GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Comparative genetic analysis of trichome-less and normal pod genotypes of *Mucuna pruriens* (Fabaceae)

S.S. Dhawan[1], G.K. Rai[1], M.P. Darokar[2], R.K. Lal[3], H.O. Misra[3] and S.P.S. Khanuja[4]

[1]Biotechnology Division, Central Institute of Medicinal and Aromatic Plants, Council of Scientific and Industrial Research, Lucknow, India
[2]Molecular Bioprospection Division,
Central Institute of Medicinal and Aromatic Plants, Lucknow, India
[3]Plant Breeding and Genetics,
Central Institute of Medicinal and Aromatic Plants, Lucknow, India
[4]NutraHelix Biotech Pvt. Ltd., Mehrauli, New Delhi, India

Corresponding author: S.S. Dhawan
E-mail: sunita.dhawan@cimap.res.in / sunsdhawan@gmail.com

Genet. Mol. Res. 10 (3): 2049-2056 (2011)
Received January 14, 2011
Accepted May 6, 2011
Published September 15, 2011
DOI http://dx.doi.org/10.4238/vol10-3gmr1264

ABSTRACT. Velvet bean (*Mucuna pruriens*) seeds contain the catecholic amino acid L-DoPA (L-3,4-dihydroxyphenylalanine), which is a neurotransmitter precursor and used for the treatment of Parkinson's disease and mental disorders. The great demand for L-DoPA is largely met by the pharmaceutical industry through extraction of the compound from wild populations of this plant; commercial exploitation of this compound is hampered because of its limited availability. The trichomes present on the pods can cause severe itching, blisters and dermatitis, discouraging cultivation. We screened genetic stocks of velvet bean for the trichome-less trait, along with high seed yield and L-DoPA content. The highest yielding trichome-less elite strain was selected and indentified on the basis of a PCR-based DNA fingerprinting method

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(RAPD), using deca-nucleotide primers. A genetic similarity index matrix was obtained through multivariant analysis using Nei and Li's coefficient. The similarity coefficients were used to generate a tree for cluster analysis using the UPGMA method. Analysis of amplification spectra of 408 bands obtained with 56 primers allowed us to distinguish a trichome-less elite strain of *M. pruriens*.

Key words: *Mucuna pruriens*; Velvet bean; L-DoPA; RAPD profiling; Genetic divergence; Molecular polymorphism

INTRODUCTION

Mucuna pruriens L., also known as Kewanch or Velvet bean or the Cowhage, is a member of the family Fabaceae (Leguminosae) consisting of different species of climbing vines and shrubs, which are commonly termed as legumes, and found worldwide in tropical areas. The genus *Mucuna* covers approximately 100 species of annual and perennial legumes, including the Velvet bean (Buckles, 1995). Velvet bean is basically a soil improving crop that is also used as a cover crop in citrus, peach, etc. *M. pruriens* has been used as a green manure and weed control crop and also as a rotation crop with corn, cotton and sugarcane (Duggar, 1899; Bailey, 1907; Bort, 1909). The *Mucuna* plant is a self-pollinated, climbing shrub with long vines (Duke, 1981). Sastrapradja et al. (1974) showed the occurrence of N = 11 in *M. pruriens*. Pods are covered with loose hairs, which can cause a severe itching sensation. The trichomes present on the pods contain mucunain and serotonin and because of their presence, pods cause itching, blisters and dermatitis (Sastry and Kavathekar, 1990). Lubis et al. (1979, 1980) suggested that multigenic factors were involved in the production of seed colors. They reported that two genes (R and N) were responsible for determining the characteristics of pod hairs. According to Ayurveda, *Mucuna* seeds are also used as an astringent, laxative, anthelmintic, aphrodisiac, alexipharmic, and tonic. The root is used as a remedy in facial paralysis and nervous disorders. Decoction of the roots purifies the blood, cures rheumatism, asthma, cough, stone in the bladder, and improves vitality. It is also used for fevers, edema and elephantiasis.

Mucuna pruriens seeds contain the amino acid L-DoPA (L-3,4-dihydroxyphenylalanine), glutathione, lecithin, gallic acid, glycosides, nicotine, prurenine, prurenidine, and a dark brown viscous oil. The occurrence of the catecholic amino acid 3-(3,4-dihydroxyphenyl)-L-alanine (L-DoPA) in this plant has attracted attention for use in L-DoPA production (Bell et al., 1971; Daxenbichler et al., 1972). L-DoPA is a neurotransmitter precursor, which is used for the treatment of Parkinson's disease and mental disorders. However, the high demand for L-DoPA is largely met by the pharmaceutical industry through extraction of the compound from wild populations, but commercial exploitation of this compound is hampered because of its limited availability. All parts of *Mucuna* possess valuable medicinal properties (Caius, 1989), and therefore *Mucuna* is in heavy demand by industry. After the discovery that *Mucuna* seeds contain L-DoPA, its demand in international markets has increased many fold and motivated Indian farmers to start its commercial cultivation. Conventional methods of propagation of this plant are limited to seeds, which creates problems because the trichomes present on the pods cause

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uncontrolled itching. Therefore, improvement is required in terms of higher yields of L-DoPA content as well as trichome-less pods. In the present study, we have applied a DNA fingerprinting technique, random amplified polymorphic DNA (RAPD), to differentiate the three accessions of *Mucuna* as well as for genetic characterization of the selected accession, which is a high yielder of L-DoPA and has a very specific feature of absence of hairs on the pods.

MATERIAL AND METHODS

Plant material

Genetic stocks of *Mucuna* were screened primarily for trichome-less and high L-DoPA content in seeds. Thirty-five genotypes were evaluated for the selection of elite accessions for L-DoPA content, presence or absence of trichomes on pods and high yield of seeds (Table 1). Genotype selection based on single seed of the highest weight separated up to S_4 generation followed by the best L-DoPA content. By applying this single seed descent breeding method, three promising genotypes were selected and evaluated under large scale trials (Plot size: 100 m²) along with check. Young leaves were collected from fully grown vines for DNA isolation.

Isolation of DNA from leaf samples

DNA was extracted from leaf tissues by homogenization of samples using a pestle grinder followed by the protocol described by Khanuja et al. (1999). For large scale DNA isolation, approximately 1 g (1000 mg) young leaves was taken, ground to fine powder in liquid nitrogen and transferred to a centrifuge tube (10 mL). Then, 4 mL extraction buffer along with 1% PVP and 0.5% β-mercaptoethanol were added in each sample and incubated for 1 h at 60°C with occasional swirling. Later, the mixture was extracted with an equal volume of chloroform/isoamyl alcohol (24:1) and centrifuged at 10,000 rpm for 10 min at room temperature. The upper aqueous layer was carefully transferred to the fresh tube containing 1.5 mL 5 M NaCl and mixed gently. Then, 0.6 volume isopropyl alcohol was added and kept for 30 min at room temperature. DNA was either scooped or the mixture was centrifuged at 12,000 rpm for 10 min at 30°C. The pellet was dissolved in high salt TE (500 µL), transferred to a microcentrifuge tube and extracted again with 500 µL chloroform isoamyl alcohol. To the upper aqueous phase, 1 mL absolute alcohol was added to precipitate the DNA. DNA precipitate was pelleted down by centrifugation at 10,000 rpm at room temperature for 10 min and subsequently the pellet was washed with 70% ethanol (1 mL). The pellet so obtained was dried under vacuum and dissolved in 200 µL sterile double-distilled water. The isolated DNA samples were quantified by measuring absorbance at 260 nm and visualized by agarose gel electrophoresis along with a molecular weight marker.

RAPD-PCR amplification

The polymerase chain reaction (PCR) was carried out in a 25-µL volume and the re-

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action mixture in each tube contained 0.2 U Taq polymerase (Banglore Genei), 100 µM each of dNTPs, 1.5 mM $MgCl_2$ and 5 pmol decanucleotide primers (Operon Biotechnologies Inc.). The amplification was carried out in a thermal cycler (DNA Engine PTC 200, MJ Research, USA) programmed for one cycle at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 37°C for 1 min, an extension at 72°C for 2 min, and a final extension step at 72°C for 5 min, all with a minimum ramp time. The amplified products were separated on 1.2% agarose gel containing ethidium bromide for 2.5 h at 50 V. A negative control was included in all the reactions, which consist of all the reaction components except template DNA. The gels were observed on a UV transilluminator and digital photographs were taken using a gel documentation system (Pharmacia Biotech). A total of 56 decanucleotide primers were employed in this study, which had two series of Operon decanucleotide primers, viz. OPA (1-20) and OPB (1-20), from Operon Biotechnologies Inc. (USA), and the other primers (MAP 1-20) were designed at CIMAP and synthesized from Banglore Genei (Khanuja et al., 2000).

Statistical analysis

Statistical analysis was performed for the calculation of genetic similarity according to the formula developed by Nei and Li (1979). The similarity of fragment profiles produced by the primers for each individual genotype was compared with that of every other individual in a pairwise fashion and the similarity index (SI) between each pair was calculated according to the formula SI = 2NAB / (NA + NB) developed by Nei and Li, where NAB is the number of shared amplified fragments for specimen A and B, and NA and NB are the total number of amplified fragments for specimen A and B, respectively.

RESULTS AND DISCUSSION

Various accessions (35) of *M. pruriens* were evaluated in the field for different characteristics (Table 1). Of these, three accessions (M-4, M-5, and M-37) were selected on the basis of the highest L-DoPA content. Two of these selections (M-4, M-37) were trichome-less, while the third accession (selection M-5) had trichomes on the pods. Interestingly, selection M-37 was observed with one more peculiar characteristic, i.e., seed color; the seeds were dull white with a black tinge besides early maturity. Figure 1 shows the characteristics of *M. pruriens*.

As evident from RAPD profiles, the three accessions selected (M-4, M-5, and M-37) of *Mucuna* could be distinguished at the molecular level. The comparative analysis of all accessions was established on the basis of RAPD profiles generated by using decanucleotide primer kits of MAP, OPA and OPB. Figure 2 shows the characteristic profile of the selected accession M-37 of *M. pruriens* with MAP primers (02, 03, 04, 06, 08, 09, 11, 12, 13, 14, 15, 16), whereas Figure 3 clearly depicts the genetic differentiation of selected accession M-37 using MAP primers 06, 08, 15, 16, 18, and 19. The data scored on RAPD polymorphism and band sharing were aimed at analyzing the genetic similarity matrix obtained through multivariate analysis using Nei and Li's coefficient. The similarity coefficients were used to generate a tree for cluster analysis using the UPGMA method as evident from the phenogram (Figure 4).

Table 1. Comparative details of the presence or absence of trichomes on pods, seed color and L-DoPA content of *Mucuna pruriens* accessions.

S. No.	Accession number	Trichome on pods (presence/absence)	Seed color	L-DoPA content in seeds
1	M-1	Absent	White	4.51
2	M-2	Absent	Dark black	5.50
3	M-3	Absent	Black	5.52
4	M-4	Absent	Black tinged	6.13
5	M-5	Present	Light black	6.63
6	M-6	Absent	Brown	5.52
7	M-9	Absent	White	3.02
8	M-10	Absent	White with black tinged	4.13
9	M-11	Absent	White with black tinged	3.93
10	M-12	Absent	White	1.71
11	M-13	Absent	White with black tinged	5.03
12	M-14	Absent	Black	3.26
13	M-15	Absent	White with black tinged	2.46
14	M-16	Present	Dark black	2.70
15	M-17	Absent	White	2.60
16	M-19	Absent	Black	3.41
17	M-20	Absent	White with black tinged	4.18
18	M-21	Absent	Brown	2.89
19	M-22	Absent	White with black tinged	1.91
20	M-23	Absent	Black	4.11
21	M-24	Absent	White with black tinged	1.88
22	M-25	Present	White	3.48
23	M-26	Absent	Brown	4.74
24	M-27	Absent	Dark black	5.23
25	M-28	Absent	White	2.23
26	M-29	Absent	Black	3.98
27	M-30	Absent	White	1.58
28	M-31	Present	Black	1.41
29	M-32	Absent	White	4.88
30	M-33	Absent	Dark black	5.50
31	M-34	Absent	White	5.65
32	M-36	Absent	Brown	4.42
33	M-37	Absent	Dull white with black tinged	6.18
34	M-38	Absent	Brown	3.92
35	M-39	Absent	White with black tinged	4.42

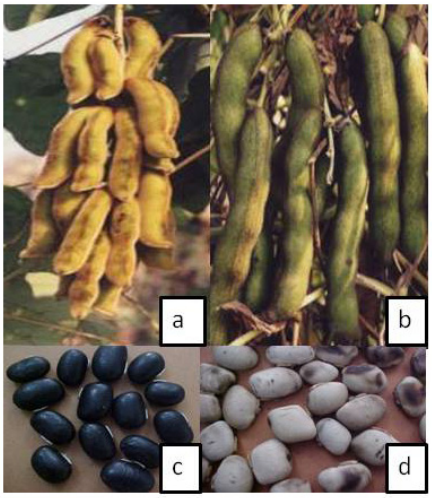

Figure 1. Characteristics of *Mucuna pruriens*. **a.** Pods with trichomes of accession number M-4. **b.** Trichome-less pods of selected accession M-37. **c.** Seeds of accession M-4. **d.** Seeds of selected accession M-37.

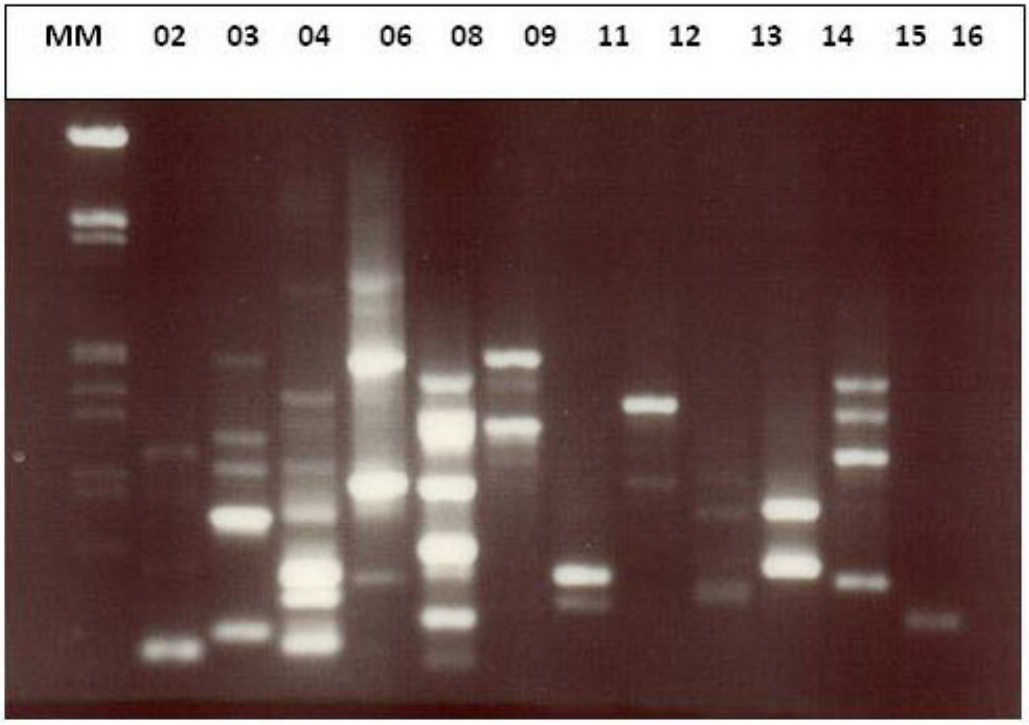

Figure 2. Characteristic RAPD profile of *Mucuna pruriens* accession M-37 with MAP primers. MM = molecular weight marker.

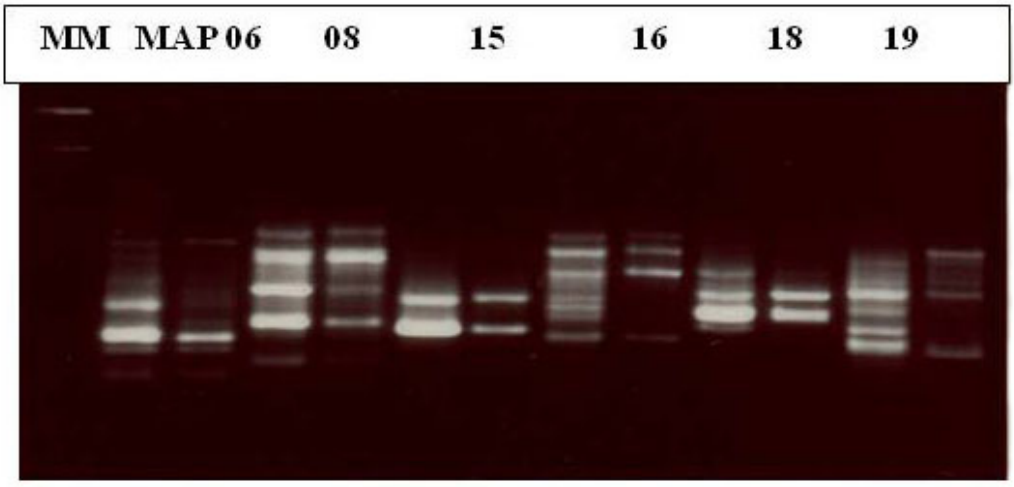

Figure 3. Differentiating RAPD profile of selected accession M-37 and M-4 with MAP primers. MM = molecular weight marker.

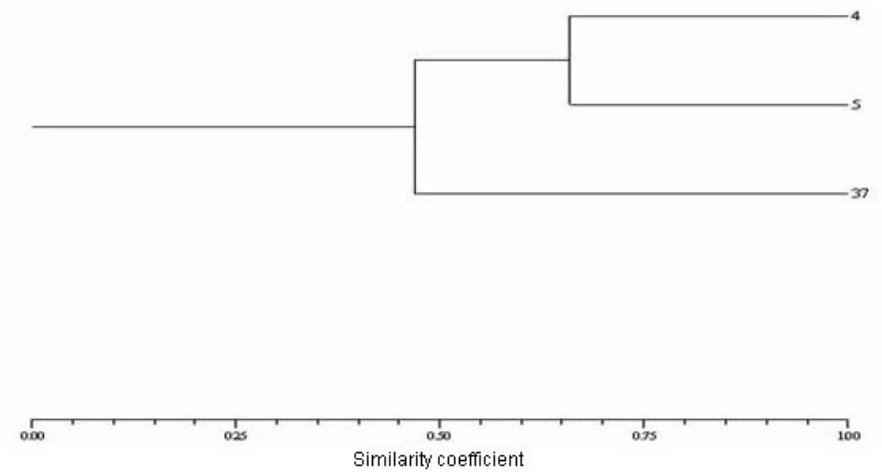

Figure 4. Dendrogram showing distinct identity of selected accession M-37 of *Mucuna pruriens* compared to other accessions.

A total of 408 bands were generated by using three primer sets. The primer number MAP 08, 17 and OPA 04 produced the highest number of bands (11 each), whereas primers OPB 04, 08 and 14 scored the lowest number of bands (4 each). Primers MAP 03, OPB 01, 12, and 19 did not respond. As revealed by the band matching analysis of RAPD pattern, primers MAP 01, 02, 18, OPA 03, 04, 16, and OPB 09, 17, 20, upon amplification, showed up to 100% polymorphism, while MAP 08, 17 and OPA 08 produced about 90% polymorphic bands. As evident from the phenogram, accession M-37 was distinctly separated from other selections, M-4 and M-5, which is in accordance with morphological characters such as trichome-less pods and dull white color with black tinged seeds, M-4 and M-5 were grouped in the first cluster and accession number M-37 forms a separate group. In addition, M-37 was also observed to be a fast growing climber with light greenish stem and pods with early maturity compared to other accessions (Table 2).

Table 2. Growth attributes of three selected accessions of *Mucuna pruriens*.

S. No.	Accession number	Growth in nature	Days of flowering (50%)	Stem color	Pod color	Maturity
1	M-4	Medium growing climber	90	Green	Green	130-140
2	M-5	Slow growing climber	100	Yellow	Yellow	160-170
3	M-37	Fast growing climber	90	Green	Green	130-140

Multilocus RAPD fingerprinting (RAPD-PCR) is widely used in analyzing population and genetic relationships between and among species, establishing taxonomic status and evolutionary studies. In addition, RAPD-PCR can be used for studying genotype fusion at various levels, including those of individuals (parentage tests) and populations (identification of hybrid populations, species and subspecies) (Hadrys et al., 1992). Khanuja et al. (1998) used this profiling technique for isolating somaclone of altered genotypes in *Mentha arvensis*. RAPD markers have proved to be highly efficient for studying the consequences of hybridization, especially when other markers (e.g., allozymes or microsatellites) are not sensitive enough to identify hybrid genotypes. Saikia et al. (2000) assessed the diversity among *Taxus wallichiana* accessions collected from northeast India using RAPD analysis. Capo-Chichi et

al. (2001) reported genetic variability within the cultivated species of *Mucuna* by using AFLP (amplified fragment length polymorphism) and classified different taxa on the basis of level of variation. Shasany et al. (2005) used RAPD and AFLP markers to identify inter- and intra-specific hybrids in *Mentha*.

In summary, the analysis of amplification spectra of 408 bands obtained by 56 primers clearly demonstrated the differentiation of selected accessions with trichome-less pods and established the effectiveness of this molecular tool for establishing the distinct identity of selected accessions of *Mucuna pruriens*.

ACKNOWLEDGMENTS

We gratefully acknowledge the support and facilities provided by the Director of CIMAP and financial assistance from the Council of Scientific and Industrial Research, India.

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