Bioitechnological Communication

BBRC Bioscience Biotechnology Research Communications

Biosc.Biotech.Res.Comm. Vol 13 No (4) Oct-Nov-Dec 2020 Pp 1906-1913

Optimization of Biomass Culture Yield in the Callus Culture of Mucuna pruriens Using Synergetic Combination of Auxins and Cytokinins

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ABSTRACT

The *Mucuna pruriens* (L.) DC is an important legume cover crop and it is commonly known as cow-age or cowitch or velvet bean or Alkushi, a medicinal plant traditionally used in Indian medicine which belongs to the family Fabaceae. Almost all the parts of the plant are reported to contain L-3,4-dihydroxy phenylalanine (L-DOPA). L-Dopa have high medicinal value and necessary characteristics features like high content of protein, easy digestibility similar to other pulses like soybean, rice bean, and lima bean. Therefore, it is also considered as rich source of food. Studies on its micro-propagation are up taken on high rate. In the present study, a rapid and efficient protocol for the large-scale propagation of a potential medicinal plant, *Mucuna pruriens* (L.) DC, through *in vitro* culture of different explants is described. Here we report a rapid and reliable method for high fidelity micro-propagation. The explant used under the study includes the node, internode, shoot tip and seed that were inoculated on the MS basal media after proper surface sterilization. The explants were incubated on the MS basal media supplemented with variable concentration of the growth regulators to determine the optimal concentration for each type of explant. The study concluded that the combination of auxin and cytokinin are very much efficient for the higher yield micropropagation of explants under the *in vitro* conditions. The higher concentration of these growth regulators could also stun the growth of growing callus hence optimum concentration knowledge is a must to get maximum propagating callus from any explants used under the study. Further study could focus on applying these optimum culture conditions for the propagation of explants of *Mucuna pruriens* (L.) DC and analyze their secondary metabolites which confers commendable medicinal importance.

KEY WORDS: MICROPROPAGATION, EXPLANT, *MUCUNA PRURIENS* (L.) DC., AUXIN, CYTOKININ.

INTRODUCTION

3-(3,4-dihydroxyphenyl)-L-alanine (L-DOPA) is one of psychoactive drug component which has its source from many natural food products and herbaceous plants. It acts as a precursor for many neurotransmitters likes dopamine, norepinephrine (noradrenaline), and epinephrine (adrenaline) collectively known as catecholamines. D. One of the examples of herbs that is a rich source of L-Dopa is *Mucuna pruriens* (L.) DC seeds. It is commonly called velvet bean belongs to the family Fabaceae and is a commercially important medicinal plant found in bushes and hedges, at damp places, ravines, and forests of Western and Eastern Ghats of India. India is one of the natural centers of origin of the *Mucuna* in the world. Rich genetic diversity coupled with wide-ranging traditional knowledge on various usage practices offers great scope for biotechnological improvement of the *Mucuna* species

Article Information:
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Received 20/10/2020 Accepted after revision 19/12/2020
P-ISSN: 0974-6455 E-ISSN: 2321-4007
Thomson Reuters ISI Clarivate Analytics
Web of Science ESCI Indexed Journal

Identifiers & Pagination:
Vol 13(4) E-Pub 31st Dec 2020 Pp- 1906-1913

Published by Society for Science & Nature India
DOI: http://dx.doi.org/10.21786/bbrc/13.4/41

of India for diverse applications. Besides its role in the formation of a variety of neurotransmitters, it has an impressive position in the treatment of Parkinson's disease and mental disorder. This species possesses valuable medicinal properties, and in the markets across the world, there is a heavy demand for *Mucuna* seeds containing L-DOPA (Eilittä et al., 2002, Ali et al., 2005; Alam and Anis, 2019).

Its demand in international markets has increased many folds in recent years. Since it is being used as a source of L-DOPA by many pharmaceutical companies in India, a continuous supply of L-dopa producing seeds are very much in demand. These seeds are generally collected from forests, leading to the problem of quality control especially of % of L-dopa content. The other hurdle in the collection and in its cultivation is the presence of trichomes (hairs) on the pods, which give a very strong itching sensation. Therefore, attention has been diverted to *in vitro* culture of *Mucuna* similar to *Stizolobium* which is also a rich source of L-DOPA. Micropropagation is one of the innovative methods of asexual propagation that has proved to be effective for *in vitro* propagation of medicinal and aromatic plants and for commercial exploitation of valuable plant-derived pharmaceuticals. *In-vitro* cell culture techniques are one of the innovative and effective methods to produce medicinal and aromatic plants for commercial exploitation of valuable plant-derived pharmaceuticals.

Table 1. Response of Node (N) / Internode (IN), Shoot-tip (ST) and Seed explants of *Mucuna pruriens* (L.) DC. on MS basal media supplemented with effective growth hormone during the explant preparation

Growth Regulators (mgl^{-1})	Explants	Number of Explants Cultured	No. of Responded Plant	% of Response	Response
2,4-D ($3mgl^{-1}$)	Node	10	8	80	Callusing
	Internode	10	6	60	Loose callus
	Shoot-tip	10	7	70	Crystalline callus
	Seed	10	7	70	Apical Bud
NAA ($4mgl^{-1}$)	Node	10	8	80	Well-developed callus
	Internode	10	7	70	Callusing
	Shoot-tip	10	7	70	Callus
	Seed	10	6	60	Apical Bud
BAP ($2.5mgl^{-1}$)	Node	10	7	70	Callusing
	Internode	10	6	60	Hydrated callus
	Shoot-tip	10	8	80	Shoot development
	Seed	10	5	50	Budding
Kn ($2.5mgl^{-1}$)	Node	10	8	80	Multiple shoot development
	Internode	10	6	60	Callusing
	Shoot-tip	10	6	60	Clear callus
	Seed	10	6	60	---

These techniques are under spot light for research and studied thoroughly with various combinations like plant hormones, nanoparticles and many such strategies (Rout, 2002; Faisal et al., 2005, Alam and Anis, 2019). In view to above mentioned difficulties and some of the strategies employed earlier, callus culture has been an alternative and efficient source for the production of secondary metabolites. The *in vitro* technology could be a cost-effective means of high-volume production of the elite planting material throughout the year, without any seasonal constraints (Raaman et al., 2013; Oviedo-Silva et al., 2018; Alam and Anis, 2019; Rakesh and Praveen, 2020).

MATERIAL AND METHODS

For the collection of plant material, the healthy wild plants of *Mucuna pruriens* (L.) DC. were collected from the local area of Hathwa and plain area of Kuchikot of District Gopalganj. The experimental plant is found in these areas among the hedges and bushes of other plants and the plant gives flowers and fruits in the period of October-March. The plant samples were collected, cleaned thoroughly and sorted.Explant material and sterilization: In the present work four different explants were taken from the plant including the node, internode, shoot tip and seed. The seeds were collected from the young pods of 5 months old plant and rests were collected from the in vivo grown plants and all of these were used as the explant. All the segments taken as explants were surface sterilized by cleaning thoroughly under the running tap water for 30 minutes and then were washed with a solution of labolene (5-6 drops in 100ml of water) for 5 minutes followed by washing with

distilled water. These cleaned explants were treated with 70% ethanol for 2 minutes followed by treatment of 0.1% mercuric chloride treatment for 5 minutes under the aseptic conditions. Finally the explants were washed 7-8 times with sterile distilled water to remove traces of any sterilizing agent.

Table 2. Response of different explants of Mucuna pruriens (L.) DC. on MS basal media supplemented with variable conc. of 2,4-D growth regulator with a culture period of 21 days and 10 replicates

Conc. (mgl^{-1})	Explant	No. of explants cultured	No. of respond explants cultured	%of Response	Av. Fresh wt. of callus (in gm)
1.5	Node	10	06	60	1.83
	Internode	10	05	50	1.54
	Shoot tip	10	07	70	1.96
	Seed	10	06	60	1.89
2.5	Node	10	06	60	1.98
	Internode	10	06	60	1.75
	Shoot tip	10	07	70	1.98
	Seed	10	06	60	2.01
3.5	Node	10	06	60	2.89
	Internode	10	05	50	2.76
	Shoot tip	10	08	80	2.89
	Seed	10	06	60	2.76
4.5	Node	10	07	70	3.05
	Internode	10	05	50	2.89
	Shoot tip	10	09	90	3.89
	Seed	10	07	70	3.85
5.0	Node	10	04	40	1.56
	Internode	10	03	30	1.23
	Shoot tip	10	06	60	1.80
	Seed	10	04	40	1.69

Culture media and conditions: The analytical grade chemicals obtained from Hi-Media laboratories and hormones and vitamins from Sigma-Aldrich chemicals were used for preparing the stock solutions and subsequent media preparation. Murashige and Skoog's (1962) salt with 3% (w/v) sucrose was used as basal medium excepting seed germination medium, which completely lacked sucrose. After adding the growth regulators, the pH of the medium was adjusted to 5.7±0.1 followed by gelling with 0.8% of agar in case of solid medium. The media was autoclaved at 121°C for 20 minutes. All the cultures were incubated in a growth chamber maintained at a temperature of 25±2°C, relative humidity, 70-80% and photoperiod of 16:8 h duration under photon flux density of 50 µE mol m-2s-2 provided by day light fluorescent tubes.

Explants preparation: All the sterilized explants materials were used for the preparation of explants that can be used for callus induction. The surface sterilized materials were germinated on MS basal medium without sucrose and the seedlings were allowed to grow for 12-14 day (d) until the auxiliary buds become prominent. The explants were then prepared as per the technique described by Jayanand et al. (2003). Auxiliary buds measuring 0.8-1.0 cm lengths were aseptically inoculated onto MS medium supplemented with various shoot induction hormones.

Initiation of Callus: The auxiliary buds obtained were aseptically transferred on the MS basal media containing various concentration of 2,4-D (1.5, 2.5, 3.5, 4.5, 5.0mg/L), NAA (2.0, 3.5, 4.5, 5.5, 6.5mg/L), BAP (0.5, 1.5, 2.5, 3.5, 4.5mg/L), and Kn (1.5, 3.5, 5.5, 6.0, 7.5mg/L) for the callus induction process. Primary callus was established from cotyledonary leaf explants. For secondary callus production, a small portion of primary callus was excised by using sterile knife holder and was sub-cultured periodically once in three weeks. The secondary callus was used for all the experimental studies. A standard approach of Latin square method was followed for screening of media to establish optimum culturing of callus by manipulating the concentration of auxins (2,4-D and NAA) and cytokinins (BAP and Kn) alone and in combinations (Collin and Edwards, 1998).

Callus growth: The growth measurement of callus was determined by standard method. The growth of the callus and its biomass was measured in terms of fresh (FW g/L) and dry weight (DW g l^{-1}). FW of callus was measured after removing the excess moisture and agar adhering to the callus surface using blotting paper. DW of callus was

determined by drying the callus in hot air oven at 60°C for 24 hr and was expressed in g l^{-1} DW culture.

Acclimatization: Plantlets with well-developed shoots and roots were removed from the culture medium, washed gently under running tap water and transferred to plastic pots containing sterile garden soil supplemented with the combination of growth regulators, under diffuse light (16:8 h photoperiod) conditions. Potted plantlets were covered with a transparent polythene membrane to ensure high humidity and watered every three days with half-strength MS salt solution for two weeks. Polythene membranes were opened after two weeks in order to acclimatize plants to field conditions. After four weeks, acclimatized plants were transferred to pots containing normal soil and maintained in a greenhouse under normal day length conditions.

Table 3. Response of different explants of Mucuna pruriens (L.) DC. on MS basal media supplemented with variable conc. of NAA growth regulator with a culture period of 21 days and 10 replicates

Conc. (mgl^{-1})	Explant	No. of explants cultured	No. of respond explants cultured	%of Response	Av. Fresh wt. of callus (in gm)
2.0	Node	10	07	70	2.43
	Internode	10	05	50	2.10
	Shoot tip	10	08	80	2.89
	Seed	10	07	70	3.01
3.5	Node	10	08	80	2.13
	Internode	10	06	60	1.98
	Shoot tip	10	07	70	3.52
	Seed	10	06	60	3.89
4.5	Node	10	07	70	2.52
	Internode	10	07	70	2.76
	Shoot tip	10	08	80	3.95
	Seed	10	07	70	2.89
5.5	Node	10	08	80	3.85
	Internode	10	06	60	3.65
	Shoot tip	10	09	90	2.56
	Seed	10	08	80	3.85
6.5	Node	10	06	60	2.89
	Internode	10	05	50	2.65
	Shoot tip	10	06	60	2.80
	Seed	10	06	60	2.69

RESULTS AND DISCUSSION

For the present work, four different explants were selected including the nodes, internodes, shoot tips and seeds of the plant *Mucuna pruriens* (L.) DC. First of all the selected parts were sterilized and inoculated on the MS basal media in order to analyze their response and to use them for the preparation of explants for callus induction. For the following work ten replicates from each one was cultured and their response on the media was recorded. All of them were cultured on MS basal media supplemented with different concentration of different growth regulator. The response of nodes, internodes, shoots tips and seeds on the different media are summarized in the table no.1. The results obtained showed that all the selected parts have shown the response in the range of 60-70% on all the four growth regulator media. The characteristic of the resulting callus is also mentioned. These growing calli were used for the explant's preparation and callus induction on the primary culture (Oviedo-Silva et al., 2018).

All the explants now prepared from the different parts were carried forward for the process of callus induction in order to obtain the primary culture. These explants were inoculated on the MS basal media that was supplemented with variable concentration of different growth regulators separately. Different concentration of each selected growth regulator was taken in order to optimize the best concentration at which the percentage response is maximum. For each explant and each hormone five different concentrations were taken with 10 replicates. The result obtained from this optimization study is listed in the table no. 2, 3, 4, 5 for the different concentrations of 2,4-D, NAA, BAP and Kn respectively. The table summarizes the result of 10 replicates giving information about the % response and average fresh weight of the callus for each (Oviedo-Silva et al., 2018).

Table 4. Response of different explants of Mucuna pruriens (L.) DC. on MS basal media supplemented with variable conc. of BAP growth regulator with a culture period of 21 days and 10 replicates

Conc. (mgl^{-1})	Explant	No. of explants cultured	No. of respond explants cultured	%of Response	Av. Fresh wt. of callus (in gm)
0.5	Node	10	07	70	1.54
	Internode	10	06	60	1.23
	Shoot tip	10	08	80	2.89
	Seed	10	07	70	3.01
1.5	Node	10	08	80	2.13
	Internode	10	06	60	1.98
	Shoot tip	10	07	70	3.89
	Seed	10	06	60	4.15
2.5	Node	10	08	80	3.05
	Internode	10	07	70	2.76
	Shoot tip	10	08	80	3.68
	Seed	10	07	70	3.85
3.5	Node	10	08	80	3.85
	Internode	10	06	60	3.65
	Shoot tip	10	09	90	3.56
	Seed	10	08	80	3.85
4.5	Node	10	07	70	2.89
	Internode	10	05	50	2.65
	Shoot tip	10	06	60	2.98
	Seed	10	06	60	2.96

The overall results obtained from this analysis and the data obtained clearly depicts that there is a very variable requirement of these growth regulator for the different explants. The concentration at which the maximum response was obtained for the different explants is as, for internode explant 2.5 mg 2,4-D, 4.5 mg NAA, 2.5mg BAP and 5.5 mg Kn. per litre of the media; for node explant 4.5mg 2,4-D, 5.5mg NAA, 3.5mg BAP and 5.5 mg Kn; for seed and shoot tip explant 4.5mg 2,4-D, 5.5mg NAA, 3.5mg BAP and 6.0mg Kn.

The results obtained depicted the optimization of the growth regulator's concentration required to obtain the better growth response of *Mucuna pruriens* (L.) DC. under in vitro conditions. The average fresh weight of the callus has also shown better response at the suitable hormonal concentration. After this study the next approach was the acclimatization step that is used for the field transfer of the plantlets obtained under in vitro process. For this the soil was collected from the area from where the sample plant was obtained. The soil was supplemented with growth regulator combination and the plantlets were transferred in the pots and kept under the regulated conditions for 25-30 days. The result obtained is summarized in the table no. 6 depicting the percentage response for all explants as well as the characteristic features of the plantlets growing in the soil (Oviedo-Silva et al., 2018).

The results obtained from the study clearly depicted that the growth regulators have played an important role in maintaining and promoting the proper growth of the explant under the in vitro environments. The importance and need of these growth regulators is clearly depicted from the study as well as there is a need for implementing these hormones in order to obtain maximum yield of the work. The stimulating effect of BAP on multiple shoot formation has been reported earlier for several medicinal and aromatic plant species (Khalafalla and Hattori, 1999). The results obtained under the study have clearly established the consistency with other findings where the addition of NAA promotes the proliferation and elongation of shoots in *Petasites hybridus, Eucalyptus grandis*, and *Hybanthus enneaspermus* (Wildi et al., 1998; Cid et al., 1999; Prakash et al., 1999). Higher concentration of NAA (1.0 µM) suppressed the shoot regeneration and resulted in basal callusing of the various hormones tested, BAP alone was more effective over Kn and other combinations of hormones (Alam et al., 2020).

Superiority of BAP over Kn for multiple shoot formation was also demonstrated in Pterocarpus marsupium (Chand and Singh, 2004). The study observed that there is no directly proportional relation between the concentration of hormones and percentage response as the better response was obtained at an optimal concentration nor too low nor too high.

Table 5. Response of different explants of *Mucuna pruriens* (L.) DC. on MS basal media supplemented with variable conc. of Kinetin growth regulator with a culture period of 21 days and 10 replicates

Conc. (mgl^{-1})	Explant	No. of explants cultured	No. of respond explants cultured	%of Response	Av. Fresh wt. of callus (in gm)
1.5	Node	10	06	60	1.64
	Internode	10	06	60	1.23
	Shoot tip	10	08	80	2.89
	Seed	10	07	70	3.01
3.5	Node	10	07	70	3.13
	Internode	10	06	60	2.98
	Shoot tip	10	07	70	3.99
	Seed	10	06	60	4.6
5.5	Node	10	08	80	3.95
	Internode	10	07	70	3.76
	Shoot tip	10	08	80	3.68
	Seed	10	07	70	3.85
6.0	Node	10	07	70	2.85
	Internode	10	06	60	2.65
	Shoot tip	10	09	90	3.56
	Seed	10	08	80	3.85
7.5	Node	10	06	60	1.89
	Internode	10	05	50	1.65
	Shoot tip	10	06	60	2.98
	Seed	10	06	60	2.96

Table 6. Response of Mucuna pruriens (L.) DC. on field transfer in various concentrations of phytohormones (Auxin and Cytokinin) documented after 25-30 days of plantation in the soil

Growth hormone (mgl^{-1})	Explants	No .of Explants Transferred	No. of respond explants	% of Response	Responses			
					Shoot	Bud	Root	Other Remarks
	Node	10	07	70	Elongated	Compact and green	2-3 root hair	Well developed with dark green leaves
	Internode	10	05	50	Dwarf	Flattened	Short Adv. root hairs	Not showed well responsive growth
NAA and BAP	Shoot-tip	10	08	80	Straight long	Green	Multiple root hairs, white	Well developed green leaves with long stem
	Seed	10	07	70 elongation	Apical Bud	Well dev.	Elongate white multiple root	Long straight stem

	Node	10	08	80	Elongated	Compact and green	2-3 Hair root	Well developed with dark green leaves
	Internode	10	06	70	Short	Flattened	Short Adv. root	Not showed well responsive growth
NAA and Kn (4.5+5.5)	Shoot tip	10	07	70	Straight long	Green	Multiple roots hair, White	Well dev. green eaves with long stem
	Seed	10	06	60	Apical elongation	Well dev. bud	Elongate and white multiple root	Long straight stem

Cytokinin concentrations beyond the optimum range adversely affected the shoot development, as the regenerated shoots became stunted and dense. The stunted nature of shoot formation corresponding to increased concentration of BAP in the medium was also reported in Orthosiphon (Leng and Lai-Keng, 2004) and *Eupatorium* (Martin, 2003). Faisal et al. (2005) worked on three different cytokinins, 6-benzyladenine (BA), kinetin (Kin) and 2-isopentenyl adenine (2-iP) by supplementing them to (MS) medium and showed that BA at an optimal concentration of 5.0µM was effective in inducing multiple shoots. Method of *in vitro* culture has also been well studied for other L-DOPA producing medicinal plant species (Oviedo-Silva et al., 2018).

Alam and Anis (2019) have worked to conclude indole-3-butyric acid (IBA) as most suitable supplement for *in vitro* culture of this plant. The combinations of hormones that showed optimum growth in our study similar effect was seen in Alam et al. (2020) where various strength of 6-benzyladenine), Kinetin and 2-isopentenyl adenine were observed to show optimum culture conditions for *in vitro* regeneration. Thus these combinations could be used in high rate growth of *M.puriens* and used for L-DOPA production (Alam et al., 2020).

CONCLUSION

In conclusion, a cell culture methodology for better propagation under *in vitro* condition is found to be highly useful for commercial production of medicinally important compounds. The present study developed an efficient and optimum callus biomass using a synergetic combination of auxins and cytokinins. The study concluded the optimal normal concentration for better and enhanced production of the plantlets from tissue culture technique. The study shows a better the large scale application of such finding for the propagation of medicinally important plant on large scale in order to obtain their metabolites or compounds for further study and uses. Further studies will be directed toward large scale production, testing the efficacy of secondary metabolites through animal cell lines and exploring market potential.

Financial support and sponsorship: There was no outside financial support and sponsorship.

Conflicts of interest: There are no conflicts of interest

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