International Journal of Botany Studies
ISSN: 2455-541X; Impact Factor: RJIF 5.12
Received: 27-07-2020; Accepted: 13-08-2020: Published: 27-08-2020
www.botanyjournals.com
Volume 5; Issue 4; 2020; Page No. 260-267

Cotyledonary node-based propagation protocol of *Canavalia gladiata* (Jacq) DC.- An underutilized legume or lesser-known legume

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Abstract
In this present work we for the first time developed an efficient and stable micropropagation protocol of *Canavalia gladiata* (Jacq) DC. an underutilised or lesser known legume via cotyledonary node explant derived from *in vitro* seedlings. Highest (73.3 %) percentage of seed germination was found using fresh seeds on half-Murashige and Skoog's (MS) medium without any plant growth regulators at 6-10 d of culture. MS + BAP 1.0 mg l^{-1} was found to be most successful for induction and proliferation of multiple shoots. *In vitro* shoot multiplication percentage was found to be 100 % with highest *c.a.* 8.7 shoots per explant by an average 4.8 cm shoot length on the above medium. *In vitro* nodal segments harvested from the first shoots of original cotyledonary node were used as explant source for further shoot multiplication. This facilitated the production of average 128 (8 shoots/CN x 4 *in vitro* nodes/shoot x 4 shoots/*in vitro* nodes) number of shoots per *in vitro* cotyledonary node after the 1st harvest at 30 d, whereas a total *c.a.* 224 shoots (8x4x4 1st + 4x4x4 2nd + 2x4x4 3rd) were harvested after 3rd culture of the mother cotyledonary node with two whole cotyledons at 75 d. Cent percent rooting was observed in *in vitro* derived shoots on ½ MS + 0.5 mg l^{-1} IAA showed 4.2 no of roots per shoot with 5.8 cm root length. Plantlets with well-developed roots were acclimatized in sand and soil (1:1) and placed in pot with normal soil.

Keywords: anti-nutritional factors, cotyledonary node, multiple shoots, underutilised legume

1. Introduction
In plant kingdom, the family 'Fabaceae' is regarded as third largest family for animal and human food [1, 2, 3]. The plants of Fabaceae were reported for their medicinal and nutritive value in leaves, stems, bark, pods, seeds and roots [4]. The *Canavalia gladiata* belongs to Fabaceae, commonly known as sword bean was distributed throughout the tropics within the Asian continent origin. Limited scale cultivations are found in the Asia, West Indies, Africa, South Africa and have been introduced into tropical parts of Australia [5, 6]. The plant is a vigorous perennial climber often cultivated as annual with epigeal type of seed germination [7, 8].
The sword bean has great nutritional and medicinal potential. The mature seeds of *C. gladiata* have been originally consumed by people of ancient India and are now consumed even by urbanized population [9, 10]. It was advocated to be a good source for extending protein value since the protein quality was equal to most edible food legumes [11, 8]. The seeds contain adequate amount of protein (21-28 %) which is generally higher (17-30 %) in quantity and secondary metabolites in higher quantities as compared to other legumes [12, 8]. Besides, it also contains principal fatty acids like palmitic acid, stearic acid, oleic acid, linoleic acid etc. which could be a good source of nutrition with providing health benefits [13]. The coats of the sword bean are a good source of antioxidant having potential benefits against oxidative stress [14]. This plant has been used as a folk medicine for anti-inflammatory purposes in Chinese herbal medicine [15]. The plant possesses biological functions such as anti-inflammatory, hematopoietic improving, hepatoprotective, anti-angiogenic activities, anti-oxidant activity and anti-angiogenic activity [16, 17, 14]. This crop is known to contribute immensely to the food security, income generation and environmental services.
So, this legume plant can meet the future requirements of protein for combating increasing population. Despite containing above useful health ingredients the plant is not commonly used as food because of the presence of some anti-nutritional factors such as concavalin A [18], protease inhibitor [19], tannins, phytates and L-canavine [20] etc. Due to the above properties the plant remains as an underutilized crop. Therefore, it is essential to remove the undesirable compounds of this plant to recover the nutritional quality and effectively utilize it as human food. Various simple conventional methods such as dry heating, roasting, boiling, soaking in water, alkali and acid solvent extraction, germination and fermentation were used to inactivate/reduce the above-mentioned anti-nutrients [21, 22, 6, 23]. These above methods were not so effective. Therefore, our objective was to develop plants with reduced anti-nutritional properties. As conventional propagation by seed does not maintain the clonal fidelity and it is a well-known fact that the germination rate of leguminous plants is quite low in natural habitats [24, 25]. Thus, production of quality planting material within a short period of time with low anti-nutritional factors for commercialization is an issue. So, micropropagation is an alternative which has potential to circumvent the problems by rapid and continuous production of quality planting material with reduced anti-nutritional properties.
In recent years, for high frequency *in vitro* regeneration cotyledonary nodes derived from axenic seedlings have been successfully used as a source of explant in many leguminous plant species including *Accacia sinuate* [25], *Lathyrus sativus* [26] and *Acacia mangium* [27] as they are

more responsive than older explants [28]. During the past years, only a few reports of plant regeneration via tissue culture of *C. gladiata* has been documented which includes adventitious bud formation from 5 days old axenic seedling [29] and shoot induction from axenic node [30]. However, the shoot regeneration percentage and numbers were very low therefore our aim was to develop a fast and consistent plant propagation procedure for *Canavalia gladiata* through high frequency axillary shoot regeneration from axenic cotyledonary nodes.

We may use this rapid axillary plant regeneration protocol as a base for transgenic development which will lead to overcome the underutilized properties of *C. gladiata*. Besides, the protocol will be helpful for production of large-scale planting material for food industries and pharmaceuticals.

2. Materials and Methods

2.1 Seed germination and explant source

Fully ripen pods were locally collected from a single plant, identified and dried under sunlight for 15 days. Healthy seeds were harvested from the dried pods for recent use and the dried pods were stored for further experimental use of seeds.

The fresh and stored seeds were put under running tap water for 30 minutes then 15 minutes treatment with 5 % (v/v) solution of Teepol (Reckilt Benckiser Ltd., India) and for proper removal of detergent from the seeds rinsed five times with distilled water. Subsequently, the seeds were treated with 0.1 % (w/v) aqueous solution of mercuric chloride ($HgCl_2$, Hi-media, Mumbai, India) for 20 minutes followed by five washes with sterile distilled water for surface sterilized. Then the surface sterilized seeds were inoculated on different strengths (full, ½ and ¼) of Murashige and Skoog's [31] basal medium without any growth regulator kept in 400 ml tissue culture jars (K.D. Traders, India) for germination. The media was fortified with 3.0 % sucrose and solidified with 0.6 % agar power. Germination percentage and days required for seed germination was evaluated for different strengths of basal media. The media were autoclaved for 17 min at 121 ˚C and 104 kPa prior to that the pH was adjusted to 5.8 ± 0.1. The inoculated culture jars were placed in a plant tissue culture room at 25 ± 1 ˚C temperature, less than 16h photoperiod, 35-50 µM m^{-2} s^{-1} photon flux density provided by cool white fluorescent tube (Phillips, India) with 55-60 % relative humidity.

2.2 Multiple shoot development

Multiple shoot culture was established using cotyledonary node explants excised from 13-14 days old *in vitro* germinated seedlings that were inoculated on the MS basal media supplemented with various concentrations of BAP or Kin (0.1- 3.0 mg l^{-1}) or TDZ (0.1- 2.0 mg l^{-1}) individually where MS media without any growth regulator was set as control. Cotyledonary nodes were also inoculated on MS+1.0 mg 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 + 1.0 mg 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.

2.3 Root regeneration in *in vitro* regenerated shoots

Well developed *in vitro* shoots (4.0-4.5 cm length) were harvested and inoculated into the 60 ml culture tubes (Borosil, India) containing MS, ½ MS, ¼ MS, ⅛ MS and ½ MS with IAA or IBA (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.

2.4 Plantlet acclimatization and soil establishment

The *in vitro* derived plantlets were removed from the culture tubes cautiously, washed carefully with tap water to get rid of agar to avoid contamination. Then, the thermo cool glasses were wiped with alcohol and filled with autoclaved sand and soil mixture with different proportions (1:1; 2:1; 1:2). The plantlets were planted in the thermo cool glasses with care and enough water were poured in the glass till the sand and soil mixture became fully wet. Then the thermo cool glasses were covered with polythene bags and kept in culture room. After 2 days small holes were done in polythene bags to supply water to the plantlets and for their adaptation to environmental humidity. The polythene bags were completely removed from the thermo cool glasses on 12th day. After a week the plantlets were transferred to the clay pots with garden soil kept under shade outside the laboratory. After 10-12 days the plants were transferred from pot to field under natural environment.

2.5 Data recording and statistical analysis

Seed germination experiment comprises of five culture jars containing two seeds per Jar. Shoot regeneration experiment includes one explant/bottle and 5 replicas for each treatment. For *in vitro* rooting the experiment consisted of five tubes, each replication consisting of one shoot/culture tube. Mean data was pooled from a total three separate experiment. Data was analyzed using study of variance (ANOVA) for a completely randomized design (CRD). Each trial was repeated three times at 7 days interval. Data were recorded after 30 days by visual observations of cultures showing shoot proliferation and the number of shoots/explants, shoot length, root number and root length etc. Duncan's New Multiple Range Test (DNMRT) was used to separate the means for significant effect [32].

3. Results and Discussion

3.1 Seed germination

Three different strengths of MS basal media without any growth regulators were tested for seed germination. It was observed that ½ MS medium gelled with 0.6 % agar showed highest percentage of seed germination. The seed germination was initiated at day 06, highest 73.3 % at day 10 for fresh seeds but seed germination initiated at day 11, highest 60.0 % at day 15 in stored seeds. In MS, lesser time was required for seed germination (6-9 days) with a lower percentage of germination (56.6 %) whereas, in ¼ MS days required for germination increased with decreased percentage of seed germination (Table 1, Fig. 1a). The ½ MS without any growth regulator was found suitable for highest percentage of seed germination in *Lawsonia inermis* [33] and *Withania somnifera* [34]. The use of freshly collected seed of *C. gladiata* was previously reported by Ozaki [23], about germination on MS without plant growth regulator and requirement of 5 days for germination initiation, not mentioned about the percentage but Suresh *et al*. 2015 [30] did not mention about any of the parameter where seed germination is a pre-requisite for their explant source. The high percentage of seed germination in this study may be attributed to use of seeds within short period between collection and experimental use. Both Nayak *et al*. (2013) [34] in *Withania somnifera* and Moharana *et al*. 2017 [33] in *Lawsonia inermis* suggested about the use of current season seeds to obtain maximum germination.

3.2 Multiple shoot regeneration

The cotyledonary nodes (CN) derived from 13 -14 day's old axenic seedling (Fig. 1a) were cultured on different PGRs to get the morphogenic response and the results were documented in table 2 where, MS basal medium without any plant growth regulator was not able to regenerate shoot or multiple shoots. Out of various concentrations of BAP, Kin and TDZ, the maximum shoot initiation, multiplication and elongation was observed on MS + BAP 1.0 mg l^{-1} by the CN explants with two whole cotyledons of *C. gladiate.* Cent percentage of shoot multiplication frequency with highest shoot number (*c.a.* 8.7) and shoot length of 4.8 cm was observed on the above medium (Table 2; Fig. 1b) while lesser number of shoots were observed in explant without two cotyledons (Fig. 1c). MS augmented with Kin at a concentration of 2.0 mg l^{-1} showed 86.6 % of shoot regeneration with 1.5 averages shoot numbers. MS + TDZ (1.0 mg l^{-1}) developed 2.9 shoots with 66.66 shoot regeneration percentage (Table 2; Fig. 1 d). Additions of auxins to the best shoot multiplication medium reduced shoot number markedly (Table 2). The average number of shoots i.e., 2.0 and 3.8 were found per explants on MS + BAP 1.0 mg l^{-1} + NAA 1.0 mg l^{-1} and MS + BAP 1.0 mg l^{-1} + IAA 0.5 mg l^{-1} (Fig. 1e) respectively. However, there was no change found in shoot regeneration percentage. BAP was found as the most preferred PGR for multiplication of shoots over TDZ and Kin. BA/BAP was also found more effective than TDZ and Kin for multiple shoot initiation in several plants including *Pterocarpus marsupium* [35], *Balanites aegyptiaca* [36], *Terminalia bellerica* [37] and *Wrightia Tomentosa* [38]. The effectiveness of BAP for shoot induction was may be due to the capacity of plant tissues to use BAP more gladly than other artificial growth regulators [39, 40]. Further it is considered that the ribosides and nucleosides in the BA/BAP are more stable than the other cytokinins and this may also be one of the possible reasons for its better performance [41, 42]. But the report of Suresh *et al*. (2015) [30] suggested about the use of BA (1.0 mg l^{-1}) and IAA (1.0 mg l^{-1}) for the best shoot proliferation with shoot regeneration response 70 % from axenic nodal explants of *C. gladiate.* [29] also used BA (1.0 mg l^{-1}) and NAA (0.01 mg l^{-1}) for adventitious shoot development from immature leaflet of 10 days old axenic seedling. Addition of auxins in cytokinins supplemented MS medium that failed to enhance axillary shoot proliferation in the present study. Alike trend has also been reported in other species including *Acacia sinuata* [25] and *Lawsonia inermis* [43, 44]. Whereas, for the optimum result the whole explant with emerging shoot buds was sub-cultured on the same shoot multiplication medium which promoted activation, rejuvenation and conditioning of meristems as reported by Shekhawat *et al*. 1993 (*Prosopis cineraria*) [45], Moharana *et al*. 2017 (*Lawsonia inermis*) [33]. There is a need to speed up the shoot multiplication within less time for the business purpose. Thus, after the 1st harvest of *in vitro* shoots each primary cotyledonary node explant was sub-cultured twice where we obtained 4.6 and 2.3 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 (Table 3). Similar observation was observed by Moharana *et al*. 2017 [33] while working on CN of *Lawsonia inermis*. But differing 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* [25]. *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 128 (8 shoots/CN x 4 *in vitro* nodes/shoot x 4 shoots/*in vitro* nodes) number of shoots per *in vitro* cotyledonary node after the 1st harvest at 30 d, whereas a total *c.a.* 224 (8x4x4 1st + 4x4x4 2nd + 2x4x4 3rd) number of shoots were harvested after 3rd sub-culture of the mother cotyledonary node with the help of *in vitro* nodal multiplication at 75 d (Table 3). In our case, higher number of shoots were obtained than the other reports of Suresh *et al*. 2015 [30] (only 1-2 shoot regenerated from axenic node) and Ozaki *et al*. 1993 [29] (one shoot from imature leaflet of 10 d old axenic seed ling). So far, *in vitro* micropropagation of *Canavalia gladiata* using cotyledonary node explants has not been attempted.

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 [33]. It was observed that shoot regeneration frequency percentage remained the same with a variation of shoot number and length (Table 4). Highest shoot number 8.7 & 5.1 with average shoot length 4.7 & 1.8 cm resulted in CN with two whole cotyledons & CN without any cotyledon respectively on MS + 1.0 mg l^{-1} (Table 4, Fig. 1 b & c). Similar type of response was observed with different form of cotyledonary node in *Lathyrus sativus* [26], *Vigna unguiculata* [46] *Lawsonia innermis* [33]. A late shoot initiation response and production of a few shoots were observed after complete removal of both the cotyledons. Barik *et al*. (2004) [26] and Moharana *et al*. (2017) [33] also reported similar type of observations in *Lathyrus sativus* and *Lawsonia innermis* respectively.

3.3 Rooting and acclimatisation of *in vitro* regenerated shoot

In vitro rooting of shoots was achieved on ½ MS medium and then after, ½ MS medium fortified with IBA and IAA for better root formation. Only one root of very short length (approximately 0.5-1.0 cm in length) was observed on ½

MS. Highest root induction frequency (100 %) with maximum root number (*c.a.* 4.2) of root length (5.8 cm) was observed on ½ MS + IAA 0.5 mg l^{-1} within 10-15 days after inoculation (Table 5; Fig. 1 f). In our experiment it was observed that PGRs are essential for rhizogenesis in *C. gladiata*. Similarly, Ozaki (1992) [29] reported about the requirement of 1.0 mg l^{-1} NAA and 0.01 mg l^{-1} BA in ½ MS for optimum response of root regeneration whereas Suresh *et al*. 2015 [30] did not try the rooting experiment. Ozdemir 2017 (*Mentha spicate*) [47] and Penchala *et al*. 2015 (*Wrightia tomentosa*) [38] also used IAA for maximum number of shoot regeneration from micro shoots derived from cotyledonary node.

Plantlets were successfully acclimatized by transferring it to thermo cool glasses containing soil and sand in 1:1 and eventually established in earthen pots containing garden soil (Fig. 1 g & h). The continued existence rate of the plantlets after moving to soil: sand (1:1) was 100 %, and 62.5 % of the plants transferred to garden soil survived. Yadav *et al*. (2009) [48], Vengadesan *et al*. (2002) [25] and Venkatachalam and Kavipriya (2012) [49] also used soil:sand :: 1:1 for maximum percentage of acclimatization of *Vigna radiata*, *Acacia sinuate* and *Arachis hypogaea* respectively. Addition of sand and soil in appropriate ratio in the planting substrate was vital for acclimatization practice. Presence of appropriate amount of sand may be liable for water holding capacity of the planting substrate and proper aeration [50].

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

Basal media strength	Fresh seeds		02 months stored seeds	
	Mean % of germination	Days for germination	Mean % of germination	Days for germination
MS	56.7^{c}	6-9	43.3^{d}	12-14
½ MS	73.3^{a}	6-10	60.0^{b}	11-15
¼ MS	43.3^{d}	8-12	33.3^{e}	13-16

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 3 replications (2x5x3= 30)

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

MS + PGRs (mg L^{-1})	% of explant response	No of shoots / explant	Shoot length (cm)
Control (MS)	0	0	0
MS + BAP			
0.1	0	0	0
0.25	66.7^{d}	4.5^{d}	0.7^{kj}
0.5	100.0^{a}	7.1^{ab}	3.4^{d}
1.0	100.0^{a}	8.7^{a}	4.8^{a}
2.0	86.7^{b}	5.6^{c}	4.1^{b}
3.0	80.0^{c}	3.9^{e}	3.9^{bc}
MS + Kin			
0.1	0	0	0
0.25	0	0	0
0.5	0	0	0
1.0	0	0	0
2.0	86.7^{a}	1.5^{ml}	1.6^{i}
3.0	80.0^{c}	1.0^{n}	0.9^{j}
MS + TDZ			
0.1	66.7^{d}	1.6^{lk}	1.6^{i}
0.25	100.0^{a}	1.8^{jk}	2.3^{h}
0.5	100.0^{a}	2.4^{i}	3.1^{efg}
1.0	66.7^{d}	2.9^{g}	3.3^{ed}
2.0	66.7^{d}	2.8^{hg}	3.2^{def}
MS + BAP + IAA			
1.0 + 0.25	86.7^{b}	2.9^{g}	3.3^{ed}
1.0 + 0.5	100.0^{a}	3.8^{ef}	3.4^{d}
1.0 + 1.0	100.0^{a}	1.5^{ml}	3.3^{ed}
MS + BAP + NAA			
1.0 + 0.25	0	0	0
1.0 + 0.5	0	0	0
1.0 + 1.0	100.0^{a}	2.0^{j}	3.3^{ed}

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).

Table 3: Harvest of multiple shoots during sub-culture of *C. gladiata* cotyledonary node with two whole cotyledons on MS + 1.0 mg L^{-1} BAP

MS + PGRs (mg L^{-1})	Percentage of explant responded	Initial culture (1st harvest)		I subculture (2nd harvest)		II subculture (3rd harvest)	
		Shoots / explant	Shoot length in (cm)	Shoots / explant	Shoot length in (cm)	Shoots / explant	Shoot length in (cm)
Control (MS)	0	0	0	0	0	0	0
MS+BAP							

0.25	66.7^{d}	4.5^{ed}	0.7^{j}	2.6^{i}	0.7^{j}	1.7^{ml}	0.6^{jk}
0.5	100^{a}	7.1^{b}	3.4^{d}	3.3^{g}	2.7^{f}	2.0^{k}	2.2^{hi}
1.0	100^{a}	8.7^{a}	4.8^{a}	4.6^{d}	3.4^{d}	2.3^{j}	2.9^{ef}
2.0	86.7^{b}	5.6^{c}	$4.^{1b}$	3.0^{h}	3.1^{e}	1.8^{kl}	2.5^{fg}
3.0	80.0^{c}	3.9^{f}	3.9^{bc}	2.3^{j}	2.9^{ef}	1.5^{mn}	2.3^{gh}

Different letters in a column as superscripts indicate significant difference between the means ($P\leq0.05$; Duncan's new multiple range test). One explant/ Jar, 05 jars/ treatment and 3 replications (1x5x3=15).

Table 4: Influence of cotyledon in cotyledonary node on shoot multiplication and elongation

MS + BAP (mg L^{-1})	CN with two whole cotyledons			CN with two proximal halves of cotyledons			CN with single whole cotyledon			CN without any cotyledon		
	% of response	No. of shoots	Shoot length (cm)	% of response	No. of shoots	Shoot length (cm)	% of response	No. of shoots	Shoot length (cm)	% of response	No. of shoots	Shoot length (cm)
0.25	66.7^{d}	4.5^{f}	0.7^{no}	66.7^{d}	3.2^{lm}	0.8^{n}	53.3^{e}	2.6^{op}	0.7^{no}	53.3^{e}	2.1^{q}	0.8^{n}
0.5	100.0^{a}	7.0^{b}	3.4^{d}	100.0^{a}	3.8^{hij}	3.3^{de}	100.0^{a}	3.6^{jh}	2.8^{fgh}	100.0^{a}	3.3^{kl}	1.5^{lm}
1.0	100.0^{a}	8.7^{a}	4.7^{a}	100.0^{a}	4.3^{fg}	3.4^{d}	100.0^{a}	4.0^{h}	2.9^{fg}	100.0^{a}	5.1^{d}	1.8^{k}
2.0	86.7^{b}	5.6^{c}	4.1^{b}	86.7^{b}	3.5^{hk}	2.9^{fg}	86.7^{b}	3.3^{kl}	3.0^{f}	86.7^{b}	5.0^{e}	1.6^{kl}
3.0	80.0^{c}	3.9^{hi}	3.9^{bc}	80.0^{c}	2.8^{no}	2.6^{hi}	80.0^{c}	2.9^{n}	2.3^{j}	80.0^{c}	5.0^{e}	1.6^{kl}

Different letters in a column as superscripts indicate significant difference between the means ($P\leq0.05$; Duncan's new multiple range test). One explant/ Jar, 05 jars/ treatment and 3 replications (1x5x3=15).

Table 5: Rooting of *in vitro* shoots

Basal media + Auxins (mg L^{-1})	% of rooting	Roots / shoots	Root length (cm)
MS	0	0	0
½ MS	50.0^{e}	1.0^{e}	0.5^{g}
¼ MS	0	0	0
1/8 MS	0	0	0
½ MS +IAA (0.25)	50.0^{e}	0.5^{f}	1.2^{f}
½ MS + IAA (0.5)	100.0^{a}	4.2^{a}	5.8^{a}
½ MS + IAA (1.0)	70.0^{c}	1.0^{e}	4.0^{c}
½ MS + IBA (0.25)	33.3^{f}	2.0^{c}	5.2^{b}
½ MS + IBA (0.5)	96.6^{b}	2.5^{b}	3.7^{de}
½ MS + IBA (1.0)	66.6^{d}	1.5^{d}	3.9^{cd}

Different letters in a column as superscripts indicate significant difference between the means ($P\leq0.05$; Duncan's new multiple range test). One shoot/ tube, 10 tubes/ treatment and 3 replications (1x10x3=30)

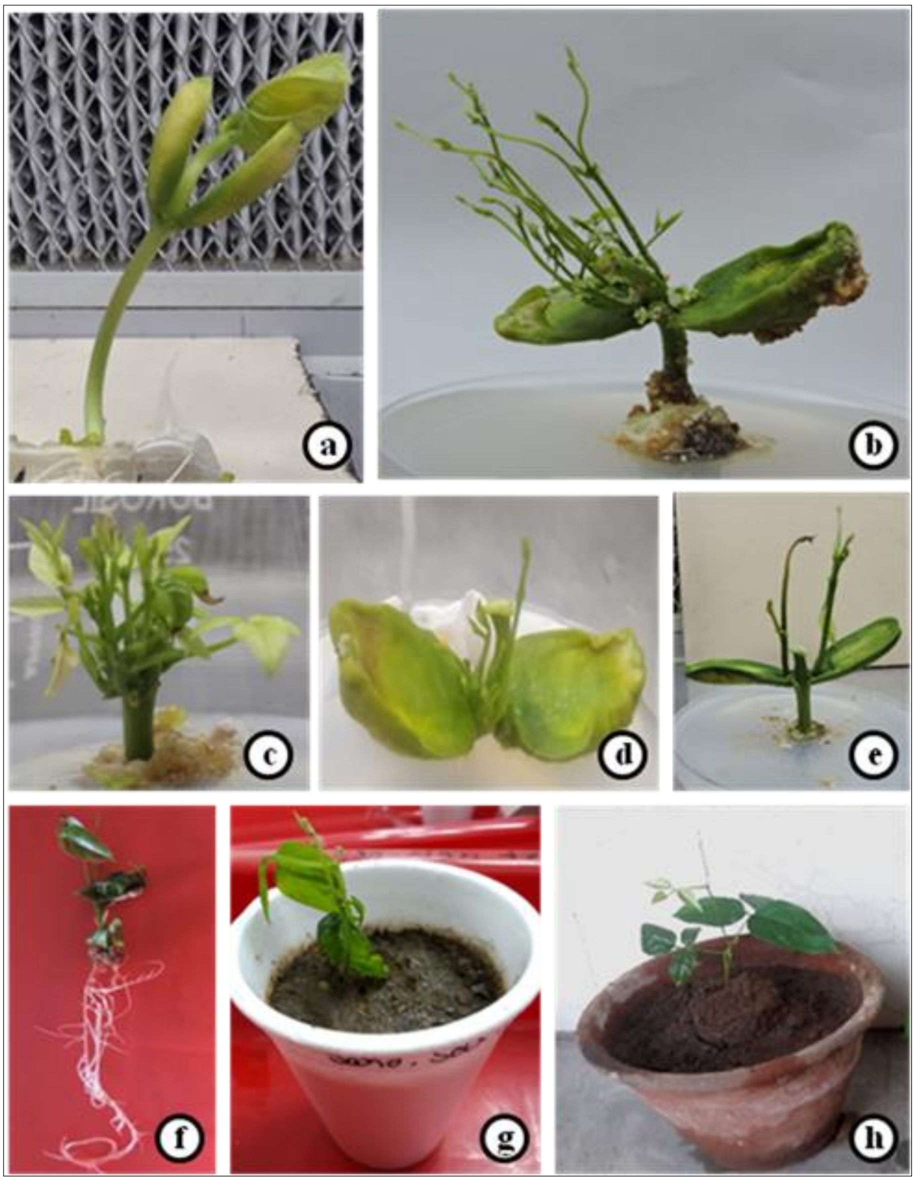

Fig 1 a: Germinated seedling on ½ MS b. Multiple shoots on MS + BAP (1.0 mg L^{-1}) in CN explant with two whole cotyledons c. Multiple shoots on MS + BAP (1.0 mg L^{-1}) in CN explant without cotyledons d. Multiple shoots on MS + TDZ (1.0 mg L^{-1}) in CN explant with two whole cotyledons e. Multiple shoots on MS + BAP (1.0 mg L^{-1}) + NAA (1.0 mg L^{-1}) in CN explant with two whole cotyledons f. Rooting of *in vitro* shoots on ½ MS + IAA (0.5 mg L^{-1}) g. Acclimated plantlet in 1: 1 :: sand: soil h. *C. gladiate* established in earthen pots containing garden soil.

4. Conclusion

In this paper we developed a reproducible efficient protocol for *Canavalia gladiata* using cotyledonary node as explant for the first time. By using this protocol there can be produced thousands of elite clones with same genotype of mother plant. It's a dietary legume whose conservation and reduction of toxin level can be done using the above protocol.

5. Acknowledgment

We thank the unnamed reviewers for their precious annotations to improve the manuscript. We offer our sincere gratitude to Dr. S. K. Naik, Reader in Botany, Ravenshaw University, Cuttack for the research support. Fund support under COE in Environment and Public Health of Odisha under OHEPEE (HE-PTC-WB-02017) and DST-FIST program, Govt. of India to Department of Botany, Ravenshaw University, Cuttack-753 003 is gratefully acknowledged (SR/FST/LSI-017-2010). There is no conflict of interest among the authors.

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