PROGRESS REPORT
(Period from 25.02.2009 – 02.10.2009)

Title of the Project	Genetic improvement of Velvetbean (*Mucuna* spp.) for anti-nutritional factors: Standardization of L-Dopa estimation, its evaluation and taxonomic studies among (on hand) Indian accessions – A Pilot Study
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STATUS OF THE MILESONES AS ON THIS REPORTING PERIOD

Sl. No.	**Milestones**	**Status**	
1.	Appointment of Staff	Completed	
2.	Purchase of Equipments	a. UV Visible Sepc.	Has been received from KHT
		b. Lab-top computer	Purchased
		c. Gel Scanner	Will be purchased during 3rd quarter
3a.	Identification of accessions for G X E interaction study	Towards this, initially a preliminary screening of L-Dopa content was carried out in 26 accessions with modified Daxenbichler (1971) method. Five accessions which showed significant difference in L-Dopa content were finally selected for plantation (**Table-1**).	
3b.	Plantation	Plantation was carried out during Kharif season (period from 1st August – 15th September) at all the five identified locations**. Seeds of individual accessions were planted in 8" plastic pots (except Bangalore) filled with local soil and arranged in Completely Randomized Block Design (CRBD) in five replications. In Bangalore, they were directly planted in the soil. All the Plantations are now being maintained with the package - practice best known in the respective areas of plantation.	
		****Name of the Place**	**Date of Plantation**
		Bangalore (plain)	31st July 2009
		Basarikatte (western ghats)	08th August 2009
		Moornad (hilly region)	15th August 2009
		Gulbarga (semi-arid zone)	22nd August 2009
		Rupaimoole (coastal)	05th september2009
4.	Developing reliable method for L-Dopa estimation:		
4a.	Validation of equipment:	Standard L-Dopa solution in the concentration range of 100-1000 µg was prepared in 10 replicates and quantity of L-Dopa for 1 OD value was estimated. For each sample, values were obtained in quadruplicates. The mean values of	

		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.
4b.	Validation of L-Dopa extraction method: TLC analysis of isolated sample	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.
4c.	Analyzing the seeds processed with different processing methods	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. Despite substantial change in the levels of L-Dopa, the ranking of the genotypes remained same in all the 8 processing methods **(Table-2)**. 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.
5.	Standardization of taxonomic studies using ITS typing	Standardization of the method has been tried out initially using 10 accessions (5 belonging to different species + 5 belonging *Mucuna pruriens*). 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. The detailed method used for ITS amplification is given in **Annexure-2**
6.	Standardization of AFLP for diversity analysis among on hand accessions	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.
7.	Budget Expenditures	Financial statement enclosed as Annexure – 3.

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[jk]
21.	500102KA	1.93±0.009[jk]
22.	500152AP	1.92±0.010[jk]
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]

[Y]Values 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 PROCESED SEEDS BY DAXENBICHELR (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: Undefatted 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 55^0 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 55^0 C and powdered for extraction and estimation of L-Dopa.

2. AUTOCLAVING:

25 gm seeds were autoclaved at 1.05 Kg/cm^2 pressure (121^0C) in distilled water (bean: water ratio of 1:7 w/v) for about 30 minutes. Later it was dried thoroughly at 55^0 C and powered 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 55^0 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 bought down to 55^0 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 cm^3 of 1X Tris EDTA buffer (pH 8.0).

b. Internal Transcribed Spacer (ITS) amplification

Amplification of ITS region was carried out in 0.025cm^3 reaction mixture containing 0.2mM dNTP's, 10mM Tris-HCl, 1.5mM $MgCl_2$, 50mM KCl, 0.1% Triton X-100, 1.0 U Taq DNA polymerase , 0.2µM 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 94^0C, 2 min. at 36^0C and 2 min. at 72^0C, 38 cycles of 1 min. at 94^0C, 1 min. at 36^0C and 2 min. at 72^0C were performed. The last cycle was followed by 7 min. extension at 72^0C.

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

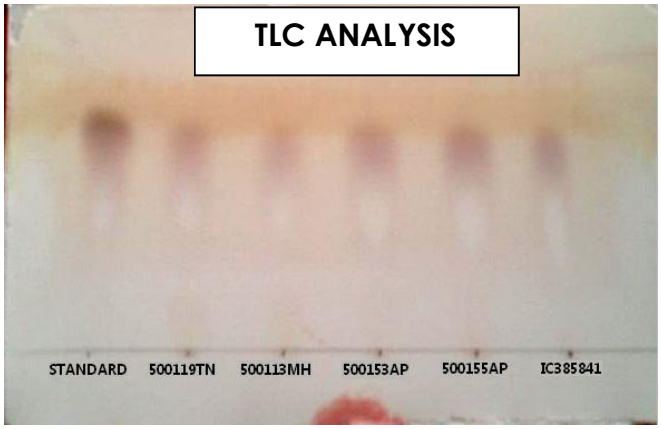
PCR amplicons of ITS regions were eluted from the agarose gel using gel elution kit (Bangalore Genie) by manufacturer's instruction. Sequencing was done at Bangalore Genei on an ABI 3100 sequencer.

PLANTATION AT BASARIKATTE

PLANTATION AT MOORNAD

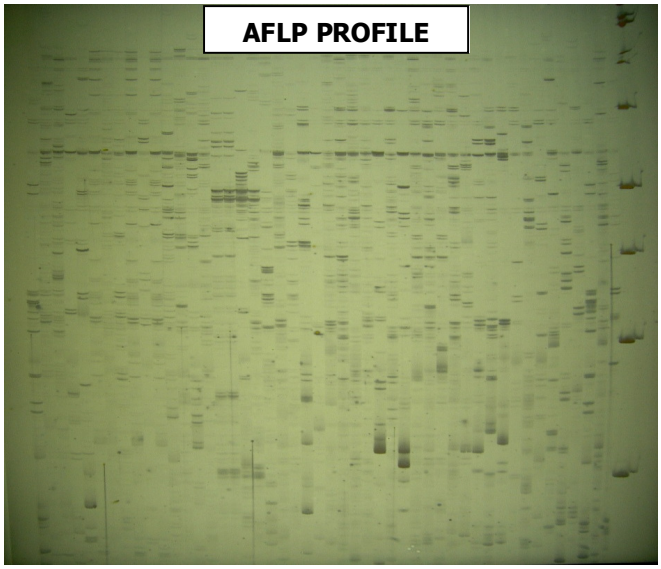
PLANTATION AT GULBARGA

TLC ANALYSIS



PLANTATION AT BANGALORE

AFLP PROFILE



ITS PROFILE