Agriculture

Somatic Embryogenesis and Quantification of Levodopa in *Mucuna pruriens* (L.) DC - A medicinal legume

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Abstract

Secondary metabolite production can be achieved by using Plant cell culture method as a choice. The plant *Mucuna pruriens* (L.) DC an annual legume is rich in an exceptionally important amino acid called L-Dopa, 3-(3,4-dihydroxyphenyl)-L-alanine. L-Dopa is a very important compound from the point of pharma and medicine. The present study reports the somatic embryogenesis induction in *Mucuna pruriens* (L.) DC. The study includes the collection of nodal and internodal samples from the mature plant and with them, the explant was prepared by culturing on MS basal medium. The shoot tip explants obtained from them was then carried out for the induction of callus formation on MS medium along with containing the different hormones like 2,4-D, NAA, 2,4-D + NAA, and 2,4-D+BAP. The callus with the best features was carried forward for the somatic embryogenesis process followed by its germination in both solid and liquid medium. The well propagative somatic embryos were then taken forward for acclimatization and finally transferred to the fields. The plants were then analyzed for the presence and quantitative analysis of L-Dopa in them via extraction from tissue and quantification by HPLC method. The conclusion driven from the study is that the somatic embryogenesis is a very better way for large scale and high yielding propagation of *Mucuna pruriens* (L.) DC as wells as it yields the plantlets with good L-Dopa concentration, which is itself a pharmacologically important compound.

Keywords: *Mucuna pruriens* (L.), levodopa, Capo chichi

Introduction

The family Fabaceae undercovers the genus *Mucuna* and under this family around 150 different species of the annual and perennial legumes belonging to the pantropical distribution are found. One of the important member of this family, *Mucuna pruriens* (L.) DC, is under intense investigation due to the potential application of this plant to be used as green manure, food crop, cover, and feed crop (Capo chichi *et al*., 2003). The plant is known to show a vibrant growth in vivid agro-climatic conditions and they produce biomass in a large amount of up to 10-12 t Dm ha-1 and they also give a good seed yield of 2000 kg/ha-1/year. Although it has such important attributes the farmers for cultivation due to lack of its use as food and feeds poorly adopt the plant. The major bottleneck for its consumption is the availability of an anti-nutritional factor in its seeds, called Levodopa (L-3, 4 dihydroxy phenylalanine). The L-Dopa is considered as an important medicinal plant and it is implemented for treating the Parkinson's disease, along with the good effects, when consumed as a portion of food it produces some dangerous side effects like anorexia and nausea (Tebett, 2000). L-DOPA-3-(3,4-dihydroxyphenyl)-L-alanine, occurs naturally and is considered as the dietary supplement. L-DOPA is also considered as the psychoactive drug that is present in some specific types of herbs and food. This compound is synthesized biosynthetically by the two essential amino acids that are L-phenylalanine and L-tyrosine. As collectively called the Catecholamines, L-DOPA acts as the precursor of the neurotransmitters like epinephrine (adrenaline) dopamine, and norepinephrine (noradrenaline). The decarboxylation of L-DOPA causes the formation of Dopamine. The marketed products of L-DOPA include the tablets which are formulated under the brand names like Sinemet, Atamet, Parcopa, and Stalevo (Ali and Haq, 2006). Across the world, the marketing for L-DOPA is estimated to be 250 t/year that overall gives the total market volume to around $101 billion/year (Koyanagi *et al*.,2005). The seeds from *Mucuna pruriens* (L.) DC as well as the allied species of this plant are the major natural source for L-DOPA. They are being used widely for obtaining L-DOPA as its synthesis via chemical process is highly cost-consuming and also come across the problems like the disadvantage of the racemic mixture inhibiting the activity of dopa decarboxylase in the human body (Krishnaveni *et al.,* 2009).

It is a well-known fact that the compound L-DOPA occurs in the seeds of different plant species and these species include *Vicia faba, Mucuna pruriens,* and *Lupinus* species (Daxenbichler *et al*. 1971). The reports have shown that L-DOPA gets accumulated in the solid culture media used for the propagation of *Mucuna pruriens* (L.) DC calli (Brain, 1976). All parts of this plant acquire a certain concentration of L-DOPA and this makes this plant to be under the higher demands even in the international drug market. The production of L-DOPA is usually carried out by the consumption of the wild population of this plant and this has led to the limitations in the population of this plant in its natural habitat. The plant is known to germinate by seeds, naturally and its rate of germination and seed viability is very poor, creating a major drawback in plant population. To overcome such problems, an effective alternative method for the propagation and conservation of this plant could be the callus and in vitro organ culture techniques (Lahiri *et al*., 2006), these methods as important for the implementation to conserve this plant with

such economical importance as there are limitations in the traditional methods.

Material and Methodology

Collection and sterilization

The collection of healthy of *Mucuna pruriens* (L.) DC. was done from the local area of Hathwa and plain area of Kuchikot of District Gopalganj. The plants were collected, cleaned thoroughly, and sorted. The parts selected for the explant preparation were nodes and internodes, which were separated carefully from the collected samples. They were cleaned thoroughly for 30 minutes using the tap water followed by a 15 minutes treatment with 5% (v/v) of labolene detergent and again washed thoroughly with water. Nodes and internodes were surface sterilized by dipping them in an aqueous solution of 0.1% (w/v) of mercuric chloride for 5 minutes then double rinsed with double distilled water thoroughly. Now these sterilized nodes and internodes samples were inoculated aseptically on Murashige and Skoog (1962) basal medium for the germination. From the 7-8 days old culture the shoot tips were excised and used as explants for the callus induction.

Media and culture conditions

The MS salts were used as macro- and micro-nutrient, 3% sucrose (w/v) as carbon source, and 0.8% of agar used as solidifier for all culture media. Before autoclaving at 121°C (15 lbs) for 20 min, the pH of the culture media was adjusted to 5.8. incubation of all cultures was done under the controlled environment provided in the culture room having 50 µmol m^{-2} s^{-1} light emitted through a white cool fluorescent lamp (40 W, Philips, India). The duration for photoperiod was 16/8 h (day/night) and the temperature is maintained at 25±2°C.

Callus induction

The explants obtained from the node and internode cultures were now used for the callus induction process. For this, the explants inoculation was done on the MS medium that was enriched with 3% sucrose (w/v) and for the solidification of the medium 0.8% agar was used. The MS basal media was also supplemented with variable concentrations and a combination of the growth hormones. The different concentrations included 2,4-D (2.24-45.50 µM), NAA (2.22-7.34 µM) and the combination used were of 2,4-D (4.26-22.62 µM) + BAP (3.5-10.13 µM) and 2,4-D (2.26-25.62 µM) + NAA (0.5-15.43 µM). For each type of hormonal treatment, the 10 different setups were prepared to determine the best concentration at which the response of callus growth is best.

Somatic embryo induction and germination

The Somatic embryogenesis in the callus generated from both the explants was initiated by sub-culturing the callus in both the solid and liquid cultures having different and decreasing concentrations of 2,4-D. The media used for this purpose had the additional components like 1mg L^{-1} of glutamine and some growth adjuvants that include 2 g l^{-1} of casein hydrolysate, 2 g l^{-1} of malt extract and 5% v/v of the coconut milk. For the germination of the embryo, the embryo at the light green cotyledonary stage that was formed after 25-30 days, were transferred on both the full and half-strength MS solid medium. The embryo germination medium was supplemented with the antioxidants to prevent the browning effects on the embryo culture and the most commonly used antioxidants are 0.25-1.0% of charcoal and 0.0-0.1 mg l^{-1} of ascorbate.

Hardening of plantlets

To maintain the cultures in the proper form they were sub-cultured at an interval of four weeks and for this purpose, the same culture media and culture conditions were applied. When the plantlets of 3-4 cm height with well-developed roots were ready after 20 days, they were plucked gently from the medium and thoroughly washed for 2-3 minutes with running water to remove medium traces. The pots were prepared for the transfer of these plantlets onto them, for this, the pots were filled with an autoclaved mixture of compost and peat moss in 1:1 ratio. The plantlets were transferred carefully on the pots and to maintain the humid environment these pots were covered with polythene bags. As the initial hardening of these plantlets gets completed in the growth chamber that is after 2 weeks, then before transferring these plantlets in the field they were transferred to another pot (15 cm) for 30 days. These pots were filled with a mixture of soil and sand in the ratio of 1:1 after autoclaving.

Extraction of L-DOPA from plants

The methodology described by Myhrman (2002) was used for the extraction of L-DOPA from the samples by applying certain appropriate modifications to the requirement. For this appx. 1 gm of tissue was taken and crushed into a fine paste using mortar and pestle with the addition of 10 ml distilled water. For obtaining the extract the final content was boiled for 10 min and then cooled. The cooled extract was collected in centrifuge tubes and centrifuged at 5000 rpm for 10 minutes. The supernatant liquid collected from this was taken as the extracted L-DOPA and used for further analysis and estimation.

Analysis and quantification of L-DOPA

The extracts obtained from all the different samples were used for the detection of L-DOPA in them using TLC analysis carried along with the standard compound on a thick silica gel plate (60 F254) of 20x20 cm dimension and 0.25 mm thickness. The mobile phase that is the mixture of isopropanol: ethyl acetate: water: acetic acid (20:19:10:1) was added in an unsaturated chamber (Brain 1976, Huizing *et al*. 1985). For the development of L-DOPA spots, the dried plates were sprayed with 0.5% ninhydrin that was prepared in a solvent mixture of butanol: acetone (1:1), and after this the plates were heated at 110ºC for 10 minutes for the appearance of spots. This was followed by the quantification of L-DOPA in all the extracts by the high-performance liquid chromatography (HPLC). The HPLC analysis used a C-18 column supplemented with a guard pre-column having the same material as in the main column and a dual absorbance UV detector. The samples were loaded using the manual sampler injector. The mobile used for the Isocratic elution was a combination of different solvents like water:methanol: phosphoric acid in the ratio of 975.5:19.5:1 (v/v) as also described by Siddhuraju and Becker (2001). The volume of the sample used for analysis was 20 µl and this separation was performed at RT (25◦C) keeping the flow rate of the mobile phase at 1.2 ml^{-1}. Detection of compounds was done at 282 nm followed by the determination of peak heights and areas. The sample elute was collected between the third

and fourth minutes. A standard curve was used for calculating the concentration of L-DOPA in the tissue extracts. The concentration of L-DOPA was expressed as mg g^{-1} tissue dry weight. Before the loading, all the samples were passed through the glass-filter (0.20 µm pore size) to remove any contaminant that could choke the column.

Result and Discussion

The sterilized samples of nodes and internodes were inoculated under in vitro condition and they showed the germination within 4 days on MS basal medium with no added sucrose. Among all the inoculated samples maximum positive response was obtained for both the examples. A synchronized germination pattern was exhibited by the seeds like all the shoots within 8 days attained a height of 6-7 cm. In the case of the callus induction carried out from the explanation obtained from the above samples, the callusing initiation time duration was variable with different explants. While, around 6 days the initiation of callusing was observed in the nodal sample, and the explant from the internodal sample had a slow callus formation rate as the callus was seen only after 10 days. Nevertheless, the subsequent subculturing showed that these callus from both the explant exhibited a similar proliferation rate. The two hormonal concentrations that are MS+2,4-D (13.31 µM) and MS+NAA (5.37 µM)+2,4-D (4.53 µM) were found to be the best for the proliferation. Rhizogenesis was stimulated by NAA added medium from a callus in subsequent sub-cultures. The characteristic features and the analysis of the callus carried out obtained from this stage are summarized in table no. 1. The callus showing the best propagation ability was carried further for the somatic embryogenesis.

MS basal media supported the best embryo maturation in the callus transferred to it. This process was related to the explant factor. Among all the explants used in the study, the maximum number of the embryonic structures were produced from the nodal callus. Whereas the better development and synchronization with a maximum number of embryos reaching the cotyledonary stage were observed in the explants of the internodal origin. At around 11-15th day of the period the switch or the transformation of the pre-embryonic mass into globular structures was seen. At this point of germination, it took around 10-15 days for the completion of a complete progression to reach the cotyledonary embryos stage. The embryos appeared to be progressively moving on for germination as they turned green between the 25-30th day on maturation medium. As observed within 4-5 days all the embryos developed the reddish-brown shade and the color progressively turned darker but after this nearly around 120 days these cells continued to exhibit the active cytoplasmic streaming. To resist the browning the explants were transferred to the medium having variability in their composition, hormonal concentration, and pH ranges but it was observed that the browning was irreversible even after these efforts.

Table 1: Response of different explants of Mucuna pruriens (L.) DC on MS basal media supplemented with variable conc. of different growth regulator with a culture period of 21 days and 10 replicates

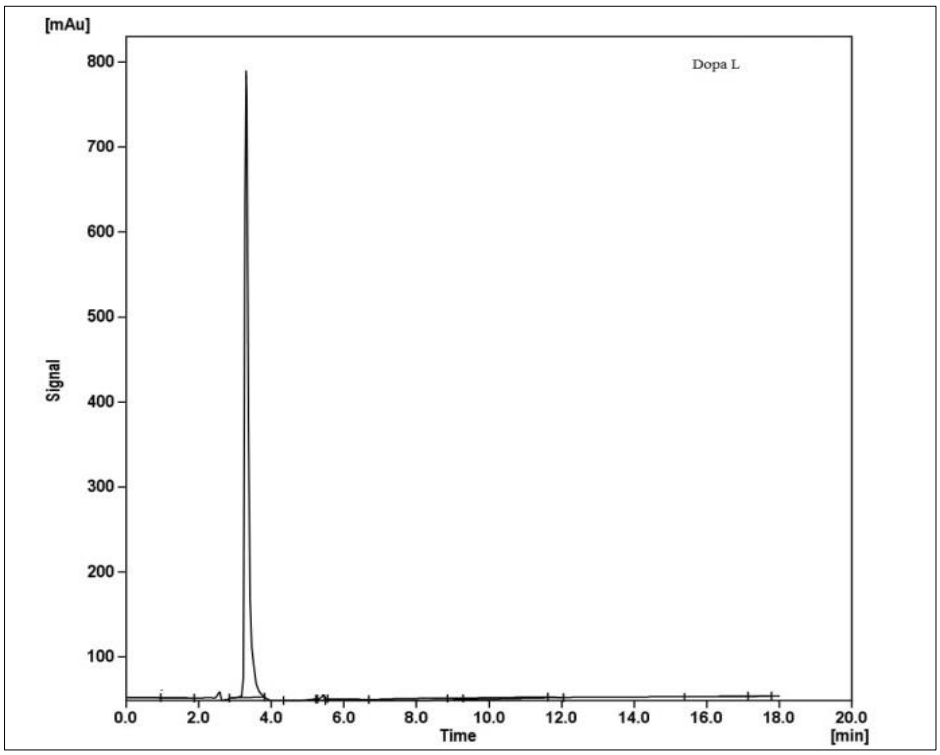
Growth regulator	Explant	No. of Explants cultured	No. of respond explants cultured	% Of Response	Av. Fresh wt. of callus (gm)	Av. Dry wt. of callus (gm)	Shoot length (cm)
2,4-D	Node	10	08	80	10.83	1.20	1.67 ± 0.58
	Internode	10	07	70	12.54	1.32	1.58 ± 0.00
NAA	Node	10	05	50	11.98	1.11	1.00 ± 0.00
	Internode	10	06	60	12.01	2.03	1.10 ± 0.29
2,4-D + BAP	Node	10	06	60	11.98	1.50	1.16 ± 0.29
	Internode	10	06	60	10.75	1.00	1.33± 1.04
2,4-D + NAA	Node	10	08	80	10.98	1.32	1.79 ± 0.58
	Internode	10	07	70	10.75	1.03	1.67 ± 0.00

A low survival rate of up to 60-70% was observed in the plantlets that were transferred directly to sand: soil mixture (1:1) from the rooting medium. To surpass this low survival rate of the plantlets, before transferring them on the sand: soil (1:1) mixture they were first transferred to an autoclaved mixture containing peat moss and compost mixture in equal proportion and maintained for 14 days in a growth chamber. These plantlets were kept in the sand: soil mixture pot for proper hardening for 30 days before finally transferring to the field. This application led to the 95% survival rate of the plants. Then the survived healthy plants were successfully transplanted to the soil.

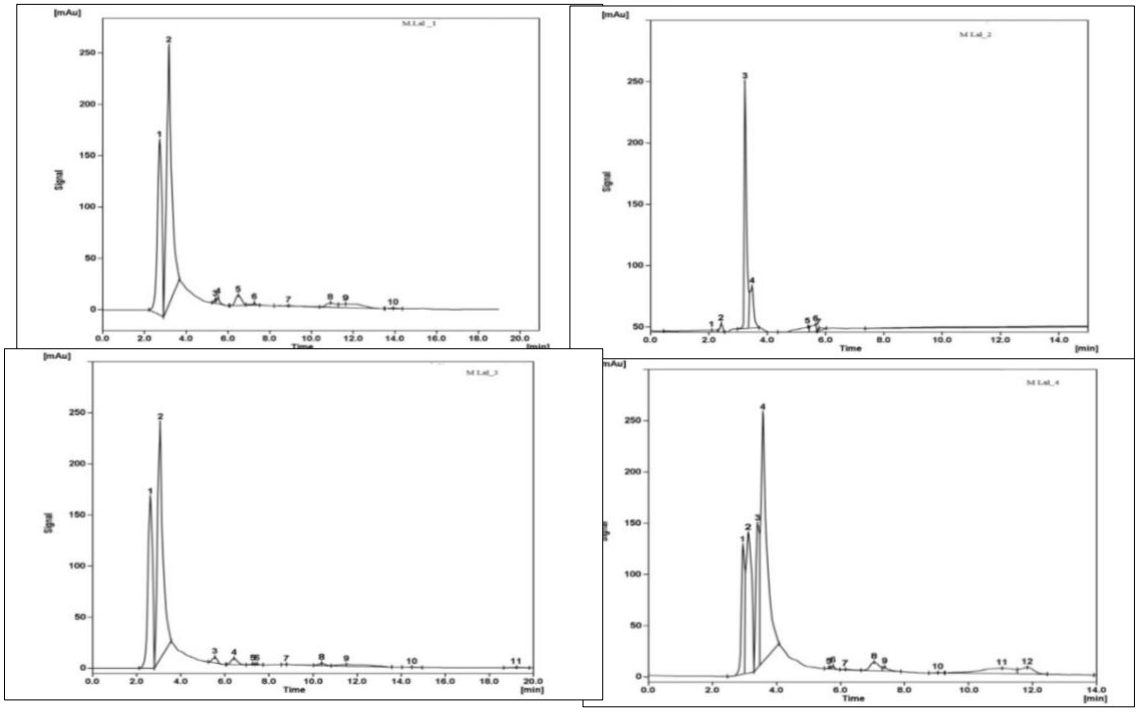
The plants obtained were used for the extraction and analysis of L-DOPA in them, for this, the extract prepared was undertaken the TLC and HPLC analysis along with a standard compound. The TLC plates when observed showed a spot in the sample extract that was similar in its mobility and color with the reference L-DOPA compound and its Rf value was 0.62. The comparative description of the chromatogram of standard (graph no. 1) and the sample extracts (graph no. 2-5) showed the presence of L-DOPA in them. The standard L-DOPA gave a retention peak at 3.27 minutes and all the samples extract when analyzed showed a remarkable peak at this particular retention time. The concentration of L-DOPA was calculated with the help of the standard curve and the result is summarized in table no.2. The concentration at which maximum callus induction was obtained also gave the extracts with the maximum L-DOPA concentration in them.

Table 2: Concentration of L- DOPA in the extract of Mucuna pruriens (L.) DC plant has grown on variable conc. of different growth regulator

Growth regulator	Explant	Concentration of L-DOPA mg/gm of tissue
2,4-D	Node	7.23 ±0.32
	Internode	6.45 ±1.20
NAA	Node	2.36 ±0.32
	Internode	4.59 ±1.23
2,4-D + BAP	Node	5.43 ±0.23
	Internode	4.63 ±1.11
2,4-D + NAA	Node	8.34 ±0.22
	Internode	9.03 ±1.22



Graph 1: Chromatogram of L-DOPA standard compound (1mg/ml)



Graph 2-5: Chromatogram of extracts obtained from different plant samples of Mucuna pruriens (L.) DC showing the presence of L-DOPA in them; 1- from nodal explant (MS+2,4-D); 2- form internodal explant (MS+NAA); 3- from the nodal and 4- from the internodal explant (2,4-D + NAA)

The lack of implementation of transgenic technology for the improvement of genetic makeup in plants is because there is no availability of potential systems for regenerating a variety of grain legumes (Chandra and Pental, 2003). An ideal and effective tool is represented by the system of embryogenesis for an in vitro production and selection of such transgenic plants (Christou, 1997). Few grains are under the use of this type of effective method and these grain legumes include *Glycine max* (Finer and Nagasawa, 1988), Chickpea (Kiran *et al*., 2005), and Horsegram (Varisai *et al*., 2004). All these cases have majority shown the use of 2,4-D for induction of embryogenesis in callus that has resulted in an established outcome (Anbazhagan and Ganapathi, 1999), and the present finding also showed a better result at this. The prevalence of 3,4-dihydroxy phenylalanine (L-DOPA), a non-essential amino acid is a characteristic feature of the species belonging to the genus *Mucuna*. Other grain legumes that are also been reported to contain L-DOPA include *Vicia, Baptista,* and *Lupinus* (Daxenbichler *et al*., 1971). In the case of *Mucuna*, the accumulation of L-DOPA is prominent and major in the seeds appx 4-6% whereas the stem root and leaf also contain it in lower concentration (Szabo and Tebett, 2000). The synthesis of L-DOPA starts with the precursor of tyrosine in the step conversion process that is catalyzed by the tyrosine hydroxylase enzyme (Griffith and Conn, 1973). For the synthesis of black pigment melanin the L-DOPA act as the precursor molecule (Brain, 1976). There is a considerable amount of evidence available that suggests that under the in vitro conditions L-DOPA accumulates in the cells of *Mucuna pruriens* (L.) DC (Huizing *et al*., 1985). The process is known to be highly influenced by different hormones and some physical factors. The previous research findings have shown that positive influence is imparted by BA (Huizing *et al*., 1985) while the synthesis is inhibited by 2,4-D (Brain, 1976). Due to its high stability as soon as the L-DOPA is synthesized it gets converted to the category of a metabolite called O-quinones and the majority among them it forms dopamine and melanin are formed (Wichers *et al*., 1983). The physical evidence and observation of this phenomenon recognized by the browning of the callus preceded by a characteristic red shade on both callus and suspension cultures of *M. pruriens*, as observed in the present study as well. The antioxidants supplemented in the medium ail to prevent the browning of the callus and this may be due to the delayed supplementation of the reagents in the reaction mixture.

Conclusion

The present findings report a practicable and reproducible micropropagation protocol for *Mucuna pruriens* (L.) DC using shoot tip explants. The study provides us with an efficient and high fidelity performance to carry the mass propagation of *Mucuna pruriens* (L.) DC var. *utilis* explants. The can be successfully implemented to initiate the production of uniform, viable, and healthy plants having maximum survival rates. The large scale cultivation and also the *in vitro* manipulations can be achieved by the following study, along with this, it also reveals the accumulation of L-DOPA in the *in vitro* propagation. The study suggests that now there is a need for a comprehensive study to establish and determine the functions of L-DOPA and the changes in the enzymatic activities that are found to be involved with the process of oxidative stress developed during the *in vitro* process of development in *Mucuna*. These studies would provide an insight into the molecular mechanism that lies behind the embryo-browning and it will also provide us with the possible ways that could help to regulate and establish an efficient embryogenic system in *Mucuna pruriens* (L.) DC.

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