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INFLUENCE OF SILVER NITRATE IN ENHANCING THE *IN VITRO* SHOOT REGENERATION IN *MUCUNA PRURIENS* (L.) DC. - A MULTIPURPOSE MEDICINAL LEGUME

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ABSTRACT: An efficient micropropagation protocol from shoot tip explants of *Mucuna pruriens*, an annual leguminous climber was established. Shoot tip excised from seven days old aseptically grown seedlings were inoculated on Murashige and Skoog (MS 1962) medium containing different concentration of benzyladenine (BA) alone or in combination with an auxins (NAA, IBA, IAA) for multiple shoot induction. BA (2.5 µM) was found to be optimum for inducing maximum shoots (7.67 ± 0.33) per explant. The augmentation of auxins in combination with optimized dose of BA gave antagonistic response towards shoot bud regeneration and resulted in basal callusing. Augmentation of $AgNO_3$ (25 µM) with best cytokinin combination considerably improved the shoot quality with maximum shoot numbers (10.67 ± 0.88) and also gave better elongation (3.97 ± 0.12 cm) at multiplication stage in shoot tip explants. The best condition for rooting was half-strength MS medium solidified with agar and with 0.03 µM indole-3-butyric acid (IBA) producing 4.67 ± 0.33 roots per microshoot after 28 days. After rooting, the plantlets were transferred to plastic pots filled with sterile Soilrite where 90 % grew well and all exhibited normal development.

KEYWORDS: Acclimatization, Shoot tip explant, Micropropagation, Plant growth regulators.

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1. INTRODUCTION

Mucuna pruriens (L.) DC. (Fabaceae), commonly known as velvet bean, is an important tropical legume found in bushes and hedges in tropical and subtropical regions of India. It is a highly

valuable medicinal plant, all parts of the plant possess valuable medicinal properties. An application of leaves, root, raw pods and seeds is well known in numerous diseases such as diabetes, cancer, arthritis and exhibit activities like analgesic and antipyretic [1,2]. In Ayurveda, it is recognized as a potent aphrodisiac [3]. It is used as a supplement of protein diet to increase muscle mass [4]. It is harvested as fodder and used as green cover crop [5]. Apart from these, *Mucuna* has an enormous worldwide demand for one of its active constituent, L-Dopa (a precursor of dopamine), used to cure Parkinson's disease [6]. The natural habitat of the species has been decreasing at an alarming rate, due to huge collection from the wild habitat. The species is mainly propagated through seeds which poses problems due to the high allergic properties of pods that cause uncontrolled itching while handling the seeds. Therefore, there is an urgent need to develop an alternative technique for its propagation and sustainable use. Now-a-days, micropropagation system is mainly used for the bulk production of stock material for further increase in biomass production. Micropropation has proved to be an effective technique for in vitro propagation of medicinal plants and for commercial exploitation of valuable plant derived pharmaceuticals [7,8,9,10]. Cytokinins and auxins are the regulatory and functional phytohormones in plant tissue culture which play crucial role in stimulation and growth of meristemic zone of axillary bud to divide and lead to form various plant parts. The objective of the study reported here was to develop an efficient method for rapid *in vitro* propagation of *M. pruriens* using shoot tip explants via optimization of basal media, pH, cytokinin, auxins and an adjuvant, followed by successful establishment of regenerated plants.

2. MATERIALS AND METHODS

Establishment of aseptic seedlings and explant preparation

Seeds collected from the University Botanical garden were washed carefully under running tap water for 30 min, treated with 5 % (*v/v*) labolene detergent for 10 min, and washed through running tap water. Surface disinfestation was carried by dipping the seeds in aqueous solution of 0.1 % (w/v) $HgCl_2$ for 4 min. and rinsed with double distilled water for at least three times. The seeds were inaculated on MS (Murashige and Skoog 1962) [11] based medium for germination. Shoot tips (ST) excised from 7 d old aseptic seedlings were used as explants.

Media and culture conditions

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

Shoot initiation and proliferation

MS medium augmented with different concentration (0.5, 2.5, 4.5, 6.5 or 8.5 µM) of BA for shoot bud induction and optimized concentration of BA (2.5 µM) as control with combination of IAA,

IBA and NAA (0.05, 0.10, 0.50, 1.00 or 1.50 µM) or different concentration (10, 15, 20, 25, 30, 35, 40 or 45 µM) of $AgNO_3$ were used for shoot proliferation and multiplication. Cultures were transferred onto fresh media after every 21 days. The frequency of shoot number per explants and shoot length were noted on 28 and 56 days of culture.

Rooting

In vitro raised healthy microshoots (4 cm) were transferred to different strengths (1, 1/2, 1/3 or 1/4) of MS and ½ MS medium in combination with various concentrations (0.1, 0.2, 0.3, 0.4 or 0.5 µM) of IBA for root initiation. Data was recorded on rooting percentage, root number and root length after 28 days of shifting to rooting media.

Acclimatization

Vigorous and healthy plantlets (5 cm) were detached from the culture vessel, properly washed to avoid contamination through agar and implanted in thermocol cup having Soilrite. The potted plantlets were covered with translucent poly-bags to ensure relative humidity and watered every 4 day of interval with half-strength MS salts without vitamins and sucrose for 14 days. Poly-bags were opened after 2 weeks in order to adjust the potted plants to field condition. Adapted plants were relocated to pots containing garden soil and established to greenhouse conditions under natural sun light.

Statistical analysis

All the experiments were conducted with a minimum of 15 replicates per treatment. The experiment was repeated three times. The data was analyzed statistically using one-way analysis of variance (ANOVA) by SPSS *ver* 16 (SPSS Inc., Chicago, USA). The significance of differences among means was carried out using Duncan's multiple range test at $P = 0.05$. The results are expressed as mean ± SE of three experiments.

3. RESULTS AND DISCUSSION

Shoot tip explants failed to respond morphogenetically to a growth regulator free MS medium. However, multiple shoots were formed in those treatments supplemented with cytokinin with different frequencies. BA (2.5 µM) showed the best response in terms of shoot induction and it is considered to be one of the most useful cytokinin for shoot regeneration. Multiple shoots were induced within 3-4 weeks of culture on shoot induction medium. There was a linear correlation upto the optimal level (2.5 µM) with percentage shoot development and number of shoots per explant. The regeneration frequencies and number of shoots declined with the increase in the concentration of cytokinin beyond the optimal level. Reduction in shoot number at concentrations higher than optimal level has been reported for several medicinal plants [12,13,14]. An antagonistic influence of auxins (IAA, IBA and NAA) with optimized concentration of BA was evident when combination of optimal concentration of BA (2.5µM) with different concentrations of IAA, IBA and NAA (0.05, 0.10, 0.50, 1.00 or 1.50 µM) were tested. There was a decline on shoot proliferation due to callus

formation and root induction as observed when higher concentration of IAA, IBA and NAA (0.10-1.50 µM) was applied. To overcome these problems, different concentrations (10-45 µM) of silver nitrate were supplemented in combination with optimized BA (2.5 µM). Among the various strength of silver nitrate tested 25.0 µM gave the maximum number of shoot (10.67 ± 0.88) with an average shoot length (3.97 ± 0.12 cm) in 90 % cultures after 56 d of inoculation, while on increasing the $AgNO_3$ concentration beyond the optimum level a reduction in other growth parameters including shoot number and length was observed.

Table 1: Effect of cytokinin on multiple shoot regeneration from Shoot tip explants on MS medium, after 56 days of culture

Cytokinin (µM) BA	Percent Response	No. of shoot per explant (Mean ± SE)	Shoot length (cm) (Mean ± SE)
0.00	00	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}
0.50	85	4.67 ± 0.33^{bc}	1.77 ± 0.09^{c}
2.50	90	7.67 ± 0.33^{a}	2.40 ± 0.06^{a}
4.50	88	5.33 ± 0.33^{b}	2.03 ± 0.07^{b}
6.50	80	4.00 ± 0.58^{cd}	1.60 ± 0.12^{c}
8.50	75	3.33 ± 0.33^{d}	1.23 ± 0.03^{d}

Values represents means ± SE. Means followed by the same letters within columns are not significantly different (P = 0.05) using Duncan's multiple range test

Cytokinins effectively remove the meristemetic shoot apical dominance, thus the addition of BA at optimum level in culture media was found to be beneficial in shoot induction and proliferation. Similar results have been documented in various reports [15,16,17,18]. Application of cytokininns in tissue culture varies according to plant species, types of explant used and types of culture practices. MS media in combination with various cytokinis at optimal concentration along with optimized auxins was most promising and show a mutual regulatory effect on in vitro shoot multiplication and elongation. It means exogenous application of phytohormones in MS media at optimal level resulted positive corelation in most of the repots [19,20]. In the present study, higher induction and proliferation of shoot bud was achieved on control media at optimized level augmented with BA (2.5 µM) for ST explants. The dominance of BA above the other cytokinins in micropropagation system has been well documented in a number of studies like in *Withania somnifera* [21], *Althaea officinalis* L. [22]. One of the probable descriptions for the better response achieved on BA is that ribosides and nucleotides are naturally stable in BA compared to other cytokinins [23]. Cellular differentiation and organogenesis in micropropagation have been found to be controlled by intraction among cytokinin and auxin applications. It is apparent that not only auxin and cytokinin

concentration but the magnitudes of one to other are contributing factor in cell cycle, cell division and differentiation. In the present finding, ST explant gave a non-significant result when exogenous auxins are supplied in control media, where an antagonistic response was recorded on shoot multiplication and elongation. Lowest concentration of IAA (0.05 μM) in combination with optimized concentration of cytokinin was most effective in all the treatment for induction and proliferation of shoots. The benefits of adding auxins at lower concentration in the culture media were to nullify the influence of cytokinins on axillary shoot elongation [24,25,26,27,28]. Of all the treatments tried, the highest regeneration (85%) with maximum (7.67 ± 0.333) number of shoots was obtained at 2.5 μM BA and 0.05 μM IAA (Table 2). The possible combination of BA and auxins for in vitro shoot induction and proliferation has been documented in numerous pharmaceutical plants such as *Salvia splendens* [29], *Vitex negundo* [30]. Cytokinins are generally acknowledged as promoter in bud formation in numerous *in vitro* organogenesis. On the basis of our result, auxins (IAA, IBA, and NAA) had shown adverse result in shoots multiplication by stimulating callus formation at the basal end of explants. A possible explanation towards callus formation is that, in leguminous plant species endogenous auxins level is high. An exogenous application of auxins at various concentrations in control media gave a negative result on shoot multiplication while profuse callus induction and root formation was found. Thus the outcome suggests that BA was sufficient for *in vitro* micropropagation of *M .pruriens*. Similar results are also documented in some plants [31,32]. In order to improve regeneration frequency, effect of $AgNO_3$ was studied with optimized BA concentration on MS medium. Among the various combinations tested, 25.0 μM $AgNO_3$ gave the maximum number of shoot with elongated shoot length (Table. 3). Addition of $AgNO_3$ in combination with phytohormones are known to enhance shoot number in numerous reports [33,34,35]. A synergistic influence of $AgNO_3$ with optimized cytokinin was evident when combination of optimal concentration of BA (2.5 μM) with different concentration of $AgNO_3$ were tested. Addition of $AgNO_3$ (25.0 μM) enhanced the rate of shoot proliferation producing best frequency (90 %) of shoot formation and total number (13.00 ± 0.57) of shoots per explant. The possible explanation may be that the silver ions act as competitive inhibitor of ethylene binding to the active site of phytohormones, thus regulate ethylene concentration and help in synthesis of new hormone at active site [36,37].

Fig. 1:

A. Induction of multiple shoot in shoot tip explant on MS medium containing BA (2.5 µM) after 56 days of culture.
B. Shoot proliferation and elongation on MS + 2.5 µM BA + 25.0 µM $AgNO_3$ after 56 days of culture.
C. Rhizogenesis in microshoots on ½ MS + 0.3 µM IBA after 28 days.
D. Acclimatized plantlets in Soilrite (two months old)

Table 2: Effect of optimum concentration of BA (2.5 µM) with various auxins on shoot regeneration from shoot tip explants on MS medium after 56 days of culture

Auxins (µM)			Percent Response	No. of shoots per explant	Shoot length (cm)
IAA	IBA	NAA		(Mean ± SE)	(Mean ± SE)
0.00			00	0.00 ± 0.000^{k}	0.00 ± 0.000^{l}
0.05			85	7.67 ± 0.333^{a}	2.40 ± 0.058^{a}
0.10			75	4.67 ± 0.333^{cd}	1.70 ± 0.100^{c}
0.50			70	4.00 ± 0.00^{de}	1.30 ± 0.000^{de}
1.00			62	2.67 ± 0.333^{gh}	1.10 ± 0.115^{efgh}
1.50			50	1.67 ± 0.333^{ij}	0.93 ± 0.033^{hij}
	0.05		82	6.33 ± 0.333^{b}	2.17 ± 0.067^{b}
	0.10		72	4.00 ± 0.000^{de}	1.43 ± 0.067^{d}
	0.50		65	3.00 ± 0.000^{fg}	1.20 ± 0.058^{defg}
	1.00		58	2.00 ± 0.000^{hij}	0.97 ± 0.067^{ghij}
	1.50		48	1.67 ± 0.333^{ij}	0.77 ± 0.067^{jk}
		0.05	80	5.33 ± 0.333^{c}	1.87 ± 0.145^{c}
		0.10	68	3.67 ± 0.333^{ef}	1.27 ± 0.033^{def}
		0.50	60	2.33 ± 0.333^{ghi}	1.03 ± 0.133^{fghi}
		1.00	45	1.67 ± 0.333^{ij}	0.83 ± 0.067^{ijk}
		1.50	40	1.33 ± 0.333^{j}	0.63 ± 0.067^{k}

Values represents means ± SE. Means followed by the same letters within columns are not significantly different (P = 0.05) using Duncan's multiple range test

The *in vitro* regenerated shoots produced roots (Fig. 1C) when transferred to half-strength MS medium containing different concentration of IBA. Presence of IBA (0.3 µM) in ½ strength MS medium facilitated better rhizogenesis with fairly good length (4.30 ± 0.20 cm) and number (4.67 ± 0.33) of roots per shoots (Table 4) after 28 days. Similar results have been reported in several plants [38,39,40,41,42]. The rooted plantlets were successfully hardened off inside the growth room in selected planting substrate for 8-12 weeks and eventually established in natural soil (Fig. 1D). Among the three different type of planting substrate examined, 95 % of plants survived in Soilrite and about 90 % survived following transfer from Soilrite to natural soil. There was no detectable variation among the potted plants with respect to morphological and growth characteristics.

Table 3: Effect of the optimum concentrations of BA (2.5 µM) with various concentrations of Silver nitrate on multiple shoot regeneration from shoot tip explants on MS medium, after 56 days of culture

$AgNO_3$ (µM)	Percent Response	No. of shoot per explants (Mean ± SE)	Shoot length (cm) (Mean ± SE)
00	00	0.00 ± 0.000^{g}	0.00 ± 0.000^{f}
10	75	7.67 ± 0.333^{d}	2.40 ± 0.058^{cd}
15	80	8.00 ± 0.000^{cd}	2.50 ± 0.115^{cd}
20	82	9.33 ± 0.333^{c}	2.93 ± 0.296^{bc}
25	90	13.00 ± 0.577^{a}	3.97 ± 0.120^{a}
30	85	10.67 ± 0.882^{b}	3.27 ± 0.291^{b}
35	78	8.33 ± 0.333^{cd}	2.67 ± 0.219^{c}
40	70	5.33 ± 0.333^{e}	2.00 ± 0.100^{d}
45	60	3.33 ± 0.333^{f}	1.40 ± 0.058^{e}

Values represents means ± SE. Means followed by the same letters within columns are not significantly different (P = 0.05) using Duncan's multiple range test

Table 4: Effect of the MS medium and IBA on root formation in *in vitro* raised micro shoots after 28 days of culture

Treatments (µM)		Percent Response	No. of roots per explants (Mean ± SE)	Root length (cm) (Mean ± SE)
Media	IBA			
Agar		0	0.00 ± 0.000^{f}	0.00 ± 0.000^{g}
MS		60	1.00 ± 0.000^{def}	1.60 ± 0.100^{de}
½ MS		78	1.67 ± 0.333^{cde}	2.30 ± 0.100^{cd}
⅓ MS		50	0.67 ± 0.333^{ef}	1.00 ± 0.500^{ef}
¼ MS		40	0.33 ± 0.333^{f}	0.53 ± 0.533^{fg}
	½ MS + 0.10	82	2.33 ± 0.333^{c}	2.93 ± 0.219^{bc}
	½ MS + 0.20	85	3.33 ± 0.333^{b}	3.47 ± 0.145^{b}
	½ MS + 0.30	90	4.67 ± 0.333^{a}	4.30 ± 0.200^{a}
	½ MS + 0.40	80	2.00 ± 0.577^{cd}	2.70 ± 0.289^{bc}
	½ MS + 0.50	75	1.67 ± 0.333^{cde}	2.40 ± 0.100^{cd}

Values represents means ± SE. Means followed by the same letters within columns are not significantly different (P = 0.05) using Duncan's multiple range test

4. CONCLUSION

The present findings report a practicable and reproducible micropropagation protocol for *M. pruriens* using shoot tip explants obtained from aseptic seedlings. The results have shown antagonistic interaction between auxins and cytokinin but showed synergistic effect with $AgNO_3$ when supplemented with cytokinin in *in vitro* regeneration. The positive response of silver nitrate appears to be highly effective and could be used as a means of rapid propagation of *Mucuna prurien*- a medicinal plant with significant commercial utility.

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CONFLICT OF INTEREST

There is no conflict of interest.

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