Crotalaria* species and 28 (48%) generated polymorphic amplicons. Using these transferred DNA markers, the genetic diversity of *Crotalaria* from four species was assessed, their phylogenetic relationships were analyzed and even some misclassified accessions were identified and renamed (Wang et al. 2006). For genetic studies of *Crotalaria* species, new genomic and genetics resources need to be developed by sequencing and mutagenesis (Fig. 3.2).

3.6 Some Dark Sides and Their Addressing

Pyrrolizidine alkaloids ingested in sufficient amount can be toxic to animals and birds. Various animal species have different susceptibility to pyrrolizidine alkaloids. Susceptibility was determined to be highest

for pigs, followed by chickens, horses and cattle, rats, mice, sheep and the least susceptible were goats (Hooper 1978). Many *Crotalaria* species have been found to be toxic to animals particularly because of high pyrrolizidine alkaloid content. In fact, Strickland et al. (1987) determined that about 50% of the *Crotalaria* species they tested were toxic. This information has been indiscriminately extended to *C. juncea*, but has not been substantiated. Exceptions are the reports of Ritchey et al. (1941) and Nobre et al. (1994). Ritchey et al. (1941) found that feeding a high dose of seeds to sheep for 26 days was toxic to them. Nobre et al. (1994) reported that feeding a diet that included 40% *C. juncea* seeds to horses for 30 days caused their death. The recent study of Hess and Mosjidis (2008) where *C. juncea* whole or ground seeds were included in the diet of broiler chicken (highly sensitive to pyrrolizidine alkaloids) showed that the seeds did not affect bird mortality. However, when seeds were fed at a feed ingredient level (2–5% of the diet) it reduced body weight and feed consumption. Inclusion at a contaminant level (0.5%) had no negative impact on the birds (Hess and Mosjidis 2008). A rat bioassay conducted by Strickland et al. (1987) indicated that neither leaves, or

Table 3.1 Seed toxicity of *Crotalaria* species introduced to the USA

Species	Common Name	Acute toxicity	Reference
<i>C. juncea</i>	Sunn hemp	No	Purseglove (1981), Rotar and Joy (1983), Williams and Molyneux (1987), Strickland et al. (1987), Hess and Mosjidis (2008)
<i>C. pallida</i>	Smooth crotalaria	No	Williams and Molyneux (1987)
<i>C. retusa</i>	Wedge-leaf rattle box	Yes	Hooper and Scanlan (1977), Williams and Molyneux (1987)
<i>C. spectabilis</i>	Showy crotalaria	Yes	Johnson et al. (1985), Williams and Molyneux (1987)

stems or seeds of *C. juncea* were toxic; however, other *Crotalaria* species were toxic. Showy crotalaria (*C. spectabilis*), wedge-leaf rattle box (*C. retusa*) and smooth crotalaria (*C. pallida*) were introduced into the USA as green manure crops to improve soil fertility (Williams and Molyneux 1987).

There are two types of evidence indicating that *C. juncea* seeds do not cause acute toxicity as reported for other *Crotalaria* species (Asres et al. 2004) and particularly some species introduced to the USA such as *C. spectabilis* (Williams and Molyneux 1987) (Table 3.1). The first set of evidence comes from the reports that found low pyrrolizidine alkaloids levels or lack of them in seeds and other plant parts in a large number of populations developed in several countries (Adams and Gianturco 1956; Pilbeam and Bell 1979b; Strickland et al. 1987; Williams and Molyneux 1987; Ji et al. 2005; Nurhayati and Ober 2005). Second, toxicological studies conducted with animals (Purseglove 1981; Rotar and Joy 1983), particularly chicken that are very susceptible to pyrrolizidine alkaloids, demonstrated lack of acute toxic effects (Williams and Molyneux 1987; Hess and Mosjidis 2008). However, the results of Hess and Mosjidis (2008) and the reports of Ritchey et al. (1941) and Nobre et al. (1994) point out that seeds should not be considered foodstuff and they should not be included in large amounts and for long periods of time in an animal diet.

3.7 Recommendations for Future Actions

Most *Crotalaria* species are weedy, but a few have been used as a crop with agricultural, economic and pharmaceutical importance. They can be used as cover crops for providing green manure and preventing soil erosion, as rotation crops for nitrogen fixation, as forage crops for grazing, as a source of long fiber to produce high-quality paper, as intercepting crops for

nematode control, and as medicinal plants for chemical compound extraction. The following aspects are recommended for future actions.

1. *Expanding the germplasm stored in banks:* *Crotalaria* species are distributed worldwide but their germplasm has not been well collected. For example, there are approximately 600 species, but there are only 242 accessions in the USDA collection representing about 30 species. More accessions need to be curated from different regions of the world.
2. *Generation of genomic resources:* There are very limited genomic resources (only 152 sequence entries in NCBI). As sequencing technology advances and sequence cost drops sharply, low-pass genomic sequencing (even with 2–3x genome coverage) should be initiated within *Crotalaria* species. Low-pass genomic sequencing will provide not only the genome feature of *Crotalaria* but also a plenty of DNA markers for genetic studies.
3. *Generation of genetic resources:* Application-oriented hybridization (crossing within and among species) and mutagenesis (irradiation or EMS-induced mutation) should be conducted in *Crotalaria*. New populations from hybridization and mutation populations will not only help to develop new cultivars and breeding materials but will also provide materials for genetic studies.
4. *Breeding for photoperiod insensitivity:* Photoperiod sensitivity is a major limitation for growing *C. juncea* in diverse environments. Sowing the plants at a later time in the season has the potential to increase the use of this plant as a green manure crop. Lack of sensitivity to photoperiod would allow plants to grow taller and produce more biomass when planted in late summer.
5. *Biochemical and biological analysis:* Some biochemical compounds are unique in *Crotalaria*. To exploit their utilization, these compounds need to be extracted, identified and characterized biochemically (such as using HPLC, GC, GC-mass

spectrometry, LC-mass spectrometry). Metabolomics could fill the gap in our knowledge of chemicals present in *Crotalaria*. On the basis of the compound property, bioassays need to be designed and to test biological activity, for example, elucidation of the mechanism of nematode resistance using characterized compounds from *Crotalaria* by bioassay.

6. *Further exploiting of its utilization: Crotalaria* are under-exploited species and many of its utilizations have not been well mined. Some species such as *C. medicaginea* have been reported to have mucilaginous polysaccharides in the seeds (Gupta and BeMiller 1990) that have a variety of industrial and medicinal applications. *C. ochroleuca* has the potential to be used as a forage plant (Sarwatt 1990). *C. juncea* has a rapid growth (120 days), large biomass production of the tropical types (8,900–13,000 kg ha⁻¹) and efficient nitrogen accumulation (135–285 kg ha⁻¹); sunn hemp (*C. juncea*) has the potential to be widely used as cover crop in the USA southeastern regions (Schomberg et al. 2007). Furthermore, its potential to be used as a feedstock for bioethanol production has not been explored yet. Because of its large biomass production, sunn hemp may need to be evaluated as one of the feedstock candidates for cellulosic ethanol production, particularly in tropical areas.

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Wild Crop Relatives: Genomic and Breeding Resources

Industrial Crops

Kole, C. (Ed.)

2011, XXII, 183 p., Hardcover

ISBN: 978-3-642-21101-0