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Rapid Micropropagation Protocol of *Mucuna pruriens* var. *utilis* using Cotyledonary Node Explant: A Cultivated Medicinal Edible Legume

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ABSTRACT

Background: The presence of L-dopa coupled with rich protein and amino acid marked *Mucuna pruriens* var. *utilis* as an important under-utilised legume. Therefore, it is useful to develop a method for large-scale multiplication for commercial production.

Method: Tissue culture technology is successfully utilized in propagation of plants with poor and uncertain response to conventional propagation. Murashige and Skoog's (MS) medium without any Plant Growth Regulators (PGRs) was used for seed germination and with PGRs for shoot and root multiplication.

Result: Highest 95% seed germination was found in fresh seeds at 7-8 days of culture. Shoot multiplication percentage was found to be 100% with highest *c.a.* 21.1 shoots with an average 4.8 cm shoot length on MS + BAP 1.5 mg L^{-1} per 10 days old cotyledonary node explant. A total *c.a.* 144 shoots were harvested after 3rd harvest of the mother cotyledonary node with two whole cotyledons at day 70. Rooting was best induced in *in vitro* derived shoots on ⅛ MS medium without any PGRs and plantlets were acclimatized in sand and soil (1:1) and established in pot with garden soil.

Key words: Anti-Parkinson effect, Cotyledonary node, Cultivated edible legume, *In vitro* multiple shoots, Velvet bean.

INTRODUCTION

The genus *Mucuna*, includes about 150 species of perennial and annual legumes of pantropical distribution belongs to the family Fabaceae (Leguminoceae). Many species of the genus offer an excellent source as cover crop and green manure, in addition to their traditional use as feed and food, (Janardhanan and Lakshmanan 1985; Mohan and Janardhanan 1993; Capo-chichi *et al.* 2003; Sathyanarayana *et al.* 2008) and as dye (Standley and Steyermark 1946). In addition, *Mucuna* is also traditionally used in treatment of pain and numbness of joints and irregular menstruation (Ding *et al.* 1991). Almost all the species are reported to contain an amino acid 3-(3, 4-dihydroxyphenyl)-L-alanine (L-dopa) acid that acts as precursor for the neurotransmitter dopamine, used in the treatment of Parkinson's disease (Manyam 1995).

Nine species with four varieties of the genus *Mucuna* are distributed in India, but unfortunately most of the species and varieties remained neglected (Sathyanarayana *et al.* 2008). Among the different species of *Mucuna*, one of the most important tropical legume is *M. pruriens* (L.) DC. It includes two varieties namely *pruriens* (not edible) and *utilis* (edible), which is commonly called as 'the cowhage' or 'velvet' bean found in bushes and hedges at damp places, ravines and scrap jungles throughout the plains of India (Faisal *et al.* 2006). Besides, the seeds are good source of protein as they contain higher amounts of crude protein and lipids when compared with other most of the commonly consumed legumes. Balogun and Olatidoye (2012) reported crude protein (25.65%), crude carbohydrate (42.98%), crude fibre (7.23%), ash (3.6%) and fatty acid (12.4%). in *M. pruriens* var. *utilis*. The fatty acid profile of the seeds revealed that the seed lipids contained higher concentration of palmitic acid and oleic acid. The seed protein contained higher levels of essential amino acid such as valin, tyrosine, threonine, histidine *etc.* So, Balogun and Olatidoye (2012) advocated the utilization of *utilis* seeds for domestic consumption in Nigeria. According to their investigation the seeds of this under-utilised crop can be explored as an alternative protein source to alleviate protein-energy-mal nutrition among economically weaker section of peoples of Nigeria (Balogun and Olatidoye 2012). Another report said boiled seeds with reduced L-DOPA are known to be consumed by Oceanic group of tribal's like Onges, Great Andamans, Sompens *etc.* The presence of L-dopa coupled with rich protein and amino acid marked *Mucuna pruriens* var. *utilis* as an important under-utilised legume.

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Therefore, it is useful to develop a method for large-scale multiplication for commercial production. Tissue culture technology is successfully utilized in propagation of plants with poor and uncertain response to conventional propagation. However, to the best of our knowledge, there is only one *in vitro* propagation protocols for *Mucuna pruriens* var. *utilis*, where Sathyanarayana *et al.* (2008) used axenic axillary bud to produce multiple shoots which were very low in number. So, attempt has been made here to standardize high fidelity, rapid and reliable micropropagation protocol using a special type of explant *i.e.* cotyledonary node.

MATERIALS AND METHODS

Explant source and seed germination

In this present work, the mature seeds were collected from the Botanical garden, Department of Botany and Biotechnology, Ravenshaw University, Cuttack and stored for the viability test. Three types of seeds were tested for germination *i.e.* one year old seeds, six months old seeds and fresh seeds. The one year old and six months old seeds were kept under running tap water for about 30 minutes followed by 15 minutes treatment with 5% (v/v) aqueous solution of Labolene (Thermo Fishers Scientific India Pvt. Ltd.) and rinsed 5 times with distilled water and then surface sterilized with 0.1% (w/v) aqueous solution of mercuric chloride ($HgCl_2$, Hi-media, Mumbai, India) for 20 minutes followed by five rinses with sterile double distilled water. But in case of fresh seeds, the seeds were kept under running tap water for 30 minutes and then treated with 5% (v/v) aqueous solution of labolene for 15 minutes and rinsed with tap water. Then the seeds were treated for 15 minutes with 5% (w/v) Megastin (K.P.R. fertilizers limited, Tata Nagar, India) and rinsed thoroughly with distilled water followed by surface sterilization with 0.1% (w/v) aqueous solution of mercuric chloride ($HgCl_2$, Hi-media, Mumbai, India) for 20 minutes. After that the seeds were rinsed five times with sterile double distilled water and inoculated to seed germination media. For seed germination, different strengths of Murashige and Skoog's (1962) basal medium (MS) was used *i.e.* MS, ½ MS, ¼ MS and ⅛ MS without any growth regulator. The seed germination percentage and days require for seed germination was evaluated for different strengths of basal media. At the same time seed germination percentage was compared among the three different types of seeds *i.e.* one year old seeds, six months old seeds and fresh seeds.

Multiple shoot development

Multiple shoot culture was established using cotyledonary node explants excised from 10 days old *in vitro* germinated seedlings that were inoculated on the MS basal media supplemented with various concentrations of BAP or Kin (0.5 - 5.0 mg L^{-1}) individually where MS media without any growth regulator was set as control. Cotyledonary nodes were also inoculated on MS+1.5mg L^{-1}BAP supplemented with different auxins IAA and NAA (0.25-1.0 mg L^{-1}) to check their control on several shoot development. The cotyledonary nodal explants with emerging shoots were sub-cultured at 15 days interval for shoot duplication and elongation. After yield of *in vitro* shoots, the original cotyledonary nodal stump was sub cultured twice on same shoot regeneration media for large scale *in vitro* shoot production. For further scale-up purpose, the *in vitro* nodes collected from primary shoots were cultured on MS + 1.5mg L^{-1} BAP for axillary shoot multiplication.

The shoot multiplication potential was evaluated on best shoot regeneration medium for different forms (CN with 02 whole cotyledons, CN with 02 proximal halves, CN with 01 whole cotyledon and CN without cotyledon) of cotyledonary node explant. All the tissue culture media were fortified with 3.0% sucrose and gelled with 0.7% agar. Similar culture environments as per seed germination experiment were maintained.

Root regeneration in *in vitro* regenerated shoots

Well developed *in vitro* shoots (4.2-4.8 cm length) were harvested and inoculated into the 60 ml culture tubes (Borosil, India) containing MS, ½ MS, ¼ MS, ⅛ MS, 1/16 MS and ⅛ MS with IAA (0.25-1.0 mg L^{-1}) media without any growth regulators, augmented with 1.5 % sucrose and gelled with 0.6 % (w/v) agar power (Hi-media, Mumbai). Culture environments are similar as above experiment.

Plantlet acclimatization and soil establishment

The *in vitro* derived plantlets were washed cleanly and carefully with tap water to get rid of agar gelled medium to avoid contamination. Then, the plastic glasses were wiped with alcohol and filled with autoclaved sand and soil (1:1) mixture. The plantlets were planted to the plastic with care and sufficient water has been poured to the glass till the sand and soil mixture became fully wet. Then the plastic glasses were covered with polythene bags and kept in culture room. After, 2 days small hole were done in polythene bags to give water to the plants and plantlets became adaptable to environmental humidity. The polythene bags were removed from the plastic glasses on 12th day, watered sufficiently. After, 3-4 days the plantlets were transferred to the pots containing garden soil avoiding direct expose to sunlight. After, 10-12 days the plants were transferred from pot to field.

Data recording and statistical analysis

Data were subjected to analysis of variance (ANOVA) for a completely randomized design (CRD) at 5% level of probability. Duncan's multiple range tests (DMRT; Gomez and Gomez 1984) was used to separate the means to determine significant effects. Some experimental data are presented as mean± standard error (SE).

RESULTS AND DISCUSSION

Seed germination

Seed viability was studied for the three types of seeds, the fresh seeds collected from the plant grown in the Botanical

garden, Department of Botany and Biotechnology showed 95% germination whereas, the germination 85% and 70% was observed for six months and one year stored seeds respectively. In case of six months and one year old seeds, the seeds were germinated after 22-24days and 28-30days respectively and 7-8 days required for the germination of fresh seeds on MS and other media also (Table 1). Out of four different strengths of MS basal media evaluated for seed germination full strength MS was found to be most effective (Table 1; Fig 1 A). So, cotyledonary nodes from the fresh seeds were taken for the shoot multiplication experiment. Likewise, Vishwakarma *et al.* (2009), Sanatombi and Sharma (2007) have taken fresh mature seeds of *Desmodium gangeticum* and *Capsicum annuum* respectively for better germination. Kambizia *et al.* (2006) also reported the maximum germination in current season seeds.

In our experiment, we have evaluated four concentrations of basal medium *i.e.* MS, ½ MS, ¼ MS and ⅛ MS without any PGRs for seed germination of *Mucuna pruriens* out of which MS showed highest 95% germination in 7-8 days of culture (Fig 1 A). Seed germination on MS without any PGRs was observed in *Mucuna pruriens* (Faisal *et al.* 2006). Similar results were observed in *Arachis hypogaea, Clitoria ternatea, Desmodium gangeticum, Lathyrus ochrus, Psoralea corylifolia, Thermopsis turcica* by Venkatachalam and Kavipriya (2012), Mukhtar *et al.* (2012), Vishwakarma *et al.* (2009), Saglam (2012), Shinde *et al.* (2009), Cenkci *et al.* (2008) respectively. Some workers like Indravathi and Pullaiah (2013), Parveen and Shahzad (2010) and Barik *et al.* (2007) have also used ½ MS without PGRs for seed germination in *Albizia amara*, *Cassia sophera, Clitoria ternatea* respectively.

Multiple shoot development

PGRs are numerous chemical substances that profoundly promote and influence plant growth. Addition of various PGRs to the media promotes the growth of different plant parts. The cotyledonary nodes derived from 10 day's old axenic seedling was experimented to various concentrations of PGRs to get the morphogenic response and the results were documented in the (Table 2). On MS medium without any PGRs the cotyledonary node showed only 01 shoot on either side. So, we have essentially added cytokinin for the

Table 1: Effect of basal media strength and period of storage on seed germination.

Media strength	Fresh seeds		Six months old seeds		One year old seeds	
	Mean % of germination	Days for germination	Mean % of germination	Days for germination	Mean % of germination	Days for germination
MS	95 ^{a}	7-8	85 ^{b}	22-24	70 ^{d}	28-30
½ MS	80 ^{c}	8-9	65 ^{e}	22-24	55 ^{g}	30-32
¼ MS	60 ^{f}	9-10	50 ^{h}	25-27	40 ^{j}	36-38
⅛ MS	45 ^{i}	11-12	35 ^{k}	26-30	35 ^{k}	40-42

In columns, different letters in superscripts indicate statistically significant difference between the means (P≤0.05; Duncan's new multiple range test). Two seed/ Jar, 05 jars/ treatment and 2 replications (2x5x2= 20).

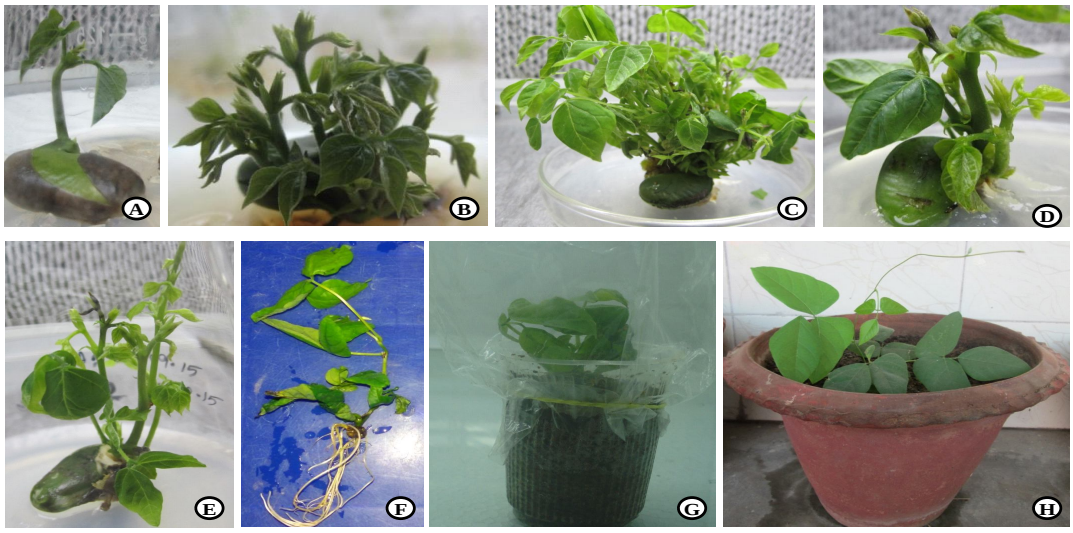

Fig 1: Germinated seedling on MS **B.** Multiple shoots on MS + 1.5 mg L^{-1} BAP in CN explant with two whole cotyledons at day 15 **C.** Multiple shoots on MS + 1.5 mg L^{-1} BAP in CN explant with two whole cotyledons at day 30 **D.** Multiple shoots on MS + 1.5 mg L^{-1} Kin in CN explant with two whole cotyledons at day 30 **E.** Multiple shoots on MS + 1.5 mg L^{-1} BAP + 0.5 mg L^{-1} IAA in CN explant with two whole cotyledons at day 30 **F.** Rooting of *in vitro* shoots on 1/8 MS at day 20 **G.** Acclimated plantlet in 1: 1 :: sand: soil **H.** *M. pruriens* established in earthen pots containing garden soil.

induction of multiple shoots from the cotyledonary node explants. Though two different types of cytokinins (BAP and Kin) were tested, the best result was recorded with BAP. To induce multiple shoot proliferation in cotyledonary node, MS supplemented with BAP at a concentration of 1.5mg L^{-1} was found to be optimum medium. The average highest (*c.a.* 21.1) number of shoots was found per explants (with maximum average shoot length 4.8 cm) with a frequency of 100% (averaging) at 30 days on the optimum medium (Fig 1B,C) whereas, MS with addition of Kin showed poor response in comparison to BAP. Among different concentrations of Kin, Kin 1.5mg L^{-1} showed highest (*c.a.* 4.8) number of shoots with 3.9 cm length (Fig 1D). Sathyanarayana *et al.* (2008) obtained 16.33 no of shoots on MS +BAP 3.5 µM using axenic axillary bud which was lower than we obtained in the first harvest in *M. pruriens* var. *utilis*. Similarly, in other varieties like *Mucuna pruriens*, Faisal *et al.* (2006) reported ½ MS supplemented with BAP (5.0 µM) and NAA (0.5 µM) was the most suitable medium for maximum shoot induction and proliferation with 17.8 shoots with 5.9 cm length. Further our results were in accordance with the findings of Indravathi and Pullaiah (2013) and Barik *et al.* (2007) who had also reported MS + BAP 1.0 mg L^{-1} was the best medium for shoot culture in *Albizia amara* (shoot number 6.84, length 3.20cm) and *Clitoria ternatea* (shoot number 5.2, length 6.4 cm) respectively.

Table 2: Multiple shoots regeneration from cotyedonary node with two whole cotyledons.

MS + PGRs mg L^{-1}	Average % of shoot response	Average no of shoots / explant	Average shoot length in (cm)
Control	73.3^{e}	1.0 ^{qr}	4.7^{ab}
	MS + BAP		
0.5	80.0^{d}	2.8	2.0^{mno}
1.0	93.3 ^{b}	9.1 ^{b}	3.1 $^{e-k}$
1.5	**100.0**^{a}	**21.1** ^{a}	**4.8** ^{a}
2.0	100.0 ^{a}	8.5 ^{c}	3.8 ^{bcdef}
3.0	100.0 ^{a}	6.7 ^{d}	3.0 $^{e-k}$
4.0	86.6 ^{c}	6.4 ^{de}	4.2 ^{abcd}
5.0	73.3 ^{e}	4.5 ^{fgh}	2.4 ^{klm}
	MS + Kin		
0.5	66.6 ^{f}	3.9 ^{ghij}	2.2 ^{lmn}
1.0	80.0 ^{d}	4.3 ^{fghi}	3.7 ^{cdefg}
1.5	93.3 ^{b}	4.8 ^{fg}	3.9 ^{abcde}
2.0	93.3 ^{b}	4.5 ^{fgh}	3.6 ^{defgh}
3.0	73.3 ^{e}	3.5 ^{hijk}	3.7 ^{cdefg}
4.0	73.3 ^{e}	3.3 ^{ijkl}	3.8 ^{bcdef}
5.0	60.0 ^{g}	3.0 ^{jklm}	3.8 ^{bcdef}
	MS + BAP + IAA		
1.5 + 0.25	80.0 ^{d}	2.9 ^{klmn}	3.3 $^{d-j}$
1.5 + 0.5	80.0 ^{d}	5.3 ^{f}	4.6 ^{abc}
1.5 + 1.0	53.3 ^{h}	1.5 ^{pq}	3.1^{e-k}
	MS + BAP + NAA		
1.5 + 0.25	73.3 ^{e}	3.3 ^{ijkl}	3.7 ^{cdefg}
1.5 + 0.5	53.3 ^{h}	3.0 ^{jklm}	3.5 ^{defghi}
1.5 + 1.0	46.6 ^{i}	2.0 ^{nop}	3.3 ^{defghij}

Different letters in a column as superscripts indicate significant difference between the means (P≤0.05; Duncan's new multiple range test). One explant/ Jar, 05 jars/ treatment and 3 replications (1x5x3=15).

Addition of auxins to the best shoot multiplication medium reduced shoot number markedly. The average number of shoots *i.e*. 3.3 and 5.3 were found per explant on MS + BAP 1.5mg L^{-1} + NAA 0.25mg $L^{-1}$and MS + BAP1.5mg L^{-1}+ IAA 0.5 mg L^{-1}respectively (Table 2; Fig 1E). However, there was change in shoot regeneration percentage *i.e.* 73.3% and 80.0% in NAA and IAA respectively. BAP alone was found as the most preferred PGR for multiplication of shoots over Kin and BAP +NAA/IAA. Similar reports were observed for multiple shoot initiation in several plants including *Pterocarpus marsupium* (Anis *et al*. 2005), *Balanites aegyptiaca* (Siddique and Anis 2009), *Terminalia bellerica* (Mehta *et al.* 2012), *Wrightia Tomentosa* (Penchala *et al.* 2015) and *Canavalia gladiata* (Behera *et al.* 2020).

There is a need to speed up the shoot multiplication within less time to fulfil the purpose. Thus, after the 1st harvest of *in vitro* shoots, each mother cotyledonary node (CN) explant was sub-cultured twice where we obtained 10.2 and 4.7 shoots in 2nd and 3rd harvest respectively. A considerable decline in the shoot number during the 3rd harvest imposed us for no more subculture of the primary explants (Fig 2). Similar observation was observed by Moharana *et al.* (2017) while working on CN of *Lawsonia inermis* and Behera *et al.* (2020) in *Canavalia gladiata*. Contrary to our results, the highest shoot number reported in the 2nd harvest as compared to 1st harvest and then consequently it decreased gradually during 3rd and 4th harvest in *Acacia sinuate* (Vengadesan *et al.* 2002). *In vitro* nodal segments obtained from the primary shoots of mother cotyledonary node were used as explants source (data not given) for further shoot multiplication. This facilitated the production of average 84.4 (*c.a.* 21.1 shoots/CN x *c.a.* 02 *in vitro* nodes/shoot x *c.a.* 02 shoots/*in vitro* nodes) number of shoots per *in vitro* cotyledonary node after the 1st harvest at day 30 whereas, a total *c.a.*144 shoots (*c.a.* 21.1x2x2, 1st + *c.a.* 10.2 x2x2, 2nd + *c.a.* 4.7x2x2, 3rd) were harvested after 3rd culture of the mother cotyledonary node with two whole cotyledons at day 70 (30 + 20 +20; Fig 2). In our case, we reported higher number of shoots while Suresh *et al.* (2015); reported only 1-2 shoot regenerated from axenic node and Ozaki *et al.* (1993) reported one shoot from immature leaflet of 10 d old axenic seedling. So far, a single report for *in vitro* micropropagation of *M. pruriens* var. *utilis* using cotyledonary node explants was reported by Sathyanarayana *et al.* (2008), obtained 16.33 no of shoots on MS + 3.5 µM BAP which was lower than what we obtained in this experiment.

During the experiment different forms of the cotyledonary node (CN with two whole cotyledons, CN with two proximal halves, CN with one whole cotyledon and CN without cotyledon) were taken into consideration for shoot multiplication because number and size of cotyledons also

influenced the shoot regeneration capacity (Moharana *et al.,* 2017). It was observed that shoot regeneration frequency percentage remained the same with a variation in shoot number and length (Fig 2). Highest shoot number of 21.1 and 3.1 with average shoot length of 4.8 and 1.8 cm resulted in CN with two whole cotyledons and CN without any cotyledon respectively on MS + 1.5mg L^{-1} (Fig 2). The results were in accordance with findings in grass pea (Barik *et al.* 2004), blackgram (Das *et al.* 1998) and mungbean (Sen and Mukherjee 1998). Complete removal of both cotyledons caused a delayed shoot regeneration response and produced fewer shoots. Similar observations were also reported in mung bean (Gulati and Jaiwal 1994; Polisetty *et al.* 1997) and sword bean (Behera *et al.* 2020). Saglam (2012) used longitudinally sliced half CN (maximum 5.92 shoots) in *Lathyrus ochrus,* while Vidoz *et al.* (2012) used longitudinally cut cotyledons along the midrib in *Lotononis bainesii*, to get the best responses.

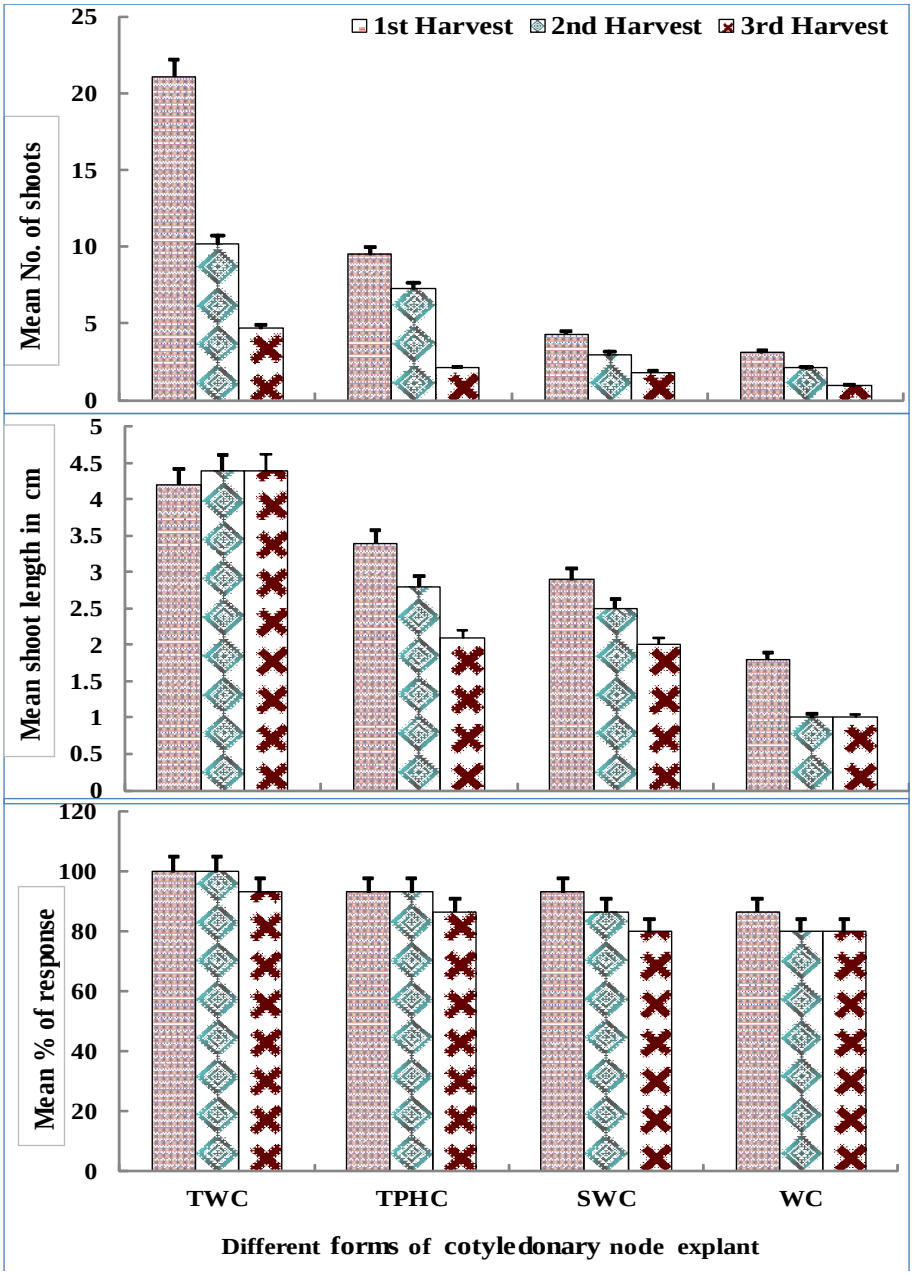

Fig 2: Influence of cotyledon in cotyledonary node on shoot multiplication during different harvest periods.
TWC : CN with 02 whole cotyledons, **TPHC:** CN with 02 proximal halves, **SWC :** CN with 01 whole cotyledon and **WC:** CN without cotyledon

Table 3: Rooting of *in vitro* shoots.

Media + auxin mg L^{-1}	Mean % of rooting	Mean No. of roots / shoot	Mean root length (cm)
MS	95 ^{b}	2.7^{fg}	5.1^{bcd}
½ MS	95 ^{c}	3.0^{f}	5.2^{bc}
¼ MS	100	3.5^{de}	5.3^{ab}
⅛ MS	**100**	**7.0**^{a}	**5.4**^{a}
$^{1}/_{16}$ MS	95.0	4.0^{cd}	5.0^{bcde}
1/8 MS+IAA (0.25)	95.0	5.0^{b}	1.2^{g}
1/8 MS+IAA (0.5)	100.0	4.2^{bc}	5.1^{bcd}
1/8 MS+IAA (1.0)	100.0	1.0^{h}	4.0^{f}

Different letters in a column as superscripts indicate significant difference between the means (P≤0.05; Duncan's new multiple range test). One shoot/ tube, 10 tubes/ treatment and 3 replications (1x10x2=20).

Rooting and acclimatisation of *in vitro* regenerated shoot

Rooting of *in vitro* regenerated shoots was experimented on five different concentrations of MS medium and out of those ⅛ MS medium devoid of any PGRs responded faster in terms of the day and facilitated better rhizogenesis. Root induction was started within 5-6 days and in 14-15 days the average root number and length were 7.0 and 5.4cm respectively (Table 3; Fig 1 F). Though ¼ MS and ⅛ MS gave same response at a time but later ⅛ MS was found to be the optimum medium in terms of root number and length. MS and ½ MS responded slower than the above mediums followed by $^{1}/_{16}$ MS and resulted in less number of roots. In our experiment, it seems as if PGRs is not essential for rooting. Likewise, another species of Fabaceae *i.e*. *Psoralea corylifolia,* developed maximum rooting (highest number 5.4 roots per explant) and highest length 10.1 cm in MS without any PGRs Shinde *et al.* (2009). But in *Mucuna pruriens*, Faisal *et al.* (2006) found satisfactory improvement in rooting on ½ MS medium containing IBA 2.0 mM with good length 5.5 cm and number 7.8 of roots per shoots. Luna *et al.* (2003) also developed maximum number of roots in *Ilex dumosa* in ¼ MS medium when supplemented with IBA 7.3 mM.

Plantlets with leaflets and well-developed roots were successfully acclimatized by transferring it to plastic glasses containing soil and sand in 1:1 and eventually established in pots containing garden soil (Fig 1 G, H). The survival rate of the plantlets after transfer to soil:sand (1:1) was 100% and 58.33% of the plants transferred to garden soil survived. To acclimatize the *in vitro* rooted plantlets of *Mucuna pruriens,* Faisal *et al.* (2006) transferred them to the plastic pots filled with sterile soilrite and 90% of plantlets survived successfully. In *Sinningia speciose,* Kashyap and Dhiman (2011) have acclimatized the *in vitro* raised plantlets by taking four different hardening media, out of which sand and soil in 1:1 showed 45.16% survival rates. Venkatachalam and Kavipriya (2012) used Soil and sand in 2:1 for successful acclimatization in *Arachis hypogaea.*

In this study, the protocol developed for *Mucuna pruriens* var. *utilis* using cotyledonary node as explants found to be most efficient reproducible and reliable one which can be used for the large-scale production and conservation of the elite variety.

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Conflict of interest

The authors state that they have no conflict of interest.

Author contributions

SKM carried out all the experiments and wrote the first draft of the manuscript. AM and DPB examined and analyzed the data. DPB supervised the entire research work and edited the final manuscript. All authors read and approved the final version of manuscript.

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