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Biochemical marker-based comparative genomic characterization of *in vivo* Varieties and *in vitro* Regenerates of *Mucuna pruriens* L., an important medicinal plant

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Abstract
The present investigation has dealt with biochemical studies of an important medicinal plant, *Mucuna pruriens* L. of Fabaceae. This plant synthesizes L-dopa, a neurotransmitter precursor, which is used in Parkinson's disease and other mental disorders. The biochemical study involves total protein analysis and activities of two isozymes in both *in vitro* regenerates and field grown plants of two varieties of *M. pruriens*, *M. pruriens* var *pruriens* and *M. pruriens* var. *utilis*. A remarkable increase in total protein content with increased number of polypeptide bands, as observed in qualitative protein profile study has been observed in the regenerates as compared to field grown plants which may be attributed to the high level of different enzyme activities. Also, differences exist in protein profile among strains of these two varieties revealing genomic diversity. The activities of both esterase and peroxidase isozymes have also been augmented in the regenerates showing more number of isoforms in comparison to field grown plants. The differential level of gene expression might have been responsible for such elevated levels of proteins and isozymes activities.

Keywords: esterase, *Mucuna*, peroxiadase, total protein

Introduction

Mucuna, a genus of annual and perennial twining herbs or shrubs under Fabaceae contains about 15 species in India [1]. *Mucuna pruriens* L., commonly known as cowhage or velvet bean, is an important medicinal plant growing in the bushes, hedges and dry deciduous forests throughout India [2, 3, 4]. The plant is a vigorous annual herb with compound trifoliate leaves. The leaflets are broadly ovate and the terminal leaflet is symmetrical and smaller than the lateral ones, which are conspicuously asymmetrical at bases [5, 6]. The two varieties of *M. pruriens* (vars *pruriens* and *utilis*) are available in India. *M. pruriens* var *pruriens* is found in wild whereas *M. pruriens* var *utilis* is a cultivated variety. These two varieties differ in seed and pod characteristics. *M. pruriens* L. (DC.) var *pruriens* possesses seeds which are black with brown spots and ovoid in shape. The mature pods contain reddish-brown irritating hairs that cause intense itching on contact with skin (pruritis) due to presence of mucunain. On the other hand, the seeds of *M. pruriens* var *utilis* Wall. ex Wight are oblong-ellipsoid and glossy with variable seed coat colour and sizes. The pods are covered with velvety non-irritant hairs [7]. As all parts of this plant possess valuable medicinal properties [8], there is an ever-increasing demand of *Mucuna* in the international drug market. This plant synthesizes a medicinally important compound, L-dopa, a neurotransmitter precursor, which is used as an effective principle for symptomatic relief of Parkinson's disease and other mental disorders [5, 9, 10].

Several biochemical changes occur during growth and development of cells, thus bringing about diversification and specialization characteristic of multicellular organisms. As cellular differentiation progresses resulting in morphological and anatomical development and functional specialization there is continuous synthesis and/or degradation of specific enzymes and structural proteins [11, 12, 13]. Tissue culture technique immensely helps in understanding the nature of differentiation [14]. The complexity of cellular and biochemical phenomena involved in the process of differentiation can be studied during tissue culture which permits interpretation and understanding at various levels of organization, from free cells to callus to cytodifferentiation and/or morphogenesis/ organogenesis. Development of any tissue involves temporal and spatial changes of cellular phenotypes and specific biochemical activities appear and disappear during morphogenesis [15]. The characterization of cell cultures and the study of transmissible characters must be directed towards biochemical and physiological properties rather than morphological characteristics [16]. As cells mature, quantitative and qualitative changes in various proteins may occur [13].

Polyacrylamide gel electrophoresis (PAGE) is an extremely useful analytical tool for separation and quantification of protein. It is a method of choice for locating any qualitative changes in protein metabolism at the cellular or tissue level. It, therefore, provides a sensitive index of basic changes occurring in the tissue [17, 18]. This method has been successfully applied for separation of plant proteins and isozymes from plants grown *in vivo*, as well as from cultured tissues by several workers [14, 18, 19].

The term isozyme was first introduced by Markert and Moller (1959) [20], to refer to multiple molecular forms of an enzyme with similar or identical substrate specificity present within

the same organism. Shaw (1969) [21] classified isozymes into two major categories: A. Those that are distinctly different molecules and are presumably produced from different genetic sites; and b. Those that result from secondary alterations in the structure of a single polypeptide species and may, in many cases, be *in vitro* artifacts. Isozymes are complex proteins comprising of paired polypeptide units (monomers), which are encoded by different gene loci. The different monomers of an isozyme differ in their isoelectric points and therefore, can be separated by electrophoresis.

Study of isozymes helps to understand the basic mechanism involved in differentiation during different stages of development [13]. Isozyme studies are also used as a tool for identification of different cultivars and strains of several plant species [22, 23, 24, 25]. Isozyme activity is also used as a suitable marker during the process of growth and development in different stages in culture [26, 27].

The present biochemical investigation was undertaken to note the variation or changes, if any, in the quantitative and qualitative protein profile and isozyme activity patterns of esterase and peroxidase in the different *in vivo* tissues as well as tissues from the regenerates of the four different strains of *Mucuna pruriens* L.

Materials and Methods

Plant Materials

The seeds of three strains of *Mucuna pruriens* var *utilis* [Strain Nos.: IC 471876 (I), IC 241679 (II), IC 392338 (III)] were obtained from NBPGR, New Delhi. On the other hand, plants of one strain of *Mucuna pruriens* var *pruriens* (IV) were collected in wild from West Midnapur district of West Bengal. The following tissues from all the four strains of *Mucuna pruriens* L. were used for biochemical studies. (a) Seeds (Cotyledons along with the embryo, without the seed coat); (b) Young and healthy leaves of the *in vivo* plants and (c) Young and healthy leaves of the *in vitro* plants, regenerated from callus.

Methods

Extraction of protein

One gram of each sample tissue was weighed and homogenized separately in 1-2 ml of cold Protein Extraction Buffer (PEB) (0.1M Tris; pH 6.8, 0.25M Sucrose, 1% PVP, 0.1% Ascorbic acid, 0.1% Cystein hydrochloride, 1mM EDTA, 1mM $MgCl_2$) at 4°C. The pH of the buffer was adjusted to 6.8 prior to autoclaving and stored at -20°C. The extraction was done in a cold room at 4°C. The homogenates were centrifuged at 15,000 rpm for 30 minutes at 4°C in an Eppendorf 5810R centrifuge. The supernatants (protein suspended in buffer) were decanted carefully and stored in small aliquots in capped vials at -20°C, until further analysis.

Quantitative estimation of protein

300 µl of protein sample (in PEB) was taken in separate microcentrifuge tubes. Double volume of chilled acetone was added to each tube for precipitation of protein. The tubes were incubated at -20°C for 30 minutes and then centrifuged at 10,000 rpm for 15 minutes in an Eppendorf 5810R centrifuge. The pellets were collected and the supernatants were discarded. The pellets were then washed two to three times with chilled acetone and finally dissolved in 300 µl of 0.15 M sodium chloride (NaCl) solution. The unknown sample proteins were quantified according to the method of Bradford (1976) [28] spectrophotometrically at 595 nm and compared against the absorbances in a standard curve using Bovine Serum Albumin (BSA) as authentic sample [6].

Qualitative study of total protein profiles by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-Page).

Qualitative estimation of protein was done by polyacrylamide gel electrophoresis [SDS-PAGE]. The thin layer horizontal gel slab (10cm x 10cm) was placed on a vertical electrophoresis unit (Techno Lab). Gel polymerized with ammonium per sulphate and consisted of 12.0% acrylamide.

The polypeptide fragments were scored and transformed into binary character matrices (1 for presence, 0 for absence). UPGMA (unweighted pair group method with arithmatic mean) cluster analysis was done with the help of Statistica 6.0 software.

Qualitative estimation of Isozyme activity by polyacrylamide gel electrophoresis (Native-PAGE)

The enzyme activity was measured by NATIVE-PAGE utilizing 8% resolving gel and 5% stacking gel after protein extraction. The protein extarct was mixed with equal volume of sample loading buffer and finally loaded into wells carefully. After electrophoresis, the gel was immersed in sodium phosphate buffer with 0.01% α-NAA and 0.01% β-NAA, till bands resolve properly to study esterase activity. The gel was immersed in sodium phosphate buffer containing H_2O_2 and guaiacol solution.

Molecular weight determination

The MW determination was carried out by SDS-PAGE of known molecular weight proteins along with the sample proteins. A linear relationship exists between the logarithm of the MW of the SDS-denatured polypeptide and it's Rf (Relative flow). The Rf is determined as the ratio of the distance migrated by molecule to that migration by a marker dye front. The Relative mobility (Rm) in electrophoresis is determined by plotting a standard curve of distance migrated vs. log10 MW for known samples and read off the log Rm of the sample after measuring the migrated distance on the same gel.

Data Analysis

Just after electrophoresis the glass plates were detached and the gels were immersed in respective buffer specific for each enzyme and then stained with specific stain. When bands and zones were visualized clearly, the gels were fixed in Methanol: Water: Glacial acetic acid (5: 4: 1) and photographed under visible light illuminator using a digital camera immediately. The positions of the isozyme bands or zones were expressed by relative mobility (Rm). In all these sample gels, the bands were scored as 1, when present and 0 in absence. A percent of disagreement value distance matrix was formed. This matrix was subjected to UPGMA (unweighted pair group method with arithmatic mean) cluster analysis using joining tree algorithm of Statistica version 5.1

software to generate a dendogram.

Results and Discussion

Quantitative estimation of total protein

The quantitative estimation of protein by Bradford's method in different tissue samples showed differences in protein content in the four different strains of *Mucuna pruriens* studied here. The protein content was found to be much higher in the seeds than in the leaves of both the *in vivo* and *in vitro* plants. The amount of seed proteins, in these four strains, ranged from 173.75 to 235.32 mg per gram of tissue. The seeds of strain IV contained the highest amount of protein (235.32 mg/g tissue) and the seeds of strain III had the lowest amount (173.75 mg/g tissue) (Table 1). Among the *in vivo* plants, the total protein content ranged from 10.15 to 12.05 mg/g tissue, with strain IV having the highest protein content and strain III having the least (Table 1). The total protein content among the *in vitro* plants varied from 12.50 to 16.00 mg/g tissue. Strain IV contained the highest protein content and strain III the least (Table 1).

The total protein content analysis revealed differences in protein amounts among the different types of tissues between these strains. In all these strains, the total protein content has been found to be quite high in the seeds as compared to the leaves of *in vivo* and *in vitro* plants. Among the different strains, the only strain of var *pruriens* contains the maximum amount of total protein in the seeds while lowest amount of protein was found in strain III of var *utilis*. It is well understood that the seeds usually contain high amount of storage proteins to nurture the developing embryos during germination. A higher amount of total protein in the *in vivo* plants of strain IV is observed than the other three strains, which might be related to a higher growth rate as observed in the field condition. The presence of higher amount of protein in the *in vitro* plants might be attributed to the higher and more enzyme activity during the process of differentiation [25, 29].

Table 1: Quantitative estimation of total protein from seeds and leaves of *in vivo* plants of four strains under two varieties of *Mucuna pruriens* L. utilizing Bradford Assay.

Variety	Strains	Tissue	Amount of protein (mg/g of tissue)*
M. pruriens var *utilis*	I	Seeds	215.00 ± 1.94
		In vivo leaves	10.50 ± 0.40
		In vitro leaves	13.00 ± 0.14
	II	Seeds	228.75 ± 1.18
		In vivo leaves	11.50 ± 0.32
		In vitro leaves	14.50 ± 0.25
	III	Seeds	173.75 ± 2.07
		In vivo leaves	10.15 ± 0.32
		In vitro leaves	12.50 ± 0.28
M. pruriens var *pruriens*	IV	Seeds	235.32 ± 2.47
		In vivo leaves	12.05 ± 0.22
		In vitro leaves	16.00 ± 0.13

* Data represent Mean ± S.E. from 5 replicates

Qualitative estimation of total protein

In the present investigation the electrophoretic profile of the total protein from seeds, *in vivo* plant tissues and *in vitro* plant tissues were analyzed.

Seed Proteins

Protein molecular weight marker from Genei PMW-B (Range: 3,000 Da to 205,000 Da) was used for analysis (Fig 1). In strain I, 18 bands were observed having molecular weights ranging from 2.36 kD to 91.70 kD. Strain II also showed 18 bands (molecular weights ranging from 2.44 kD to 91.75 kD). In strain III, 17 bands were seen (molecular weights ranging from 2.47 kD to 91.70 kD), whereas in IV, a total of 19 bands were observed (molecular weights ranging from 2.47 kD to 107.16 kD) (Fig 1a).

In vivo plant tissues

In strain I, 23 bands were seen (molecular weights ranging from 2.50 kD to 93.72 kD). Strain II showed 24 bands (molecular weights ranging from 2.50 kD to 87.15 kD). 21 bands were observed in strain III (molecular weights ranging from 2.53 kD to 85.50 kD) and in strain IV, 19 bands were seen (molecular weights ranging from 2.50 kD to 85.52 kD) (Fig 1b).

In vitro plant tissues

27 bands were observed in strain I with molecular weights ranging from 3.05 kD to 189.39 kD. In strain II, 26 bands were identified, with molecular weights ranging from 3.05 kD to 189.39 kD. 25 bands were present in the strain III having molecular weights ranging from 3.05 kD to 164.58 kD. 27 bands were also observed in strain IV with molecular weights ranging from 3.05 kD to 201.08 kD (Fig 1c).

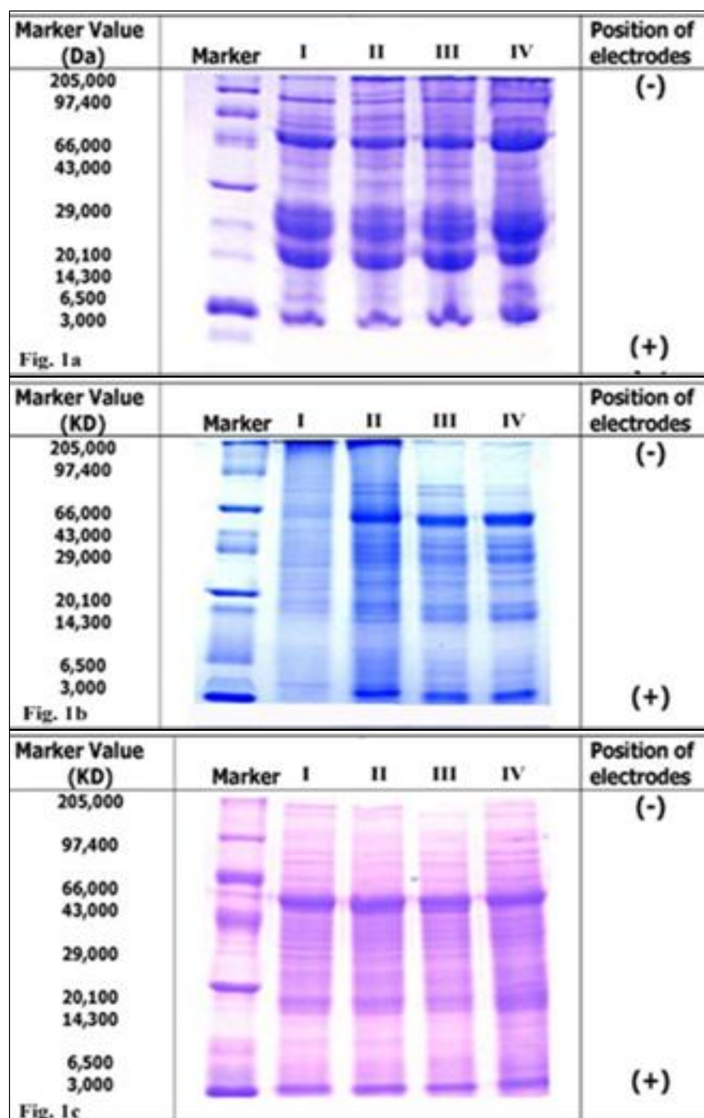

Fig 1: Total protein profiles of seed, *in vivo* plants and *in vitro* regenerates in strains of *Mucuna pruriens*. Marker: MW marker; I, II, III, IV: Strains. (a) Seeds; (b) *In vivo* plants; (c) *In vitro* plants

Qualitative difference has also been observed in one-dimensional SDS-PAGE profile of the buffer soluble total protein from different tissue types in all the four strains. The SDS-PAGE profile of the seeds, leaves of *in vivo* plants and leaves of *in vitro* regenerates have revealed differences in number and intensity of polypeptide bands in all the strains [30].

In the seed protein profile, the total number of bands varied from 17-19 and the molecular weights ranging between 2.36 kD to 107.16 kD. On the other hand, the number of bands ranged from 19-24 with molecular weights varying between 2.50 kD to 93.70 kD in the field-grown plants. This clearly indicated that the total amount and the type of protein are related to the physiological condition and surrounding environment of the plant tissue. Interestingly, in case of *in vitro* plant tissues, both the number as well as position of the bands varied to a great extent than those of the seeds and *in vivo* plants. The number of bands varied from 25-27 and polypeptides of high molecular weight (164 to 201 kD) were found to be more prevalent. These observations may suggest the difference in biochemical activity involving production of specific biochemical products during *in vitro* growth and differentiation.

In the present investigation, the only strain of var *pruriens* has revealed specific bands in the seeds (18.63 kD), *in vivo* plants (72.28 kD) and *in vitro* plants (71.07 kD). This may suggest the presence of specific protein in this variety that may be responsible for certain morphological characteristics. Thus the electrophoresis analysis of soluble proteins in these strains during stages of *in vivo* and *in vitro* growth as well as during developmental stages of morphogenesis might suggest differential gene expression at specific stages of differentiation. This may not only be used as a genetic marker but may also be used for identification of key metabolic steps during differentiation and morphogensis [19, 31].

Qualitative estimation of peroxidase activity

In the present investigation, the zymogram of peroxidase has been divided in three zones, Zone A (Cathode zone), Zone B (Intermediate zone) and Zone C (Anode zone).

In seed protein

Peroxidase activity was not detected in the seed proteins of all these strains.

***In vivo* plant tissues**

Two bands were observed in each of the three strains (I, II, IV). In strain III, only a single band was observed. All the bands were observed in the cathode zone (Zone A) (Table 2, Fig 2a).

***In vitro* plant tissues**

The number of isoforms of peroxidase in the *in vitro* plant tissues is higher than that of the *in vivo* plants. In strain I, 5 bands were in the cathode zone (Zone A) and 2 were in the intermediate zone (Zone B). In strain II, out of the 6 bands 4 were in the cathode zone (Zone A) and 2 were in the intermediate zone (Zone B). In strain III, 4 bands were in the cathode zone (Zone A) and 1 in the intermediate zone (Zone B), while in strain IV, 4 bands were in the cathode zone (Zone A) and 1 in the intermediate zone (Zone B) (Table 3, Fig 2b).

Peroxidase activity was not detected in the seed (cotyledon and embryo) proteins in all the four strains in the present study. This may be explained by the absence of any stress in the matured healthy seeds. However, a few authors have previously detected peroxidase activity in the seed coat proteins of soybean [32] and in the micropylar endosperm of tomato seeds just prior to germination [33].

Table 2: Number and position of peroxidase bands observed in the *in vivo* plant tissues in different strains of *Mucuna pruriens* L.

Variety	Strain	Number of Bands	Rm values of the bands
M. pruriens var *utilis*	I	2	0.04 0.23
	II	2	0.04 0.24
	III	1	0.29
M. pruriens var *pruriens*	IV	2	0.24 0.29

Table 3: Number and position of peroxidase bands observed in the *in vitro* plant tissues in different strains of *Mucuna pruriens* L.

Variety	Strain	Number of Bands	Rm values
M. pruriens var *utilis*	I	7	0.01
			0.07
			0.13
			0.22
			0.26
			0.34
			0.55
	II	6	0.01
			0.07
			0.13
			0.24
			0.44
			0.55
	III	5	0.010.07
			0.13
			0.23
			0.55
M. pruriens var *pruriens*	IV	5	0.01
			0.07
			0.11
			0.20
			0.55

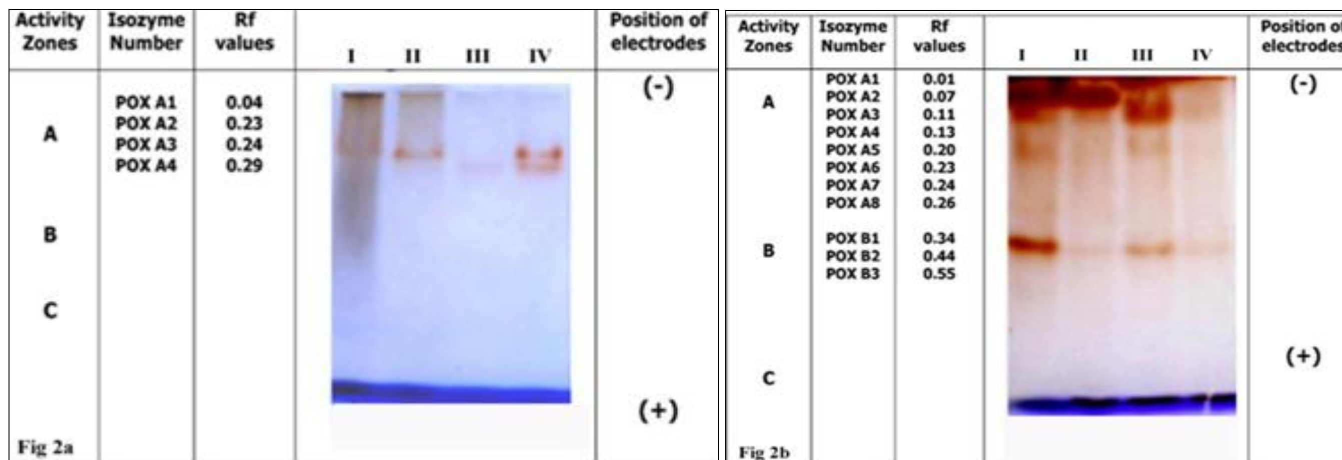

Fig 2: Zymogram of peroxidase isozymes in different strains of *Mucuna pruriens* L. (a) *In vivo* plants; (b) *In vitro* regenerates. Strains: I, II, III, IV.

In the *in vivo* plants of different strains of two varieties of *Mucuna pruriens*, the peroxidase isozymes have been distributed in the cathode zone. The two strains of variety *utilis* (I and II) showed two isoforms with almost similar Rm values while the other strain (III) revealed only one isoform. The only strain of variety *pruriens* (IV), showed two different isoforms with two different Rf values. In higher plants the pattern of peroxidase expression are mostly organ specific, and developmentally and environmentally regulated [34]. In the present study, young leaves were taken from all these strains three months after germination, and grown in similar environmental conditions. Keeping the parameters constant, the isozymes varied to a lesser extent between these strains.

The peroxidase activity has also been measured in the plants of *in vitro* origin. Interestingly, the isoforms of peroxidase has shown two distinct zones of activity that has been designated as Cathode zone (Zone A) and Intermediate zone (Zone B). In all these strains, most of the isoforms are found in zone A (4-5) and 1-2 isoforms are in zone B. In both strains III and IV, the zone B has only one band. The number as well as the intensity of the bands has been more in the regenerates as compared to that of the *in vivo* plant. This may be due to the exposure of the plants to a number of stresses under *in vitro* condition. High concentration of sucrose and nitrogen in the culture medium alongwith limited gas exchange and low light intensity during culture induces several stresses. The *in vitro* plants have to adapt to osmotic stress, mineral toxicities and oxidative stress through the action of reactive oxygen species [35, 36, 37].

It is well understood that peroxidase expression is environmentally regulated. The regulatory role of 2,4-D on peroxidase activity has been reported previously by many authors. Scandalios and Sorensen (1977) [38] have demonstrated a quantitative change in peroxidase pattern after omission of 2,4-D in the callus tissue. In the present study, the plants have been regenerated from callus tissue in a medium containing only cytokinin, though the callus tissue was induced in a medium with 2,4-D and that may be responsible for high peroxidase activity. This high activity of peroxidase might lead to a change in morphogenetic expression, thus correlating the peroxidase activity with the tissue differentiation and development [17]. The similar correlation between specific developmental events and isoperoxidase patterns was observed in cultures of maize [17], *Asparagus* [25] and *Allium* [29]. It is, therefore, suggested that the study of peroxidase isozyme may be utilized as a genetic marker to identify the properties of morphogenesis at the cellular level [40, 41, 42].

Qualitative estimation of esterase activity

The zymogram of esterase has also been divided in three zones, Zone A (Cathode zone), Zone B (Intermediate zone) and Zone C (Anode zone).

In seeds

In each of the four strains, two bands were present in the cathode zone (Zone A), and three bands were observed in the intermediate zone (Zone B) (Table 4, Fig 3a).

***In vivo* plant tissues**

Four bands were observed in each of strains I and II, and all of which are in the intermediate zone. In strain III also, four bands were observed, all in the intermediate zone. In strain IV, on the other hand, only three bands were observed in the intermediate zone (Table 5, Fig 3b).

***In vitro* plant tissues**

The number of isoforms of esterase in the *in vitro* plants was higher than that present in the *in vivo* plants. In strain I, 3 bands were in the cathode zone, 5 were in the intermediate zone and 1 was in the anode zone. In strain II, 3 bands were in the cathode zone, 5 were in the intermediate zone, and 3 were in the anode zone. In strain III, 3 bands were in the cathode zone, 3 in the intermediate zone and 1 in the anode zone. In strain IV, 3 bands were in the cathode zone, 5 in the intermediate zone and 2 in the anode zone (Table 6, Fig 3c).

Table 4: Number and position of esterase bands observed in the seed protein of different strains of *Mucuna pruriens* L.

Variety	Strain	Number of Bands	Rm values of the bands
M. pruriens var *utilis*	I	5	0.25
			0.29
			0.36
			0.41
			0.52
	II	5	0.25
			0.29
			0.36
			0.41
			0.52
	III	5	0.27
			0.29
			0.36
			0.41
			0.50
M. pruriens var *pruriens*	IV	5	0.25
			0.29
			0.36
			0.41
			0.49

Table 5: Number and position of esterase bands observed in the *in vivo* plant tissues of different strains of *Mucuna pruriens* L.

Variety	Strain	Number of bands	Rm values of the bands
M. pruriens var *utilis*	I	4	0.36
			0.41
			0.48
			0.50
	II	4	0.36
			0.41
			0.46
			0.50
	III	4	0.36
			0.41
			0.45
			0.48
M. pruriens var *pruriens*	IV	3	0.36
			0.41
			0.46

Table 6: Number and position of esterase bands observed in the *in vitro* plant tissues of different strains of *Mucuna pruriens* L.

Variety	Strain	Number of bands	Rm values of the bands
M. pruriens var *utilis*	I	9	0.21
			0.24
			0.28
			0.34
			0.39
			0.45
			0.51
			0.56
			0.77
	II	11	0.21
			0.24
			0.28
			0.34
			0.39
			0.49
			0.51
	III	7	0.56
			0.65
			0.70
			0.77
			0.21
			0.24
			0.28
			0.34
			0.39
			0.45
			0.77
M. pruriens var *pruriens*	IV	10	0.21
			0.24
			0.28
			0.34
			0.39
			0.45
			0.51
			0.56
			0.65
			0.70

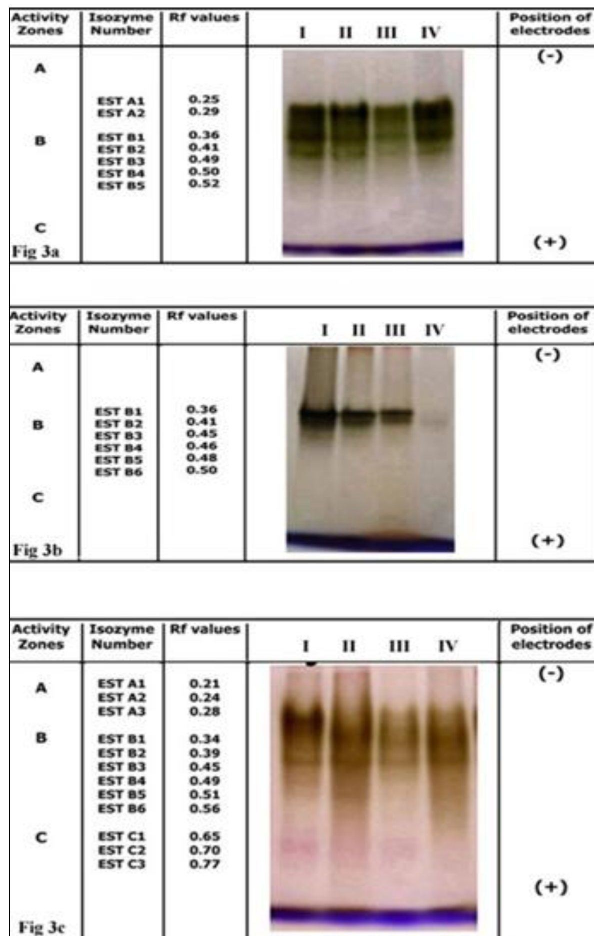

Fig 3: Zymogram of esterase isozymes in different strains of *Mucuna pruriens* L. (a) Seeds; (b) *In vivo* plants; (c) *In vitro* regenerates. Strains: I, II, III, IV.

The isozyme patterns of esterase have shown the distribution of isoforms in three distinct zones of activity- Zone A (cathode), Zone B (intermediate) and Zone C (anode). In the seeds of all the strains, a number of bands have been observed in both cathode and intermediate zones. Interestingly, all the strains revealed five bands each. On the other hand, the *in vivo* plants showed esterase bands in the intermediate zone. It was noted that the three strains of var *utilis* produced four bands each, and the only strain of var *pruriens* revealed only three bands. The plant tissues were collected from the *in vivo* plants on the same day. Therefore, the isozyme pattern may be considered as a genetic marker for identification of species and strains. The isoforms of esterase in the plants regenerated from callus tissue has been found to be much more in number than the seeds and *in vivo* plants and are distributed in all three zones. The maximum number of isoforms has been observed in strain II and the lowest in strain III of var *utilis*. In the only strain of var *pruriens* ten isoforms were noted, which is much higher than the *in vivo* plants, where only three isoforms were present. All these facts clearly suggest that the isozyme activity remains very high in the *in vitro* condition due to presence of high nutrient level, particularly the carbon source.

The isozyme patterns may, therefore, provide a unique genetic marker for genetic and physiological changes during the process of differentiation in tissue culture. The presence of some forms of esterase in cultured carrot cells and their increased activity in auxin-depleted media have earlier been observed [43]. In the present investigation, the process of differentiation of callus tissue have occurred in medium devoid of 2,4-D, though 2,4-D was required for callus induction. In the regenerated plants of all these strains of *M. pruriens* L., a burst of both the isozyme activities may be the consequence of the following facts: (i) *In vitro* cell dedifferentiation process overcomes the natural tissue-specific control over enzyme expression in these cells; (ii) specific gene expression adjustments may be involved allowing the callus to become fully adapted to artificial culture conditions, and (iii) cells under stress may concomitantly induce altered enzyme activities [44]. All these observations regarding isozyme activity in the field-grown plants as well as in the *in vitro* regenerates may suggest the use of isozyme activity as a marker for species and strain identification and for the characterization of cellular differentiation process as well.

A percent of disagreement value distance matrix revealed relationships among these strains (Table 7). The UPGMA cluster analysis, obtained from the total protein profiles and isozyme activities of different tissue types of all the four strains, clearly revealed that the strain IV of the wild variety (var *pruriens*) is distantly related to all the strains of var *utilis*. Among the variety *utilis*, the strains I and II are more closely related to each other than to strain III (Fig 4).

Table 7: Distance matrices of

Table 7a: Seed data

	I	II	III	IV
1	0.00			
2	2.00	0.00		
3	2.65	2.24	0.00	
4	3.61	3.32	2.83	0.00

Table 7b: *In vivo* leaf data

	I	II	III	IV
	0.00			
2	2.24	0.00		
3	2.83	2.24	0.00	
4	3.74	3.61	2.83	0.00

Table 7c: *In vitro* leaf data

	I	II	III	IV
1	0.00			
2	1.00	0.00		
3	2.00	1.73	0.00	
4	3.16	3.00	2.83	0.00

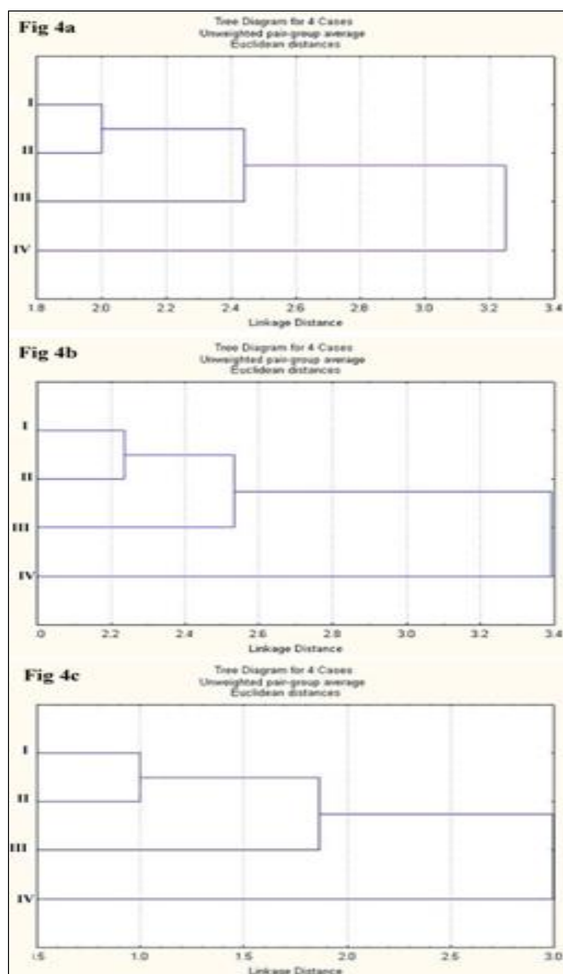

Fig 4: UPGMA cluster analysis of the protein and isozymes profiles of the seeds (a), *in vivo* plants (b) and *in vitro* regenerates (c) of different strains of *Mucuna pruriens*. Strains: I, II, III, IV.

Conclusion

The responses *in vitro* during plant regeneration in different strains of *Mucuna pruriens* have been different and might be under genetic control. In the present scope of investigation on protein and isozyme analysis, it has been interesting to note that the total protein content has been elevated during *in vitro* culture. The quantitative increase has also been reflected in the qualitative protein profile. It might be due to augmentation of different enzyme activity. It was evidenced by increased activity of peroxidase and esterase isozymes resulting in increase in number of isoforms in the *in vitro* regenerates.

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