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Heat-stable and heat-labile antinutritional profile in *Mucuna pruriens* var *utilis*: Effected by germination

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Abstract: The seeds of different germplasm of velvet bean, an underutilized tribal pulse, collected from Western Ghats, India were germinated upto 120 h with a view to assess the extent of reduction/deactivation of both heat stable and heat labile antinutrients. The extent of reduction in the contents of heat stable ANF like total free phenolics, tannins, phytic acid, L-Dopa ranged from 69.5 to 75.7%; 62.3 - 70.7%; 60.67 - 82.81%; 34.8 - 58.4%, respectively; whereas the heat labile ANF's like trypsin and chymotrypsin inhibitors reduced at the ranges between 67.5 -77.0% and 76.9 - 83.0%, respectively in the seeds subjected to prolonged germination (ie., upto120 h). Regarding lectins (phytohaemagglutinins) the reduction was registered upto 60% in all the studied germplasm except Thachenmalai (black coloured seed coat). Furthermore, significant positive correlation i.e. $R^2 > 0.791$ was observed between the contents of antinutrients (x) and the period of germination (y) in the studied germplasm. However, it was less pronounced for L-Dopa content. To sum up, prolonging period of germination has significant reduction in the contents of ANF's been noticed. Germination for five days was found to be one of the optimal, traditional and cost-effective alternate processing methods in deactivating these antinutrients for further product development to combat highly prevailing Protein Energy Malnutrition.

Keywords: Antinutrients, germplasm, germination, underutilized tribal pulse, velvet bean

Introduction

In India legumes constitute an important foodstuff and are chief economic sources of proteins in the diets of economically weaker sections of population. Now-a-days research is being geared up to exploit the protein source from underutilized grain legume seeds. Underutilized species (both plant and animal) are those with a potential, not yet fully exploited, to contribute to food security and poverty alleviation (Bhat and Karim, 2009). Among the different underutilized pulses, velvet bean is currently receiving global attention. The velvet bean, *Mucuna pruriens* (L.) DC. var. *utilis* (Wall ex Wight) (Baker ex Burck) is grown predominantly in Asia, Africa and in parts of Americas since this wild pulse is a rich source of protein (20%), carbohydrate (65%), fat (15%) and several minerals (Janardhanan *et al*., 2003). However, the digestibility and utilization of this pulse is limited due to the presence of certain antinutritional factors (ANF) or anti-metabolic factors such as heat stable ANF's viz., total free phenolics, tannins, phytic acid, L-Dopa and heat labile ANF's of protease inhibitors and lectins which exert serious problem and limit their complete utilization as a viable and cost-effective protein source.

Improperly boiled seeds of velvet bean consumed by tribals Kannikars, in Kerala known to cause increase in body temperature, skin eruptions vomiting and diarrhea (Gurumoorthi *et al*., 2008). It is attributed to the presence of high levels of L-Dopa (L- 3, 4- dihydroxyphenylalanine), the aromatic non–protein amino acid (Jabadhas, 1980). It also has been reported to produce serious hallucinations and diskinesias in addition to gastrointestinal disturbances like nausea, vomiting, and anorexia and shown to be toxic in individuals with glucose –6– phosphate dehydrogenase deficiency in their erythrocytes (Buckles, 1995; Siddhuraju and Becker, 2001). The presence of protease inhibitors in animal diets (including human diets) can lead to pancreatic enlargement, reduced digestibility, reduced absorption of amino acids and reduced availability of minerals (Gatel and Grosjean, 1990).

Though ample of attempts have been made by several researchers to reduce/deactivate these ANF's from velvet bean in the last decade, but till date achivement on complete/partial deactivation of such compounds by suitable cheap method is found to be meager. Hence, it is aimed to ascertain the extent to which both heat stable and heat labile ANF's gets reduced by subjecting the seeds to less invasive, cost effective and traditional methods like germination.

Material and Methods

Collection of seed samples

Seven different germplasm of velvet bean, *Mucuna pruriens* (L.) DC. var. *utilis* (Wall ex Wight) (Baker ex Burck), were collected from different

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agro-climatic regions of Tamil Nadu and Kerela bordering to Western Ghats. After drying, the pods were thrashed to remove mature seeds and latter they were stored in plastic containers at room temperature (25°C) until further use.

Germination

The seeds, sterilized with 0.1% mercuric chloride and washed, were soaked in distilled water at 4°C for 12 h. The soaked seeds were allowed to germinate for 24, 48, 72, 96 and 120 h at 30°C. The seeds were moistened with distilled water at regular intervals of 12 h. The sprouts were rinsed with distilled water and freeze- dried.

Preparation of seed flour

About 50 g of both germinated and unprocessed (raw seeds) seed samples were powdered in a Wiley Mill (Scientific Equipment Works, New Delhi, India) to 60-mesh size. The fine seed powder, so obtained will be referred, hereafter, as processed or unprocessed seed flour. The powdered samples were stored in screw-cap bottles until further use.

Analysis of antinutritional factors

All the germinated and raw seed materials were analyzed for the antinutritional factors and the detailed procedures given below.

Extraction and estimation of total free phenols and tannins

Total free phenols were extracted and estimated following the method of Singleton *et al*. (1999) using folin-ciocalteu's reagent. While, tannin content was estimated by the method of Antoine *et al*. (2004).

Extraction and estimation of phytic acid

Phytic acid extraction and estimation was done following the method of Wheeler and Ferrel (1971). Estimation was done on aliquot of 5 mL and the iron content present in the sample was calculated from a ferric nitrate standard curve. The phytate phosphorus was calculated from the iron results assuming a 4:6 iron: phosphorus molecular ratio. The phytic acid was estimated by multiplying the amount of phytate phosphorus by 3.55 based on the empirical formula $C_6P_6O_{24}H_{18}$.

HPLC analysis of L-Dopa

The content of L – Dopa in both germinated and raw seed flours of velvet bean was determined by the method of Myhrman (2002). Approximately 0.1 g of flour was weighed and placed in a 27 x 95 mm glass screw-top vial. 20 mL of distilled-deionized water was added, and the capped vial was subjected to sonication for 10 min (2 x 5 min, with agitation in between and followed by ultra filtration. The concentration of L-Dopa in extracts of seed flour was determined by HPLC on a Zorbax StableBond SB-C18 column (Chadds Ford, PA 19317) with a mobile phase consisting phosphoric acid buffer, 2 mM disodium EDTA and 1 part HPLC-grade methanol (9: 2:1 v/v) and adjusted to pH 3.0. L-dopa concentrations were determined with reference to standard (# D9378, Sigma-Aldrich) using Star Chromatography software (Varian). In order to report percentage of L-dopa on a dry-weight basis, percentage of moisture was determined by drying samples for 24 h at 110°C.

Assay for protease inhibitors

Trypsin inhibitor activity (TIA)

Trypsin inhibitor activity was determined according to the method of Smith *et al*. (1980) using the synthetic substrate, BAPNA (benzoyl –L- arginine p-nitroanilide and standard trypsin. Trypsin inhibitor activity (TIA) is calculated in terms of mg pure trypsin inhibited g^{-1} sample as weighed (mg g^{-1}).

Assay for chymotrypsin inhibitor activity (CIA)

Chymotrypsin inhibitor activity was determined in a similar way as seen in TIA analysis but the synthetic substrate BETNA (benzoyl-L- tyrosine p-nitroanilide) was used (Norioka *et al*., 1988).

Purification of lectins and assay for phytohaemagglutinating activity

Ammonium sulphate fractionation and further purification of velvet bean lectin through a Con A Sepharose column (1.2 cm x 18 cm) was done following the method of Cuadrado *et al*. (1997; 2000). Fractions were eluted with 0.5% methyl α-D-mannopyranoside in Con A buffer containing 0.2 M NaCl. 5 mL fractions were collected at the flow rate of 200 mL/h. All fractions recovered were tested for their ability to agglutinate human erythrocytes. Protein determination was carried out following the method of Lowry *et al*. (1951). The purified fractions of velvet bean lectin were assayed for haemagglutinating activity against human erythrocytes of A, B, O blood groups following the method of Tan *et al*. (1983). One heamagglutinating unit (HU) is defined as the least amount of heamagglutinin that will produce positive evidence of agglutination of 25 µl of a blood group erythrocyte after 3 h.incubation at room temperature. The heamagglutinating activity was expressed as heamagglutinating units (HU)/mg protein.

Statistical analysis

All the values were estimated in triplicate determinations except lectin samples. Whereas L-Dopa contents were determined in quadruplicate samples. All the data were analyzed statistically using the AGRES statistics programme version 7.01, 1994. Significant differences between mean values were calculated by analysis of variance (ANOVA) using Duncan's Multiple Range Test at $p < 0.05$.

Results and Discussion

Antinutrient profile in raw seeds

The profiles of antinutrients in raw seed samples are showed in Table- 1. Significant variation in the levels of antinutrients was observed. The content of total free phenolics, tannins, phytic acid and L-Dopa ranged from 5.96 to 5.45; 0.335 - 0.310; 0.940 - 0.709 and 5.63 - 4.44 g100 g^{-1}DM, respectively. Trypsin and chymotrypsin inhibitor activities ranged from 43.60 to 51.20 (mg of pure trypsin inhibited g^{-1} of sample) and 25.11 to 29.81 (mg of pure chymotrypsin inhibited g^{-1} of sample), respectively. The molecular weight of purified velvet bean lectin ranged from 42 to 67 kDa. Further the phytohaemagglutinating activity against human erythrocytes of 'A' and 'B' blood groups was found to be higher compared to that of erythrocytes from 'O' blood group (i.e. only upto 28 HU mg^{-1} protein) in all the germplasm of the present study.

The germplasm Thachenmalai (white- coloured) was found to contain low levels of both total free phenolics and tannins, while Mylaru (white-coloured) germplasm registered the lowest level of phytic acid. Nonetheless, the content of L-Dopa was found to be low in both the germplasm of Valanadu and Mundanthurai (black- coloured). The activities of trypsin and chymotrypsin inhibitors were found to be low in both the white-coloured germplasm of Mundanthurai and Thachenmalai.

Correlation between the contents of antinutrients (x) in different germplasm of velvet bean and period of germination (y) was also studied and it was given in the form of regression equation in Table-2. Significant positive correlation (i.e. R^2 more than 0.791) was observed between germination period and the type of antinutrients but it was found to be insignificant as in the case of L-Dopa. It was also proved statistically that $F_0 > F_e$ in all the antinutrients except for L-Dopa. The extent of reduction in the contents of antinutrients was found to be phyto haemagglutinating activity > CIA > tannins > phytic acid > TIA > total free phenols > L-Dopa.

Effect of germination on the level of antinutrients

The effect of germination on the levels of antinutrients is given in Figures 1 – 7. In general, with increasing period of germination there was a corresponding decrease in the contents of all antinutrients in all the germplasm studied and higher reduction was noticed in the seeds germinated for 120 h.

Germination at 24 h reduces the contents of total free phenolics and tannins to the extent of 49.9%, 41.43%, respectively. The contents of the same were reduced to an extent of 75.7% (total free phenolics) and 70.7% (tannins) in the seeds of 120 h of germination. Leaching of total free phenols and tannins during steeping may account for this loss. Loss of polyphenols during germination may be attributed to the presence of polyphenol oxidase and the hydrolysis of tannin- protein and tannin – enzyme complexes, which result in the removal of tannins or polyphenols.

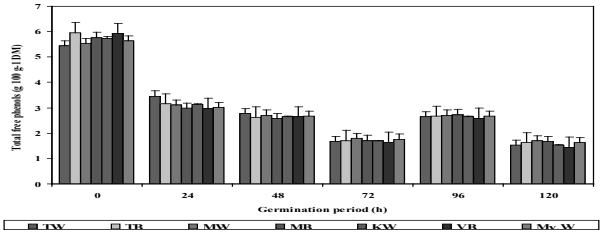

Figure 1. Effect of germination on the levels of total free phenols in different germplasm of velvet bean

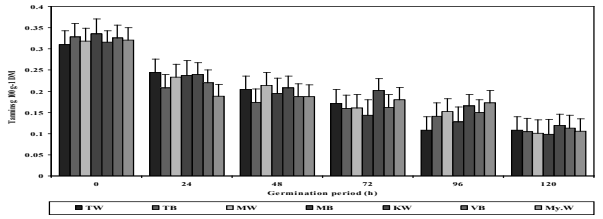

Figure 2. Effect of germination on the levels of tannins in different gerplasm in velvet bean

Gradual reduction in the contents of phytic acid was noticed upon germination in all the germplasm in the present study. The reduction in the content of phytic acid during germination at 24 h, 48 h, 72 h, 96 h, 120 h, ranged from 34.80 to 72.4%; 37.74 – 74.1%; 52.29 – 79.3%; 55.06 – 80.2%; 66.67 – 82.1%, respectively. Maximum reduction was recorded in the germplasm of Mundanthurai (white- coloured) followed by Thachenmalai (white-coloured). Nonetheless, the germplasm, Mylaru (white-coloured) exhibited insignificant reduction in the content of the same upon germination even prolonged upto 120 h (i.e., 66.67%), which might be due to poor water holding capacity and high seed coat percentage.

These findings were corroborated with earlier

Table 1. Profiles of antinutrients in raw seeds of different germplasm of velvet bean (g 100 g^{-1} DM)[a]

Germplasm	Total free phenols	Tannins	Phytic acid	L-Dopa	TIA*	CIA**	Haemagglutinating activity***		
							'A' blood group	'B' blood group	'O' blood group
TW	5.45^{d}	0.310^{c}	0.834^{c}	5.14^{c}	48.80^{e}	25.11^{f}	336	172	24
TB	5.96^{a}	0.328^{ab}	0.752^{d}	5.00^{d}	48.90^{e}	28.52^{c}	320	160	18
MW	5.54^{cd}	0.318^{bc}	0.940^{a}	5.63^{a}	43.61^{f}	26.54^{e}	332	172	28
MB	5.77^{b}	0.335^{a}	0.918^{b}	4.44^{f}	51.20^{a}	28.51^{c}	340	160	24
KW	5.74^{b}	0.316^{bc}	0.709^{e}	5.55^{a}	50.83^{b}	27.82^{d}	320	164	26
VB	5.93^{a}	0.326^{abc}	0.715^{e}	4.60^{e}	49.51^{d}	29.81^{a}	328	156	22
My. W	5.63^{bc}	0.321^{abc}	0.612^{f}	5.43^{b}	50.6^{c}	28.90^{b}	318	152	22

TIA –Trypsin inhibitor activity CIA - Chymotrypsin inhibitor activity
* - mg of pure trypsin inhibited g^{-1} of sample ** - mg of pure chymotrypsin inhibited g^{-1} of sample
*** - Erythrocytes from human blood group and haemagglutinating activity expressed in units (HU mg^{-1} protein)
a – All values are mean of triplicate determinations
Values in the same column with different letter in superscripts are significantly different statistically ($p < 0.05$).
TW: Thachenmalai (white-coloured) TB: Thachenmalai (black-coloured) MW: Mundanthurai (white –coloured)
MB: Mundanthurai (black –coloured) KW: Kailasanadu (white –coloured) VB: Valanadu (black –coloured)
My.W (Mylaru white-coloured)

Table 2. Regression equation showing relationship between antinutrients and germination period

Antinutrients	R^2	Regression[a]	r	F_0
Total free phenolics (g 100 g^{-1} DM)	0.934	$Y= 0.160 + 1.207x - 0.1932x^2$	0.996	20.49
Tannins (g 100 g^{-1} DM)	0.983	$Y =0.082 + 0.0445x - 0.03036x^2$	0.960	85.99
Phytic acid (g 100 g^{-1} DM)	0.974	$Y =0.964 + 0.3608x + 0.111x^2$	-0.910	55.47
L-Dopa (g 100 g^{-1} DM)	0.791	$Y =0.175 + 2.057x - 1.548x^2$	0.934	5.014
Trypsin inhibitor activity[b]	0.943	$Y = 0.444 + 1.61x + 0.7162x^2$	0.980	24.09
Chymotrypsin inhibitor activity[b]	0.989	$Y=5.43 + 0.195x - 1.056x^2$	0.940	134.6
Phytohaemagglutinating activity[c]	0.991	$Y =1.27 + 1.064x - 1.348x^2$	-0.91	164.5

a Y = antinutrients ; X germination period (h);
b Expressed in units of enzyme activity inhibited per mg of protein
c Expressed in units (HU mg-1 protein); R^2 = Multiple Correlation Coefficient.
F_e = 10.13 (Statistical table value); F_0 = Distribution at 5 percent points

work on germinating seeds of *Cicer arietinum* (El-Adawy, 2002) and *Cajanus cajan* (Mulimani *et al*., 2003; Oloyo, 2004). The loss in the content of phytic acid during germination may be attributed to the appearance of phytase in sprouted pigeonpea cultivars (Duhan *et al*., 2002) and partial dephosphorylation of the inositol hexaphosphate to penta, tetra, tri phosphates and other simpler forms and release of cations such as Ca, Cu, Fe, Mn, Co, Zn from the complex and setting them free (Manez *et al*., 2002).

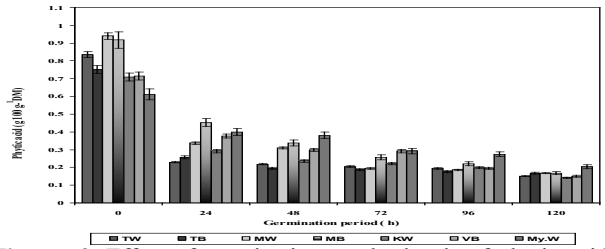

Figure 3. Effect of germination on the levels of phytic acid different germplasm of velvet bean

Maximum reduction in the contents of L- Dopa was noticed in the seeds germinated for 120 h and the reduction in the content ranged from 34.8 to 58.4%. Effective reduction in the level of L- Dopa during germination was observed in Thachenmalai (black-coloured) germplasm. In general as the period of germination increases there was a concomitant decrease in the level of L- Dopa in all the germplasm. But it was less pronounced in the germplasm of Thachenmalai (white-coloured) and Kailasanadu (white- coloured).

Germinating the seeds for prolonged period of time (120 h) considerably favours significant reduction in protease inhibitory activities (67.9 – 77.0% loss for TIA and 76.9 – 83.05 loss for CIA) in all the germplasm except for the germplasm Kailasanadu (white-coloured) (only 67.9% loss for TIA). The haemagglutinating activity also exhibits a decreasing tendency towards germination. In the present study reduction in lectin activity was observed to a significant extent or even complete deactivation upon prolonged germination period in all the germplasm. However, it was accounted to a loss of upto only 54%, 38% and 77% in Thachenmalai (black- coloured) germplasm against A, B, O blood groups, respectively.

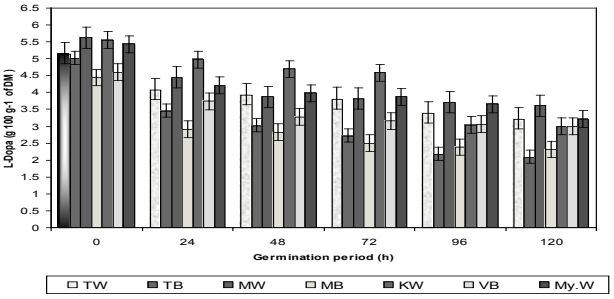

Figure 4. Effect of germination on the levels of L-Dopa in different germplasm of velvet bean

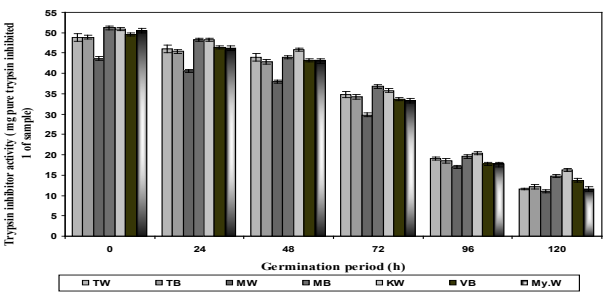

Figure 5. Effect of germination on the levels of trypsin inhibitor activity in different germplasm of velvet bean

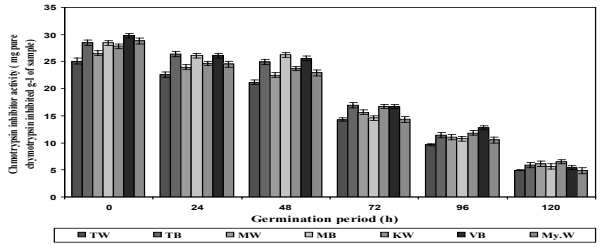

Figure 6. Effect of germination on the levels of chymotrypsin inhibitor activity in different germplasm of velvet bean

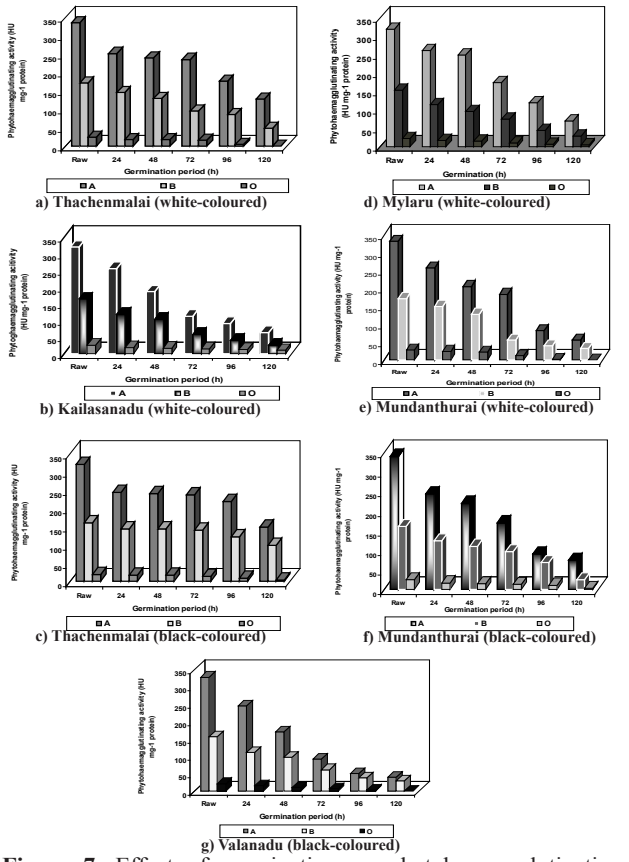

Figure 7. Effect of germination on phytohaemagglutinating activity in velvet bean gemplasm

Conclusions

Raw seeds of velvet bean registered for the highest concentration of L-Dopa (5.63%), total free phenolics (5.93%), phytic acid (0.940%) and tannins (0.335%). Drastic reduction in the contents of both heat stable and heat labile antinutritional profile was noticed in the seeds with prolonging period of germination. Antinuritional factors in seeds after five days of germination found to get deactivated / eliminated to the extent of more than 60% and upto 82.81% in all the germplasm except Thachenmalai (black-seed coat coloured). However it was found to be less effective for L-Dopa; where only 58.4% of reduction was noticed. Nevertheless, when compared to other previous studies carried out on this pulse, germination seemed to be less invasive, cost-effective, traditional methods of processing which can be easily adopted at house-hold level preparations at rural population for further product development.

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