

4b.	Validation of L-Dopa extraction method: TLC analysis of isolated sample	<p>all the 10 trails were subjected to standard deviation and standard error for determining the accuracy and precision. The SD value of 0.0176 and SE value of 0.004 between the mean values indicated equipment is performing with good precision and accuracy.</p> <p>To determine the purity of samples isolated from Daxenbichler (1971) method, five randomly selected samples were subjected to TLC analysis with L-Dopa from sigma aldrich chemicals used as standard. In all the test samples single clear band matching in colour and Rf with standard was obtained confirming the purity of isolated compound.</p>
4c.	Analyzing the seeds processed with different processing methods	<p>Seeds collected through random sampling from 10 different accessions differing in L-Dopa content were subjected to four processing methods such as: soaking, roasting, autoclaving and repeated boiling under two conditions: defatted and undefatted, using protocols described Janardhanan et al. (2003)(Annexure-1). All the experiments were repeated thrice.</p> <p>Despite substantial change in the levels of L-Dopa, the ranking of the genotypes remained same in all the 8 processing methods (Table-2).</p> <p>Thus, the results from 4a, 4b and 4c confirmed beyond ambiguity the reliability of the experimental platform developed for L-Dopa analysis using Daxenbichler (1971) method in our lab. We propose to utilize the same for screening germplasm for L-Dopa content in future.</p>
5.	Standardization of taxonomic studies using ITS typing	<p>Standardization of the method has been tried out initially using 10 accessions (5 belonging to different species + 5 belonging <i>Mucuna pruriens</i>). ITS region was successfully amplified in four different primer combinations. Though all of them gave good amplification, combination of ITS3 & 4 gave consistent results in all the three trails without any spurious bands. This region was eluted and sequenced for further analysis. Work is in progress.</p> <p>The detailed method used for ITS amplification is given in Annexure-2</p>
6.	Standardization of AFLP for diversity analysis among on hand accessions	<p>Repeatability of the protocol developed by Capo-Chichi et al. (2001) has been tried out. The method is found to be reproducible in our lab with minor modifications. Detailed AFLP analysis is planned for the ensuing quarter.</p>
7.	Budget Expenditures	<p>Financial statement enclosed as Annexure – 3.</p>

TABLE-1: RESULTS OF PRELIMINARY SCREENING OF L-DOPA CONTENT IN 26 ACCESSIONS ANALYZED USING DAXENBICHLER (1971) SPECTROPHOTOMETRIC METHOD FOR SHORTLISTING ACCESSIONS FOR PLANTING

SL. NO	ACCESSIONS	PERCENTAGE L-DOPA ^Y
1.	500153AP	3.02±0.018^a
2.	500104KL	2.86±0.01 ^b
3.	500113MH	2.76±0.00 ^b
4.	500149AP	2.58±0.205^c
5.	IC265577	2.51±0.040 ^{cd}
6.	500147Ap	2.40±0.017 ^{de}
7.	500123KL	2.38±0.035 ^e
8.	500109KA	2.32±0.009 ^{ef}
9.	500148AP	2.31±0.012 ^{ef}
10.	500162TN	2.23±0.020 ^{fg}
11.	500112KA	2.16±0.019 ^{gh}
12.	500150AP	2.15±0.015^{gh}
13.	500124KL	2.15±0.017 ^{gh}
14.	500159TN	2.15±0.012 ^{gh}
15.	500144AP	2.11±0.025 ^{hi}
16.	500110KA	2.10±0.018 ^{hi}
17.	500108KA	2.10±0.011 ^{hi}
18.	500120KA	2.01±0.015 ^{ij}
19.	500161TN	2.01±0.005 ^{ij}
20.	500119TN	1.94±0.014 ^k
21.	500102KA	1.93±0.009 ^k
22.	500152AP	1.92±0.010 ^k
23.	500155Ap	1.88±0.017 ^k
24.	500101KA	1.67±0.014^l
25.	IC385841	1.54±0.017^m
26	500103AN	1.47±0.005 ^m

^YValues are mean ± standard deviation of 4 independent experiments. Means followed by same letter are not significantly different at 1% significance level as determined by Tukey's HSD test.

Note: Accessions highlighted in yellow are the ones selected for plantation

TABLE 2: ESTIMATION OF L-DOPA CONTENT(%) IN DIFFERENT PROCESSED SEEDS BY DAXENBICHEL (1971) SPECTROPHOTOMETRIC METHOD

SL. NO.	NAME OF THE ACCESSIONS	CONTROL	PROCESSING METHODS							
			REPEATED BOILING		AUTOCLAVING		SOAKING		ROASTING	
			UDSM*	DSM**	UDSM	DSM	UDSM	DSM	UDMS	DSM
1	500153 AP	3.02±0.018	1.80±0.014	1.69±0.00	2.40±0.012	2.3±0.012	2.18±0.005	2.03±0.016	2.31±0.018	1.94±0.014
2	500149 AP	2.58±0.205	1.45±0.01	1.40±0.020	2.00±0.010	1.95±0.021	2.10±0.012	1.93±0.010	2.24±0.0	1.70±0.01
3	500123 KL	2.38±0.035	1.43±0.012	1.35±0.010	1.93±0.021	1.89±0.005	2.06±0.010	1.75±0.020	2.19±0.008	1.64±0.014
4	500162 TN	2.23±0.020	1.36±0.014	1.24±0.010	1.78±0.005	1.69±0.010	1.93±0.012	1.77±0.010	2.01±0.010	1.55±0.016
5	500159 TN	2.15±0.017	1.15±0.00	1.08±0.008	1.42±0.012	1.36±0.024	1.93±0.012	1.42±0.0	1.94±0.012	1.48±0.01
6	500120 KA	2.01±0.015	1.13±0.017	1.04±0.017	1.39±0.012	1.24±0.005	1.93±0.23	1.18±0.012	1.77±0.008	1.34±0.01
7	500119 TN	1.94±0.014	1.11±0.008	0.90±0.019	1.34±0.008	1.17±0.005	1.40±0.017	1.10±0.008	1.49±0.014	1.25±0.012
8	500155 AP	1.88±0.017	0.96±0.017	0.79±0.012	1.10±0.014	1.07±0.00	1.17±0.017	1.00±0.058	1.26±0.017	1.15±0.0
9	500101 KA	1.67±0.014	0.84±0.031	0.72±0.012	1.03±0.012	0.91±0.005	1.09±0.008	0.94±0.008	1.17±0.0	1.12±0.014
10	IC 385841	1.54±0.017	0.71±0.017	0.64±0.012	0.96±0.024	0.85±0.008	1.00±0.012	0.88±0.008	1.11±0.008	1.02±0.012

*UDSM: Undefined seed meal

**DSM: Defatted seed meal

ANNEXURE-1

PROTOCOLS USED FOR PROCESSING OF SEEDS (Janardhanan et al. 2003)

1. SOAKING:

a. SODIUM BI CARBONATE TREATMENT:

25 gm whole seeds were soaked in 0.1% Sodium bi carbonate solution for 16 hours at room temperature in bean: water ratio of 1:10 (w/v). The seeds were then dried at 55⁰ C in hot-air oven and powdered for extraction and estimation of L-Dopa.

b. SODIUM CHLORIDE TREATMENT:

25 gm whole seeds were soaked in 0.1% Sodium chloride solution for 16 hours at room temperature in bean: water ratio of 1:10 (w/v). The seeds were then dried at 55⁰ C and powdered for extraction and estimation of L-Dopa.

2. AUTOCLAVING:

25 gm seeds were autoclaved at 1.05 Kg/cm² pressure (121⁰C) in distilled water (bean: water ratio of 1:7 w/v) for about 30 minutes. Later it was dried thoroughly at 55⁰ C and powdered to use for further extraction and estimation of L-Dopa.

3. REPEATED BOILING:

25 gm seeds were boiled in distilled water in bean: water ratio of 1:10 w/v for 15 minutes on a hotplate and water decanted. This was repeated seven times for each sample before drying the seeds at 55⁰ C and powdered for the extraction and estimation of L-Dopa.

4. ROASTING:

25 gm each seed sample was mixed with 50 gm sand (to facilitate uniform distribution of heat and avoid charring) and was roasted for 15 min. in an iron cooking pan, on medium flame, for 15 min. duration. The seeds were brought down to 55⁰ C temperature and powdered for extraction and estimation of L-Dopa.

ANNEXURE-2

PROTOCOL FOR ITS REGION AMPLIFICATION

a. DNA isolation

DNA from young leaves of 10 different accessions of *Mucuna* was extracted using modified Doyle and Doyle (1990) method. To the extraction buffer containing cetyltrimethylammonium bromide (CTAB), 0.5% charcoal along with 0.2% β -mercaptoethanol were added to avoid polyphenol oxidation. After propanol precipitation, DNA was resuspended in 0.5 cm³ of 1X Tris EDTA buffer (pH 8.0).

b. Internal Transcribed Spacer (ITS) amplification

Amplification of ITS region was carried out in 0.025cm³ reaction mixture containing 0.2mM dNTP's, 10mM Tris-HCl, 1.5mM MgCl₂, 50mM KCl, 0.1% Triton X-100, 1.0 U Taq DNA polymerase, 0.2 μ M forward and reverse primers (Sigma Aldrich Chemicals Pvt. Ltd.) and 50ng of genomic DNA. Four different combinations of forward and reverse primers were used in the study namely: (1) ITS-1 and ITS-2 (2) ITS-3 and ITS-4 (3) ITS-1 and ITS-4 (White et al., 1990) and (4) PIL and P2R (Crisp et al., 2000). Amplification was performed in a Peltier thermal cycler (MJ Research, USA) using the following protocol:

After the initial cycle of 2 min. at 94^oC, 2 min. at 36^oC and 2 min. at 72^oC, 38 cycles of 1 min. at 94^oC, 1 min. at 36^oC and 2 min. at 72^oC were performed. The last cycle was followed by 7 min. extension at 72^oC.

Reaction mixture in which template DNA was replaced by distilled water was used as negative control. Amplified products were resolved on 1.2% agarose gel (1X TAE) followed by ethidium bromide staining.

c. Gel Elution and Sequencing

PCR amplicons of ITS regions were eluted from the agarose gel using gel elution kit (Bangalore Genie) by manufacturer's instruction. Sequencing was done at Bangalore Genie on an ABI 3100 sequencer.

PLANTATION AT BASARIKATTE



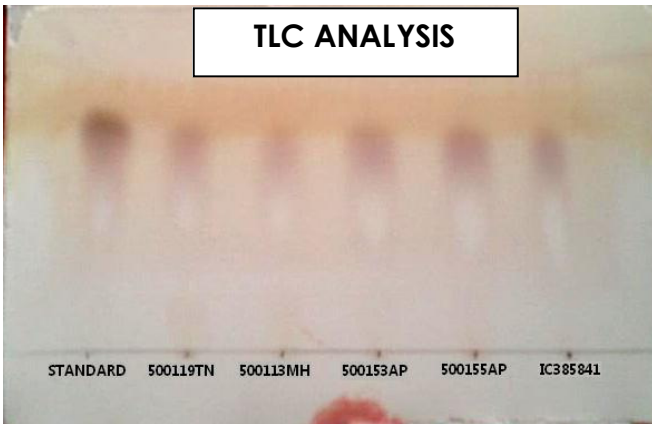
PLANTATION AT MOORNAD



PLANTATION AT GULBARGA



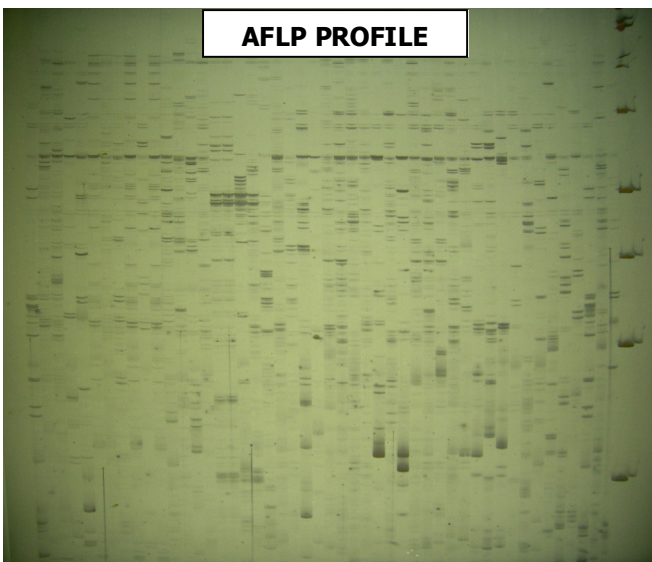
TLC ANALYSIS



PLANTATION AT BANGALORE



AFLP PROFILE



ITS PROFILE

