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Enhancement of L-DOPA Production in Micropropagated Plants of two Different Varieties of *Mucuna pruriens* L., Available in India

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Abstract

Reports on increment in L-DOPA content in micropropagated plants, regenerated through a simple technique following shoot bud multiplication in four strains of two different varieties of *Mucuna pruriens* are made. Nodal segments from *in vitro* grown seedlings were cultured on modified MS with various concentrations and combinations of BAP, 2iP, Kn either alone or with NAA. Highest shoot regeneration from the callus was achieved in modified MS fortified with BAP at 1.33 μM level. The regenerated shoots were rooted *in vitro* in half-strength of liquid MS supplemented with various levels of NAA (0.54 - 10.7 μM) or IBA (0.49 - 9.85 μM). However, roots of superior quality were obtained at 5.4 μM NAA level after two weeks in culture. The regenerants were acclimatized for 2 - 3 weeks and about 85% survival rate was observed after transferring to the field. Both chromosome number stability as well as stability in nuclear DNA contents of the regenerants of all these strains was recorded with complete absence of aneuploidy. The different strains have revealed two to three-folds increase in L-DOPA contents in the cultured plants. The L-DOPA concentration in leaves of the regenerated plants varied from11.85 - 15.42 mg/g dry weight in all these accessions. However, the highest amount was observed in the wild strain of *M. pruriens*. This is the first report on enhancement of L-DOPA content in differentiated tissue of cultured plants of both the varieties of *M. pruriens*.

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Introduction

3-(3,4-dihydroxyphenyl)-L-alanine (L-DOPA) is a naturally-occurring dietary supplement and psychoactive drug found in certain kinds of food and herbs and is synthesized from the essential amino acids L-phenylalanine and L-tyrosine. L-DOPA is the precursor to the neurotransmitters dopamine, norepinephrine (noradrenaline), and epinephrine (adrenaline) collectively known as catecholamines. Dopamine is formed by the decarboxylation of L-DOPA. In addition to its natural and essential biological role, L-DOPA is also used in the clinical treatment of Parkinson's disease and dopamine-responsive dystonia. L-DOPA is used to increase dopamine concentrations in the treatment of Parkinson's disease and dopamine-responsive dystonia since it is capable of crossing the protective blood-brain barrier, which dopamine itself cannot. Once L-DOPA has entered the central nervous system, it is converted into dopamine by the enzyme aromatic L-amino acid decarboxylase, also known as DOPA decarboxylase. The occurrence of this unusual amino acid (L-DOPA) in the seeds of different plant species like *Vicia faba, Mucuna pruriens, Baptisia* species and *Lupinus* species is well-known (Guggenheim 1913, Daxenbichler et al. 1971). Brain (1976) reported that L-DOPA was accumulated in solid media on which *Mucuna pruriens* calli were grown.

Since all parts of the plant produces L-DOPA, there is an ever increasing demand of *Mucuna* in the international drug market. Large scale extraction of L-DOPA from the wild populations of this plant has led to limited availability of this plant in natural condition. The plant usually propagates through seeds and the germination rate and viability of seeds are very poor. Therefore, *in vitro* organ culture and callus (Lahiri et al. 2006) may be an effective alternative for propagation and conservation of plants of such an economic importance in which conventional methods show limitations. Two varieties of *M. pruriens* (vars. *pruriens* and *utilis*) are available in India. *M. pruriens* var. *pruriens* is found 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. 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 (Hammerton 2003).

In the present study, an attempt has been made to induce shoot bud multiplication and subsequent rooting to produce plants rapidly in culture in two varieties (four accessions) of *Mucuna pruriens* L. L-DOPA accumulation in the seeds and leaf tissues (both *in vivo* and *in vitro* plants) of the different strains

of *Mucuna pruriens* has been analyzed to assess the increment of L-DOPA production in the *in vitro* regenerates.

Materials and Methods

Seeds of three accessions of *Mucuna pruriens* var. *utilis* [IC 471876 (I), IC 241679 (II) and IC 392338 (III)] were obtained from NBPGR, New Delhi. Plants of one accession of *Mucuna pruriens* var. *pruriens* (IV) were collected in wild from West Midnapur district of West Bengal, India. The seeds were collected from dried pods of these plants for further study.

Seeds were surface sterilized by treating with 0.1% (w/v) aqueous $HgCl_2$ solution for 15 min followed by thorough washing in sterile distilled water. The seeds were then cultured on MS with 3% (w/v) sucrose, and 0.25% (w/v) Gelrite®, adjusted at a pH of 5.7 and without any exogenous growth regulator. Modified MS with 3% (w/v) sucrose, 0.1 g/l each of glutamine and ascorbic acid along with various concentrations and combinations of different growth regulators were used for multiplication. The cultures were incubated in a culture room at 24°C ± 1°C and 55% maximum relative humidity, under a photoperiod of 16 hrs supplied by cool white fluorescent tubes providing a light intensity of 48 μmol/m^2/s. The nodal segments of the 7-day-old *in vitro* grown seedlings were cultured in modified MS with various concentrations and combinations of BAP, 2iP, Kn either alone or with NAA.

All the cultures were maintained by subculturing at every four weeks interval on the same medium and culture conditions. Each treatment was repeated three times with 5 replicates of each. Data were recorded at 30 days interval up to 150 days. The square root transformations of the data were submitted to ANOVA using the software SPSS 16, to detect significant differences between means. To ensure normality and equal variance among groups, the square root transformation of the data was used. Square root transformation for count data is one of the applications of the general data transformation theory (Sakia 1992).

For rooting, the regenerated plants were cultured on full and half-strength MS (both liquid and semi-solid) and also on modified half-strength MS (both liquid and semi-solid) supplemented with various levels of either NAA (0.54 - 10.7 μM) or IBA (0.49 - 9.85 μM).

The completely developed healthy plants were separated individually and washed carefully under tap water to remove all traces of media. The *in vitro* roots were cut to facilitate *ex vitro* root development. The plants were then potted in sterile Soilrite® mixture (mixture of horticulture grade expanded perlite, Irish

Peat moss and exfoliated vermiculite in equal ratio) at 24 ± 1°C, with spraying of MS salts solution every alternate day to acclimatize for two - three weeks. These were further transferred to soil.

Chromosome analysis of five randomly selected multiplied plants was carried out from the root tip cells following aceto-orcein squash technique (Mukhopadhyay and Banerjee 1989) to determine ploidy status. At least 10 well-scattered metaphase plates from each slide were analyzed for determining the somatic chromosome number of the regenerants. 4C and 2C nuclear DNA contents of the clones were compared with their mother plant following micro-spectrophotometry and flow cytometry, respectively. Microspectrophotometric analysis was done following Lahiri et al. (2010) using a Leitz Wetzlar Aristophot microscope. Flow cytometric analysis was done according to Johnston et al. (1999) in a Becton Dickinson FACS Calibur (Becton Dickinson, San Jose, USA), using propidium iodide as the fluorochrome.

Extraction of L-DOPA was made according to Brain (1976). 1.0 g of tissue and 4 ml of 0.1N HCl was added to it. The tissue was ground to a fine paste in a mortar and pestle. The mixture was heated on a boiling water bath for 5 min. It was cooled to room temperature and equal volume of ethanol was added. The mixture was shaken vigorously for 10 min and then centrifuged at 2000 rpm for 10 min. The supernatant was retained and the residue was again re-extracted with a further equal volume of ethanol. The supernatants were then combined and made up to a known volume with ethanol.

The extracts were analyzed for detection of L-DOPA by TLC along with a authentic sample on to a 20 × 20 cm, 0.25 mm thick, Silica gel 60 F254 analytical TLC plate (Merck Co. Ltd., Darmstadt, Germany) using a mixture of iso-propanol : ethyl acetate : water : acetic acid (20 : 19 : 10 : 1) as solvent, in an unsaturated chamber (Brain 1976, Huizing et al. 1985). The spots were developed by spraying the dried plate with 0.5% ninhydrin in 1 : 1 mixture of butanol : acetone and heating at 110ºC for 10 min. The L-DOPA content was quantified through UV-spectrophotometry (JENWAY UV/VIS 6500) by measuring the UV absorption at 280 nm (Brain 1976, Padmesh et al. 2006).

Results and Discussion

In the present study BAP, Kn and 2iP were utilized individually and in combination with NAA at different levels for shoot bud multiplication in all the four strains of *Mucuna pruriens*. The different concentrations of the cytokinins were used individually and in combination with NAA (0.54 μM). Both BAP and Kn at low concentrations showed shoot bud multiplication without any callus formation at the base, whereas simultaneous callus formation inhibited the

multiplication rate at higher concentrations. Among these concentrations, BAP 0.88 μM gave the best response in all the four strains. However, accession III of var. *utilis* produced maximum number of shoots (29.6 shoots/explant) after 150 days in culture. The only accession of var. *pruriens* also produced high number of shoots in BAP (28.6 shoots/explants). On the other hand, Kn did not support shoot bud multiplication as much as BAP at the same concentration. Overall, the response was milder than that of BAP. It was also observed that with the increase in concentration of the growth regulator, the rate of multiplication gradually decreased.

Among the three cytokinins used individually, 2iP was least effective in inducing shoot bud multiplication. The number of shoot buds induced in different 2iP in media was less than 50% of the number of shoot buds produced in similar concentration of BAP (data not shown). Using BAP + NAA, Kn + NAA, 2iP + NAA did not favour the multiplication process. Presence of NAA in the medium produced significant amount of callus at the base that inhibited the multiplication process. Callus formation initiated after about 10 - 12 days in culture. Maximum callus formation was observed in medium containing 2ip and NAA. Therefore, studies using 2iP individually and in combination of hormones were not further continued.

The ANOVA table shows that the responses of the plants to the different treatments are highly significant (Tables 1, 2). It was also observed that the interactions of time*treatment and strain*treatment were significant (Tables 1, 2). This might be due to responses of all the four strains were not the same. However, the interaction between time*treatment*strain was not significant indicating that all treatments behaved the same notwithstanding the time and the strain (Tables 1, 2). The LSD analysis (Table 2) showed that the response of the strains in the media containing BAP (0.88 μM) was significantly different from the responses to the other media during the entire period. Sathyanarayana et al. (2008) reported the highest multiplication rate using a specific concentration of BAP that differs from the present report. The genotypic differences between the strains might have been responsible for such difference in responses. Moreover, the requirement of different levels of cytokinins might be correlated with the differences in the endogenous levels of hormones in different genotypes (Mukhopadhyay et al. 2002). The efficiency of BAP in shoot bud multiplication has also been demonstrated in other plant species (Benmoussa et al. 1997).

The induction of roots from the multiplied shoots in presence of low concentration of MS (half-strength) with only NAA has been suitable. MS without any growth regulator showed a delayed and weak response. Though root formation from regenerated shoots was induced at IBA (0.98 μM), higher

concentrations of IBA (4.9 and 9.8 µM) were found to be less effective in this process. On the other hand, NAA at lower concentrations (0.54 and 1.07 µM) was not very effective for *in vitro* root induction. Large numbers of stout and healthy roots were formed at NAA (5.4 µM), after 2 weeks in culture, in all the strains studied (Fig. 1c). However, further increase in NAA concentration did not favour *in vitro* rooting. The favourable response of NAA in root induction from shoots *in vitro* has been reported for this plant (Sathyanarayana et al. 2008) as well as for other plant species (Chithra et al. 2004, Rauf et al. 2004).

Table 1. Three-way ANOVA with multiple observations in each category on shoot bud multiplication in four different strains of *Mucuna pruriens*.

Source	DF	F value	P value
Time	4	1296.70	0.0001
Treatment	7	889.50	0.0001
Strain	3	1.12	0.3415
Time*treatment	28	15.00	0.0001
Time*strain	12	0.55	0.8790
Treatment*strain	21	14.38	0.0001
Time*treatmen*strain	84	0.52	0.9999
Error	640		

Table 2. Responses of different concentrations of growth regulators on shoot bud regeneration from callus tissue of *Mucuna pruriens*.

Modified MS + GR (µM)	Mean number of shoot buds in four strains of *M. pruriens* (days)				
	30	60	90	120	150
BAP [0.44] (Treatment 1)	2.95	6.85	9.05	9.75	10.55
BAP [0.88] (" 2)	6.7	18.1	23.1	25.5	26.55
BAP [4.44] (" 3)	5.65	11.65	16.3	17.95	19.05
BAP [8.88] (" 4)	5.5	10.35	14.65	15.5	16.15
Kinetin [0.46] (" 5)	2.2	4.35	6.05	6.9	7.4
Kinetin [0.93] (" 6)	5.1	10.1	14.85	16.2	16.7
Kinetin [4.65] (" 7)	3.6	6.8	8.55	9.2	9.65
Kinetin [9.30] (" 8)	3.15	5.75	7.25	7.85	8.2
LSD values	0.7386	0.9937	1.1502	1.2228	1.2114

The high survival rate (85%) of the regenerated plants in the field also indicates the superiority of anatomical and morphological nature of the roots and the suitability of both physical and chemical environments in culture (Fig. 1d).

Chromosome number stability has been observed in all the strains regenerated plants of *M. pruriens*. They showed 2n = 22 chromosomes (Fig. 1e), as

previously reported (Lahiri et al. 2010). The 4C and 2C nuclear DNA contents, estimated by Feulgen microspectrophotometry and flow cytometry, respectively did not show much variation from the parent plants (Table 3) and thus indicated genomic stability of the regenerates. This might be due to the cells during regeneration were not exposed for long period to potent auxins. Also, the type and concentration of exogenous auxin, as well as the duration of culture might have been responsible for such stability (Mukhopadhyay and Desjardins 1994). In the present study, the multiplication medium contained only a cytokinin without any auxin which might have been responsible for maintaining stability, both at the chromosomal and nuclear DNA levels of the regenerates.

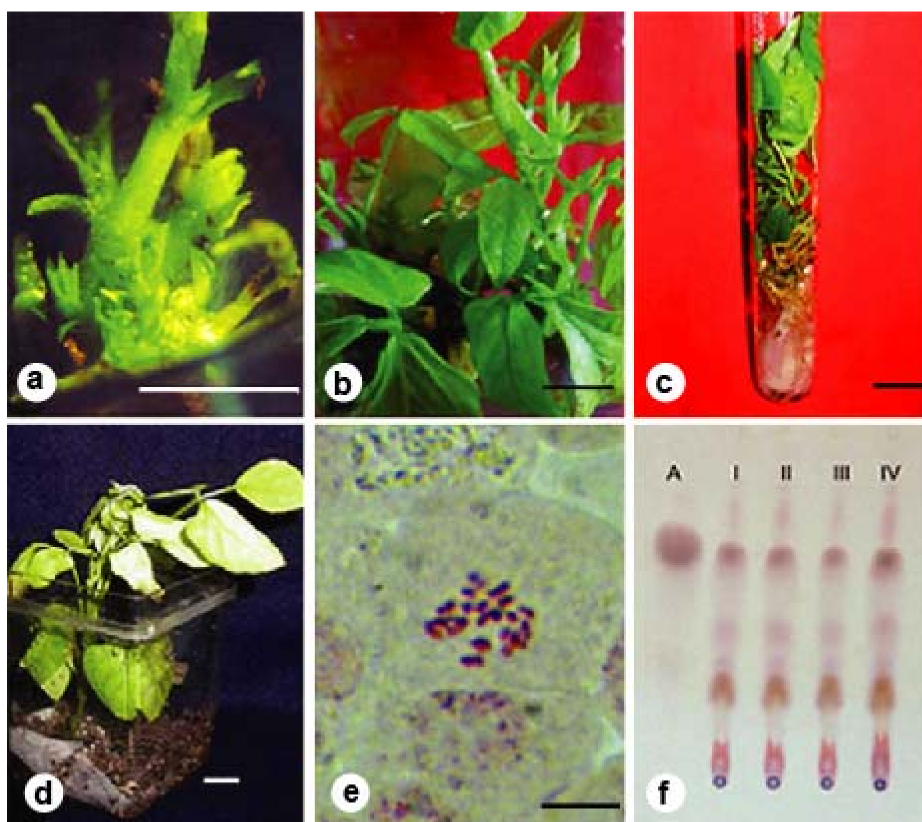

Fig. 1. Shoot bud multiplication, complete plant regeneration and L-DOPA analysis of regenerants of different strains of *Mucuna pruriens*: (a) Multiplied shoots in presence of MS + BAP (0.88 μM) after 30 days in culture (bar = 10 mm). (b) Elongated shoots in half-strength of MS (bar = 10 mm). (c) Rooting of the regenerated shoots in liquid half strength of MS basal medium + NAA (5.4 μM) (bar = 10 mm). (d) Hardening and acclimatization of complete regenerated plants in sterile Soilrite® mixture (bar = 10 mm). (e) Somatic metaphase plate of the regenerate showing 2n = 22 chromosomes (bar = 10 μm). (f) TLC plate showing presence of L-DOPA in the regenerates of all the strains. (Lane A: Authentic compound; Lane I: Strain I; Lane II: Strain II; Lane III: Strain III; Lane IV: Strain IV).

Chromatographic examination (TLC) of the extracts revealed a spot with the same mobility and colour characteristics as reference L-DOPA. The Rf value was 0.62 (Fig. 1f). The quantitative estimation by UV spectrophotometry revealed that the seeds of all the strains of *Mucuna pruriens* contained a much higher amount of L-DOPA than the *in vivo* plants and *in vitro* regenerants. In the seeds, the amount of L-DOPA among the different strains varied from 37.50 - 50.86 mg/g dry weight (Table 4). The amount was highest in accession IV of the var *pruriens* and lowest in accession III of the var. *utilis*. In the leaf tissue of the *in vivo* plants the amount varied from 4.24 - 5.52 mg/g dry weight. Here too, the amount was highest in accession IV of the var. *pruriens* and lowest in accession III of the var. *utilis* (Table 4). In the leaf tissue of the *in vitro* plants the amount of L-DOPA varied from 11.85 - 15.42 mg/g dry weight. The amount was highest in accession IV of the var. *pruriens* and lowest in accession III of the var. *utilis* (Table 4).

Table 3. 4C and 2C nuclear DNA contents of *in vivo* and *in vitro* regenerated plants in different strains of *Mucuna pruriens*.

Strain	4C nuclear DNA content (pg)*		2C nuclear DNA amount (pg) **	
	In vivo plant	*In vitro* regenerants	*In vivo* plant	*In vitro* regenerants
I	25.93 ± 0.60	26.08 ± 1.37	13.06 ± 0.08	13.14 ± 0.05
II	26.23 ± 0.56	26.16 ± 0.56	13.41 ± 0.05	13.50 ± 0.03
III	29.25 ± 0.55	29.47 ± 0.47	14.94 ± 0.10	15.03 ± 0.07
IV	26.76 ± 0.51	26.91 ± 0.49	13.54 ± 0.04	13.60 ± 0.05

*Data represent Mean ± S.E. of 25 replicates. **Data represent Mean ± S.E. of 5 replicates.

Table 4. L-DOPA contents in different tissues of four different strains of two varieties of *Mucuna pruriens*.

Plant tissue	Strain	L-DOPA content* (mg/g dry wt)	L-DOPA content (% dry wt)
Seeds	I	41.50 ± 0.26	4.15
	II	37.50 ± 0.35	3.75
	III	44.97 ± 0.47	4.49
	IV	50.86 ± 0.39	5.08
In vivo leaf tissue	I	4.88 ± 0.12	0.48
	II	4.24 ± 0.13	0.42
	III	5.10 ± 0.09	0.51
	IV	5.52 ± 0.16	0.55
In vitro leaf tissue	I	13.10 ± 0.07	1.31
	II	11.85 ± 0.11	1.18
	III	15.22 ± 0.12	1.52
	IV	15.42 ± 0.17	1.54

*Data represent mean ± S.E. of 5 replicates.

The different strains have revealed two to three-folds increase in L-DOPA contents in the regenerated plants. There are a few previous reports where L-DOPA accumulation has been studied in undifferentiated cell cultures (Brain 1976, Wichers et al. 1993, Huizing et al. 1985). The L-DOPA accumulation has been found to be influenced by some medium components including sucrose, calcium chloride, potassium dihydrogen phosphate besides L-tyrosine (Wichers et al. 1985). Sucrose in the nutrient media has an influence on production of secondary metabolites as it enhances the production of certain amino acids like L-phenylalanine (Sujanya et al. 2008). However, high sucrose level in the medium has a negative influence too and results in reduction in the secondary metabolite level (Ibrahim et al. 2009). It is also reported that L-phenylalanine level in culture is also influenced by different forms of cytokinin (Taha et al. 2008). Earlier, we also reported the efficacy of BAP in the culture medium in both promoting shoot multiplication and increased colchicine accumulation in micropropagated plants of species of *Gloriosa* and *Iphigenia* (Mukhopadhyay et al. 2008, Mukhopadhyay and Mukhopadhyay 2008). Moreover, the L-phenylalanine acts as a precursor for both L-DOPA and colchicine.

The *in vitro* plants grew in a nutrient rich condition with optimum levels of sucrose and ions required for increment of L-DOPA production (Brain 1976, Wichers et al. 1983). It has also been previously reported that light has a strong beneficial effect on the endogenous production of L-DOPA in cultured tissues (Wichers et al. 1985). Therefore, it indicates that the *in vitro* environmental condition (both physical and chemical) in the present study might have been responsible for increased L-DOPA contents in the regenerants. Moreover, the multiplication medium did not contain 2,4-D, which has a negative effect on L-DOPA production (Wichers et al. 1993).

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