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PLANT REGENERATION THROUGH AXILLARY SHOOT PROLIFERATION OF *CANAVALIA GLADIATA* (JACQ) . DC., AN ECONOMICALLY IMPORTANT UNDERUTILIZED LEGUME- A STEP FORWARD FOR THE REDUCTION OF ANTI-NUTRITIONAL FACTORS (ANFS).

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ABSTRACT

Canavalia gladiata (Jacq) DC., is a legume and considered as a good source for protein because of presence of quality protein similar to most edible food legumes and can be used as food and feed. The seeds of *C. gladiata* are known for several valuable phytochemicals, useful for food and health benefits properties. Still the plant is underutilized/ not consumed worldwide due to presence of some anti-nutritional factors (ANFs) such as Concavalin A, Protease inhibitor, tannins, flatulence factors, allergens and lysinoalanine phytates and L-canavine etc particularly in the seed. So, it is necessary to remove all these anti-nutritional compounds to eligible the seed of *C. gladiata* as food for human society to combat the nutritional deficiency. To increase the nutritional value as well to reduce anti-nutritional compounds from the seed different kind of processes such as sprouting, roasting, fermentation, de-hulling, cooking etc. have been adopted. But the above methods are not efficient enough to reduce anti-nutritional compounds. Therefore, there is a need to develop plantlets with null or reduced anti-nutritional compounds. Tissue culture method is a first and necessary strategy for any kind of further research regarding the reduction of anti-nutritional factors. Whether it is by gene editing or alteration in any regeneration influencing factors during micropropagation anything can be possible by the exploration of tissue culture technique. So, in this part of work, a simple, efficient and reliable micropropagation protocol for *Canavalia gladiata* (Jacq.) DC was developed by using *in vivo* nodal segment for the first time. The nodal segment of 3rd-6th position are found suitable for multiple shoot regeneration. The optimum shoot proliferation has been found in Murashige and Skoog's (MS) medium supplemented with BAP 2.0 mg/L showing 100% response with 5.66 average shoots/explant of average length 3.90 cm. For upscaling purpose, the *in vitro* nodes used as explant from primary shoots harvested from *in vivo* node. Again, MS medium with BAP 2.0 mg/L found as optimum medium for these *in vitro* nodes having 100% response with average shoot/ explant 2.00 of average length 4.96 cm. The seasonal influence on micropropagation has also studied where, Dec- Jan was observed as best season for explant collection showing 92% bud break followed by Feb-March. Half-MS supplemented with IBA 0.5 mg/L found as optimum medium (100% response) for rooting of *in vitro* regenerated shoots with average 4.0 roots/shoot and average root length 3.45 cm. The plantlet was acclimatized on a simple mixture of sand: soil (1: 1) and subsequently transferred to the garden under natural condition. Micropropagated plant developed through this protocol can be a step forward for further research work in reducing its anti-nutritional factor.

Keywords: Anti-nutritional factors (ANFs), *In vivo* node, Underutilized legume.

Introduction

Canavalia gladiata (Jacq) DC., is a legume commonly known as sword bean belongs to family Fabaceae known for its high protein content. The sword bean *Canavalia gladiata* is a leguminous plant originated in the Asian continent and spread throughout the tropics. Cultivated on a limited scale throughout Asia, the West Indies, Africa and South Africa and have been introduced into tropical parts of Australia (Herklots, 1972; Ekanayake *et al.*, 2000). The *Canavalia gladiata* is a perennial climber with height may vary from 4.5 to 10 meters (Purseglove, 1968; Ekanayake *et al.*, 2000). The degree of twinning, the size of seed pods, the number and the colour of the seed pods show considerable variation.

Canavalia gladiata have good source of nutritional and medicinal properties. The mature seeds and pod of *Canavalia gladiata* had been eaten by people of ancient India and these are also now consumed in certain areas of rural population (Rajaram and Janardhanan, 1994; Ekanayake *et al.*, 2000). *Canavalia gladiata* is considered to be good source for protein because of presence of quality protein similar to most edible food legumes and can be used as food and feed (Bressani *et al.*, 1987; Ekanayake *et al.*, 2000). Sometimes the bean is grown as cover, green manure or forage crop (Bressani *et al.*, 1987). The leaves also used for persistent skin rashes (Ekanayake *et al.*, 2000).

Besides the high protein content, *C. gladiata* contains 7.5% soluble sugar and 37.2% starch on fresh weight basis (Herklots, 1972). According Purseglove, (1968), fat present in sword bean is 1.6% on a fresh weight basis (Spoladore and Teixeira, 1987). The crude fat content of sword bean is 99g/kg, which is highest in commonly consuming pulses. The lipids of *C. gladiata* contain triacylglycerols (34.6 - 38.6 wt %) and phospholipids (54.8 -57.4 wt %). The principal fatty acids are palmitic (18.8 - 28.8%), stearic (0.7 - 6.8%), oleic (42.0 - 51.8%), linoleic (16.2 - 22.8%), alpha linolenic (3.0 -8.2%). On the dry weight basis, the protein content of *C. gladiata* ranges from 21-28% which is higher than average legume (Bressani *et al.*, 1987; Mohan and Janardhanan, 1992). Among three *canavalia* sp. *C. gladiata* contain higher amount of protein as compare to *C. ensiformis* and *C. virosa* (Rodrigus and Tom, 1991). The crude protein content of sword bean is high (24%) which make this good supplement of cereal diet (Ekanayake *et al.*, 2000). The cereal proteins

are particularly deficient in amino acid lysine but in contrast, it is high source of lysine (6.49%) (Ekanayake *et al.*, 2000). The other major amino acids present are glutamic acid, aspartic acid, isoleucin, leucin, tyrosin and phenylalanine (Rajaram and Janardhanan, 1994). The lipid composition suggests that these beans could be a good source of nutrition and enhancing health benefits. Bressani *et al.*, (1987) reported high levels of potassium (0.36g/100g) in sword bean. At the green pod and shelled vegetable stage, the seeds contain vitamin A, vitamin C, calcium and iron (Ekanayake *et al.*, 2000). The coats of the bean *C. gladiata* contain good source of antioxidant phenolics having potential benefits against oxidative stress (Gan *et al.*, 2016). The seeds of *C. gladiata* are known for several valuable phytochemicals such as anticancer agent, trigonelline, cytotoxic amino acids canavanine, antiviral lectin and concavalin A (Swaffer *et al.*, 1995; Jayavardhanan *et al.*, 1996; Suresh *et al.*, 2015).

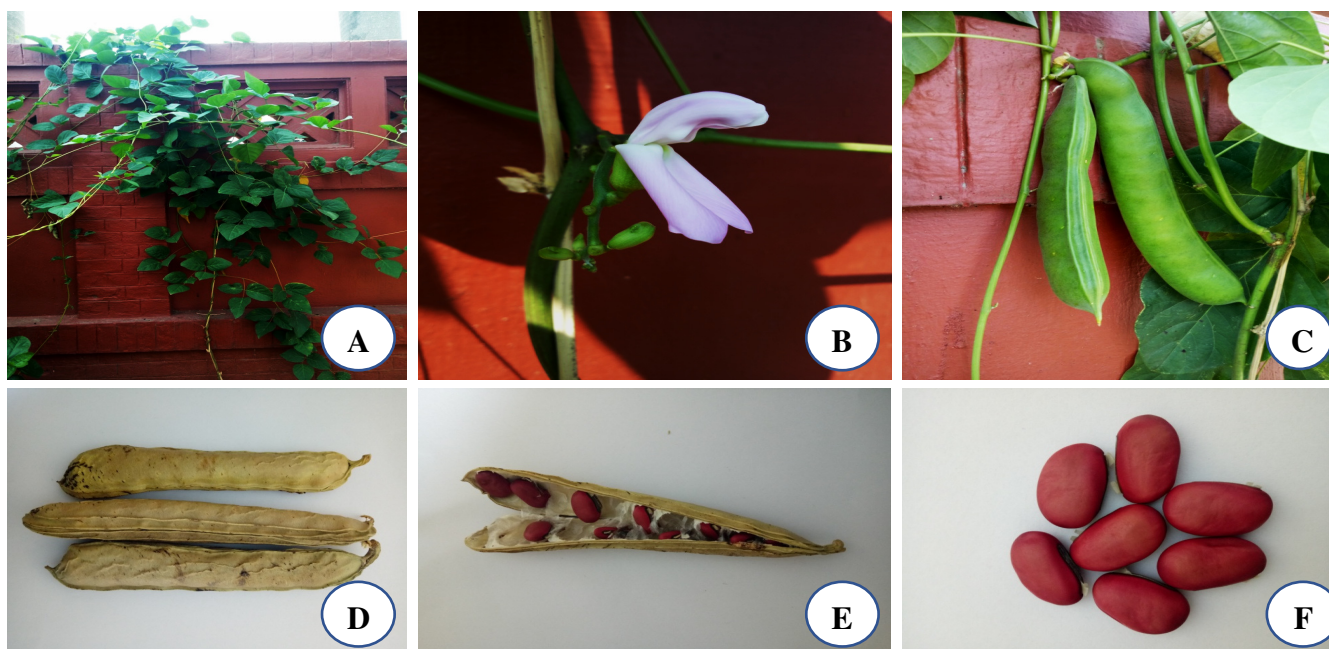


Fig. 1 (A) A young plant established in the experimental garden, Dept. of Botany, Ravenshaw University, Cuttack (B) Flower of *C. gladiata* (C) Mature green pods with ridges (D) Dried pods with ridges (E) Pods containing 8-10 seeds (F) Mature seeds

Sword bean (SB) is also a good source of medicinal properties. The *Canavalia gladiata* possess biological functions such as anti-inflammatory, haematopoietic improving, hepatoprotective and anti-angiogenic activity (Jeon *et al.*, 2005; Kumar and Reddy, 2014; Kim *et al.*, 2016; Gan *et al.*, 2016). Some studies have reported that *C. gladiata* efficient for protecting against bone loss, increase in antioxidant activity and improving cell profiles (Kim *et al.*, 2016). The seeds of *C. gladiata* exhibit antiemetic and demulcent effects and is used to stop hiccups in Chinese traditional medicine (Sridhar and Seena, 2006; Suresh *et al.*, 2015). The seeds contain 4-O-Methylgallic acid, which acts as anti-angiogenic agent and potentially inhibits endothelial cell invasion and tube formation (Jeon *et al.*, 2005). The seed coat possesses extremely high phenolic content which have antioxidant activity and may have potential health benefits. The gallic acid and its derivatives such as methyl gallate, digalloyl hexoside and digallic acid were main phenolic

compounds in the coats of the bean (Gan *et al.*, 2016). The lectins present in red sword bean have been reported as anticancer agent (Duranti, 2006; Une *et al.*, 2016) and used as biochemical tools due to their specificity and ability to recognize cancer cells (Campos-Vega *et al.*, 2010; Une *et al.*, 2016).

Although the plant contains above useful food and health benefits properties, still the plant is underutilized/ not consumed worldwide due to presence of some anti-nutritional factors (ANFs) such as Concavalin A (Ekanayake *et al.*, 2007), Protease inhibitor (Laurena *et al.*, 1994; Ekanayake *et al.*, 2007), tannins, flatulence factors, allergens and lysinoalanine phytates and L-canavine (Siddhuraju and Becker, 2001; Ekanayake *et al.*, 2007) etc. These above anti-nutritional compounds make this plant underutilized. So, it is necessary to remove all these anti-nutritional compounds to eligible the seed of *C. gladiata* as food for human society. To increase the nutritional value as well as palatability and

bioavailability of nutrients and to reduce anti-nutritional compounds from the seed different kind of processes such as sprouting, roasting, fermentation, de-hulling, cooking etc. have been adopted (Ekanayake *et al.*, 2000; Eburuaja *et al.*, 2016). But the above methods are not efficient enough to reduce anti-nutritional compounds. Therefore, there is a need to develop plantlets with null or reduced anti-nutritional compounds. Micro propagation through tissue culture method is a first and necessary method for any kind of further research regarding the reduction of anti-nutritional factors. Further, after the development of such kind of plant, large scale production going to be a crucial factor because during conventional propagation, maintenance of clonal fidelity is a bigger problem and it is known that germination of legumes is quite low in natural habitat (Dewan *et al.*, 1992; Vengadesan *et al.*, 2003). Therefore, first we have to standardized micro-propagation protocol for the plant *C. gladiata*.

To date, only a 2-3 reports are available regarding the micropropagation of *Canavalia gladiata*. Till now, to the best of our knowledge plant regeneration through adventitious bud formation from 5 days old seedling (Ozaki, 1993), shoot induction through *in vitro* node (Suresh *et al.*, 2015), *in vitro* plant regeneration from cotyledonary node (Behera *et al.*, 2020) were reported. Among the three, reports Ozaki, 1993 and Suresh *et al.*, 2015 only reported about the shoot regeneration but not about root induction where shoot multiplication number was quite low. Only Behera *et al.*, 2020 reported about both shoot and root regeneration from axenic cotyledonary node. In this part of our work, we have tried to multiple shoot regeneration and their subsequent rooting using *in vivo* node as explant.

Materials and Methods

Selection of explant and surface sterilization

The experimented plant species (*Canavalia gladiata*) was identified (by R.C. Mishra, Principal Scientist, ICAR-National Bureau of Plant Genetic Resources) and selected locally for mature seeds. Healthy seeds were collected and sown in the garden of Dept. of Botany, Ravenshaw University, Cuttack. Out of the germinated plants of *Canavalia*, one healthy plant was identified, selected and marked for our research purpose. After identification, the plant was taken care till young with healthy and enormous branching. Young and tender fresh nodal segments (10-15 cm) were collected with cut end placed in distilled water. Leaves were separated from the segments and nodes were cut into 2.5-3.0 cm segment. The nodal segments were kept under running tap water for 30 minutes followed by treatment with Labolin (Polygen, India) 10% (v/v) for 10 minutes, and then rinsed with tap water. Then treated with 0.2% (w/v) Bavistin (BASF, Mumbai, India), a fungicide for 10 minutes, then rinsed with tap water. The nodes were again treated with aqueous solution of mercuric chloride (HgCl₂, Hi-media, Mumbai, India) 0.1% (w/v) for 5 minutes followed by 3-4 times rinse with double distilled water. Then the nodes were inoculated on different shoot regeneration media.

Multiple shoot proliferation

Sterilized *in vivo* node was inoculated aseptically on Murashige and Skoog's (1962) medium (MS) and MS with different Plant Growth Regulators i.e. 6-Benzylaminopurine (BAP)/ Kinetin(Kin)/ Thidiazuron(TDZ) /Meta-topolin(mT)

(0.5- 3.0mg/L) and 1-Napthaleneacetic acid (NAA)/Indole-3-acetic acid(IAA)/ Indolebutyric acid(IBA) (0.5-1.0 mg/L) and Gibberellic acid (GA₃) (0.5- 2.0) for multiple shoot initiation and proliferation in the 250-ml conical flask (Borosil, India). The MS medium was supplemented with 3% (w/v) sucrose and gelled with 0.6% (w/v) agar powder (High Media, India). 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 flask were placed in a plant tissue culture room at 25±1 °C temperature, less than 16h photoperiod, 35-50 μM m⁻²s⁻¹ photon flux density provided by cool white fluorescent tube (Phillips, India). The aseptically inserted explant on media transfer to the culture room which was maintained the above culture condition. The inoculated nodes were sub-cultured on the same media in interval of 10-12 days. The days after which the nodes were induce shoot and the percentage of response was noted down from the day of the inoculation of node. The influence of node position and season on multiple shoot regeneration was also studied.

For scale up purpose, *in vitro* nodes excised from one-month old axenic primary shoot generated from *in vivo* node in aseptic condition were inoculated into the MS basal media supplemented with different concentrations of BAP (0.1- 3.0 mg/L) for shoot multiplication.

Rooting of *in vitro* regenerated shoots

Well developed *in vitro* shoots were excised and transferred to 60 ml culture tubes (Borosil, India) containing MS, ½ MS, ¼ MS and ½ MS media augmented with IAA (0.25-1.0 mg/l) and IBA (0.25-1.0 mg/l). The rooting media fortified with 1.5% (w/v) sucrose gelled with 0.6% (w/v) agar (Hi-media, Mumbai). The culture condition was same as above said.

Acclimatization

The *in vitro* rooted plantlets were gently washed with tap water to remove agar and to avoid contamination. Then the plastic pots were wiped with alcohol and filled with autoclaved sand: soil; 1:1 and sand: soil; 2:1 composition. Then the plantlets were carefully planted in the sand and soil mixture followed by watering until the sand soil mixture became fully wet. Then the plastic glasses were covered with polythene bags and kept in culture room. After 2 days, the upper part of the polythene bag was cut by two sides to maintain humidity and a small hole was done in the lower part for watering the plant with the help of wash bottle. This condition was kept for 1 week. Then the polythene bag completely removed and kept the plantlet in the culture room for next 2 days. After that, the plantlets were transferred to outside the lab under shade for 7 days with daily watering and subsequently transferred to larger earthen pot. At the end, after 15 days acclimated plants were transferred to field/natural condition.

Photography and Data Recording

Cultures and acclimatized plants were photographed by Canon DSLR P3000 camera. Uploading of photo to the computer was done with the help of the connecting cord. The data including average number of shoots/ explant, average number of roots/ shoot, average length of shoot and root were recorded after one month from the date of inoculation. Both shooting and rooting experiment repeated thrice. The experiments were designed with one explant/flask having

five treatments for shooting and one shoot/ tube having ten treatments for rooting.

Results and Discussion

Evaluation of growth regulators for shoot induction, multiplication and elongation

Growth, morphogenesis and development of plant tissues *in vitro* are mostly governed by the composition of culture media. The basic and principal components of plant tissue culture media are inorganic nutrients, carbon sources, organic supplements, plant growth regulators (PGRs), vitamins, amino acids and gelling agent etc. Among them PGRs are mostly required for growth, morphogenesis, shoot multiplication and elongation. The *in vivo* nodes from 3rd-6th position from shoot apex were collected because 1st and 2nd position nodes were very soft which cannot withstand the surface sterilization and nodes beyond 6th position was woody in nature found unsuitable for shoot regeneration (Fig. 2 A). The collected nodes were experimented with various concentrations of PGRs to get the morphogenic response and the results were documented in the Table 1.

In this experiment, MS basal medium was tried for multiple shoot induction, where no shoot induction was observed. So, the addition of PGRs into the MS basal media for shoot induction was found necessary. Different kinds of cytokinin like BAP, Kin, *mT* and TDZ in different concentrations were tested singularly. Out of the four cytokinins, BAP found most effective for shoot bud initiation (Table 1). Among different concentrations of BAP (0.5- 3.0 mg/L) tested, the optimum concentration of BAP 2.0mg/L was found most suitable with 100% response of explants with highest (*c.a.* 11) number of shoot buds (Fig. 2 B). BAP was also reported as suitable phytohormone for multiple shoot induction/proliferation/ multiplication from *in vivo* node in a number of medicinal legumes like like *Cassia tora* (Quraishi *et al.*, 2011), *Desmodium gangeticum* (Puhan and Rath, 2012) and *Dalbergia sissoo* (Vibha *et al.*, 2014) respectively.

Highest eleven number of shoot buds were observed on the optimum shoot regeneration medium, out of which 2-3 shoot buds become elongated during the first harvest followed by the subculture of the original explant on the

same multiplication media for elongation of other shoot bud. Again, in the 2nd harvest 2-3 rootable shoots were obtained. Finally, at the end an average 5.66 shoots with an average shoot length of 3.90 cm was obtained from a single *in vivo* node (Table 1). The other shoot buds remain as such without any elongation unsuitable for rooting.

About the other cytokinins like Kin, TDZ and *mT* (0.5- 3.0 mg/L), Kin 1.0mg/L showed 66.66% response which developed only one shoot per explant with 0.5 cm average shoot length. TDZ 0.5 mg/L also showed 100% respond, 1.33 shoots/explant and 2.2 cm average shoot length. The better result obtained by another cytokinin *mT* (2.0 mg/L) which resulted in 100% shoot regeneration response with average 1.33 shoots/explants and 3.1 cm average shoot length (Fig. 2 C) which was not higher than BAP 2.0 mg/L. So, BAP (2.0mg/L) was found as an optimum medium. The superiority of BAP may be due to the effective absorption of hormone by the tissue for shoot induction and multiplication or may be BAP is the precursor of the other natural hormone like zeatin in the tissue (Naik and Chand, 2011; Malik *et al.*, 2005). Besides, the free bases, BAP also present in form of nucleosides and nucleotides which is stable than other cytokines (Behera *et al.*, 2020; Frebort *et al.*, 2011). For further increase in shoot number auxins like IAA, IBA in their different concentrations (0.5-1.0 mg/L) were added with the optimum concentration of BAP (2.0 mg/L). Addition of IAA (1.0mg/L) resulted in 66.66% response, average 1.0 shoot/explant and average 1.5 cm shoot length. Similarly, IBA (1.0mg/L) with BAP (2.0 mg/L) resulted in 100% response, average 1.0 shoot/explant and 1.9 cm average shoot length. No further increase in shoot number was observed after the addition of auxin with different concentration.

Further addition of (1.0mg/L) GA₃ with the optimum concentration of BA (2.0 mg/L) resulted in average 1.66 shoot/explant (0.9 cm average shoot length) with 100% response. So, it is concluded that, out of 26 different concentrations and combinations best result was obtained at BAP 2.0 mg/L (Table 1). In the same plant, the superiority of BAP (1.0mg/L) in development of multiple shoots by the use of *in vitro* cotyledonary node and adventitious shoot proliferation was also previously reported by Behera *et al.* (2020) and Suresh *et al.* (2015) respectively.

Table 1 : Influence of plant growth regulators on multiple shoot initiation and proliferation of *in vivo* nodal explants of *C. gladiata*

MS + PGRs (mg/L)	Mean % of explant response	Average no. of shoots /explant	Average shoot length (cm)
Control (MS)	0	0	0
MS + BAP			
0.5	100 ^a	1 ^{def}	2.4 ^{defg}
1.0	88.66 ^{ab}	1.33 ^{de}	2.6 ^{def}
1.5	100 ^a	2.33 ^{de}	2.7 ^{de}
2.0	100 ^a	5.66 ^a	3.90 ^{bc}
2.5	83.55 ^{bc}	2.66 ^b	1.3 ^{ghijkl}
3.0	81.32 ^{bcd}	1.66 ^{cd}	4.5 ^b
MS + TDZ			
0.5	100 ^a	1.33 ^{de}	2.2 ^{defghi}
1.0	100 ^a	1.33 ^{de}	2.3 ^{defgh}

2.0	66.66 ^{ef}	1d ^{ef}	2.2 ^{defghi}
3.0	66.66 ^{ef}	1.66 ^{cd}	1.6 ^{fghijk}
MS+ mT			
0.5	66.66 ^{ef}	1 ^{def}	0.9 ^{ijklmno}
1.0	66.66 ^{ef}	1 ^{def}	1.4 ^{ghijklm}
2.0	100 ^a	1.33 ^{de}	3.1 ^{cd}
3.0	73.33 ^{cde}	1 ^{def}	2.7 ^{de}
MS+ Kin			
0.5	0	0	0
1.0	66.66 ^{ef}	1 ^{def}	0.5 ^{klmnopqr}
2.0	100 ^a	1 ^{def}	0.4 ^{lmnopqrs}
3.0	66.66 ^{ef}	1 ^{def}	0.5 ^{klmnopqr}
MS + BAP+ IAA			
2.0+ 0.5	66.66 ^{ef}	1 ^{def}	0.6 ^{ijklmnopq}
2.0+ 1.0	66.66 ^{ef}	1 ^{def}	1.5 ^{fghijkl}
MS+ BAP+IBA			
2.0+ 0.5	100 ^a	1 ^{def}	1.4 ^{ghijklm}
2.0+ 1.0	100 ^a	1 ^{def}	1.9 ^{efghij}
MS+BAP + GA₃			
2.0 + 0.5	100 ^a	1.33 ^{de}	0.9 ^{ijklmn}
2.0 + 1.0	100 ^a	1.66 ^{cd}	0.9 ^{ijklmn}
2.0 + 2.0	100 ^a	1 ^{def}	0.4 ^{mnopqrs}

Data pooled from 3 separate experiments each with 5 replicates, 1 explant/flask (1*5*3 = 15). * According to DNMR, means followed by the same letter within columns are not significantly different (P = 0.05).

For scale up purpose the *in vitro* nodes excised from primary shoots (during 1st and 2nd harvested) used as explant for further multiple shoot regeneration. As BAP found most suitable cytokinin for multiple shoot regeneration from *in vivo* node, we tried BAP in different concentration (1.0-3.0 mg/L) for the multiplication of *in vitro* nodes and the results are noted in table 2.

The *in vitro* node showed highest 100% shoot regeneration response with average shoots/explant 2.0 and average shoot length 4.96 cm on BAP (2.0 mg/L) (Fig 2 D). Upscaling of shoots was accomplished from axenic nodal explants derived from primary *in vitro* shoots on fresh medium of the same composition derived from *in vivo* nodal explants. Thus, we could obtain 36–40 shoots [1 *in vivo* node

= 05 *in vitro* shoots of 04 cm length, each *in vitro* shoot = 04 *in vitro* nodes, 1 *in vitro* node = 02 rootable shoots (05x04x02 = 40)] starting from a single node within 06–07 weeks with average shoot length was recorded to be 4.96 cm. Moharana *et al.*, (2017) used similar type of scale up process by utilising *in vitro* node from 1st and 2nd harvest for the cash crop plant *Lawsonia inermis*. Similarly, Vengadesan *et al.*, 2003 (*Acacia sinuate*), Sujatha *et al.*, 2007 (*Cicer arietinum*), Quraishi *et al.*, 2011 (*Cassia tora*), Janaki and Manoharan, 2012 (*Vigna radiate*) Jaiswal *et al.*, 2015 (*Pterocarpus marsupium*) and Bhandari *et al.*, 2021 (*Sophora mollis*) used *in vitro* node as explant for shoot regeneration where they found BAP as the suitable phytohormone.

Table 2 : Influence of plant growth regulators on multiple shoot initiation and proliferation of *in vitro* nodal explants excised from *in vivo* node of *C. gladiata*

MS + PGRs (mg/L)	Mean % of shoot response	Average no. of shoot/explant	Average shoot length (cm)
MS + BAP			
0.5	100	1.14 ^{bc}	1.9 ^e
1.0	100	1.33 ^b	2.45 ^c
1.5	100	2.0 ^a	4.0 ^b
2.0	100	2 ^a	4.96 ^a
3.0	100	1 ^{cd}	2.36 ^{cd}

Data pooled from 3 separate experiments each with 5 replicates, 1 explant/flask (1*5*3 = 15). * According to DNMR, within columns means followed by the same letter are not significantly different (P = 0.05).

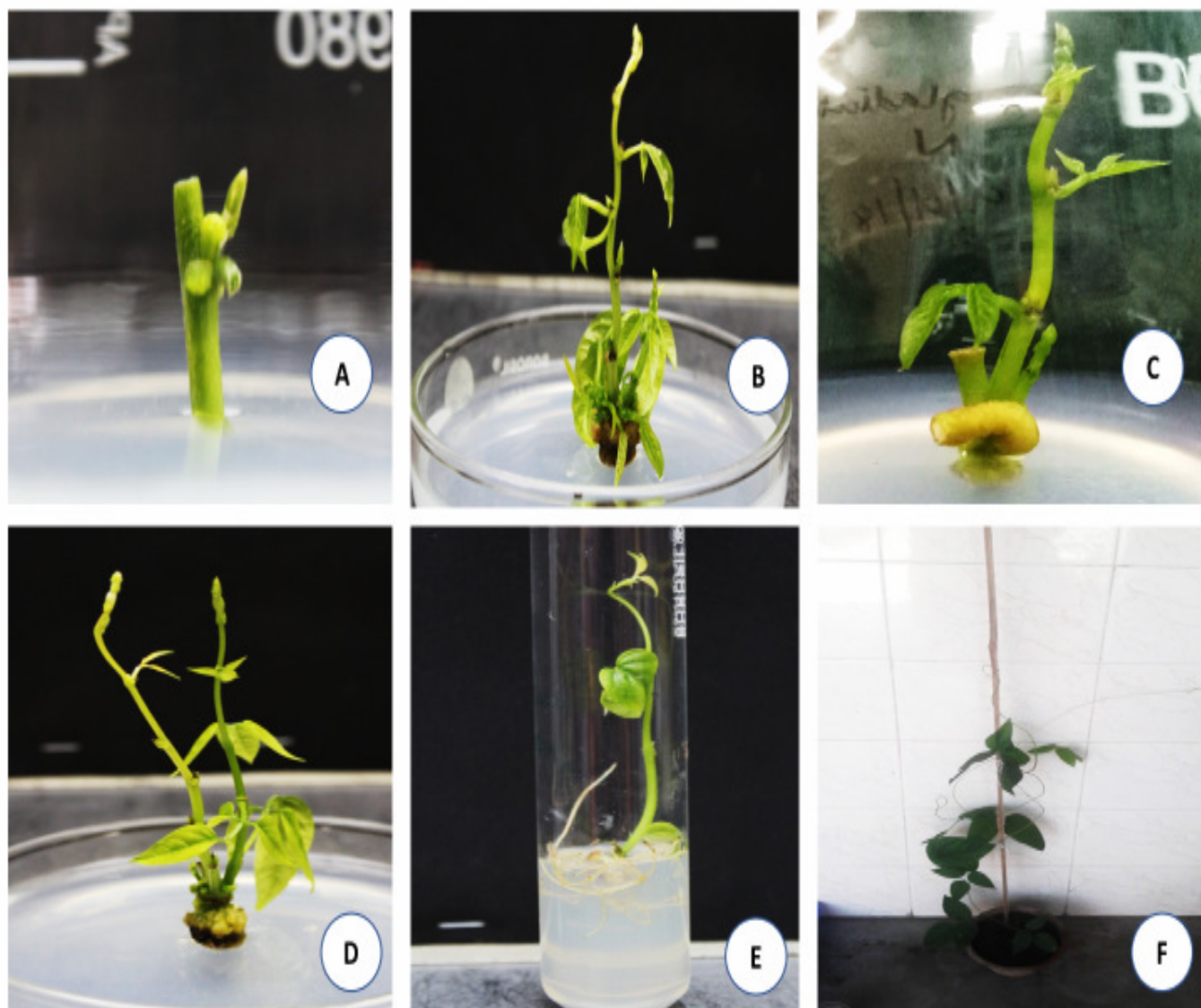


Fig. 2 (A) inoculation of *in vivo* nodal explant (B) Multiple shoots on MS+BAP 2.0 mg/l (C) Multiple shoot on MS+mT 2.0 mg/l (D) Multiple shoot of *in vitro* node on MS+BAP 2.0 mg/l (E) Rooting of *in vitro* shoots on $\frac{1}{2}$ MS+IBA 0.5 mg/l (F) Acclimated plant

Effect of seasonal variation and position of *in vivo* node on shoot bud multiplication

It was observed that bud break and axillary shoot proliferation frequency was greatly influenced by the position of the nodes from the shoot tip and season of collection in *C. gladiata*.

December–January was observed to be the best time for collection of explants for maximum frequency (92%) of bud break, where minimum loss was also observed due to infection followed by February–March with frequency percentage (80%, Fig. 3). Lower percent bud break (60%) was observed in explants collected in the months of October–November. Maximum contamination, both bacterial and fungal, of the explants was observed in the period of April–May with a 05% of explant response. Such type of seasonal variation of nodal explants on frequency of shoot multiplication was also observed in *L. inermis* by Ram and Shekhawat, (2011); Moharana *et al.* (2018).

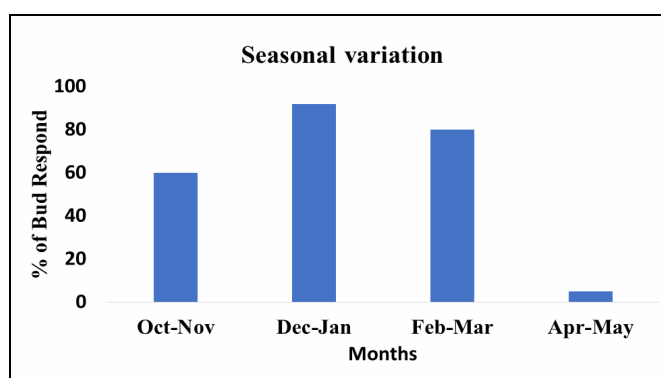


Fig. 3 : Effect of seasonal influence on *in vivo* nodal shoot regeneration

Rooting of *in vitro* shoots

The rootable shoots (4.0-5.0 cm length) regenerated due to multiplication of both *in vivo* and *in vitro* node were cut down and used for rooting. Different concentration of MS medium without any plant growth regulator i.e. MS, $\frac{1}{2}$ MS, $\frac{1}{4}$ MS, $\frac{1}{8}$ MS was tried. But any of the concentration except

½ MS showed no root development. On ½ MS only one root is formed which is only 0.5 cm and the root was weak. Therefore, ½ MS fortified with different concentration auxin (IAA/IBA) was tried for enhancement of root number and length. Among different concentrations of IAA (0.25-1.0 mg/L), 0.5mg/L showed best result with 100% response, average 03 roots/explant and average root length 3.05cm. Similarly, among different concentration of IBA (0.25-1.0 mg/L), 0.5 mg/L showed best result with 100% response with 4.0 average root number/explant and 3.45 cm average root length (Table 3, Fig. 2 E). So, among IAA and IBA, 0.5mg/L

IBA was found most suitable for root regeneration in case of *C. gladiata*. But Ozaki (1993) used another auxin NAA (1.0 mg/L) with combination of a cytokinin BAP (0.01 mg/L) for the best regeneration of root in case of *Canavalia gladiata*. Similarly, Behera *et al.* (2020) reported half-MS with IAA (0.5 mg/l) was best rooting medium Whereas Suresh *et al.* (2015) did not report anything about rooting. Similarly, IBA was found best rooting medium for *Acacia mangium* (Nanda *et al.*, 2004), *Acacia chundra* (Rout *et al.*, 2008), *Vigna radiata* (Janaki and Manoharan, 2012) and *Psoralea corylifolia* (Nabi and Shrivastava, 2017).

Table 3 : Rooting of *in vitro* shoots regenerated from *in vivo* node of *C. gladiata*

Media + Auxin (mg/L)	% of Rooting	No. of roots/ shoot	Mean root length(cm)
MS	0	0	0
½ MS	53.33 ^c	1 ^d	0.5 ^f
¼ MS	0	0	0
1/8 MS	0	0	0
½ MS + IAA			
0.25	66.66 ^b	2 ^c	1.5 ^e
0.5	100 ^a	3 ^b	3.05 ^b
1.0	0	0	0
½ MS + IBA			
0.25	66.66 ^b	3.0 ^b	1.36 ^{cd}
0.5	100 ^a	4.0 ^a	3.45 ^a
1.0	100 ^a	3.0 ^b	1.33 ^{cde}

Data pooled from 3 separate experiments each with 5 replicates, 1 shoot/ tube (1*5*3 = 15). * within columns means followed by the same letter are not significantly different according to DNMR (P = 0.05).

Acclimatization and field transfer

Plantlets with well-developed root are successfully acclimatized by transferring it in to plastic disposable glass containing soil and sand in 1:1 ratio. The survival rate of plantlets after transfer to soil: sand (1:1) was 80% and among them 62% plants were transferred to garden soil successfully (Fig. 2 F). Similarly, Behera *et al.* (2020) also documented sand and soil in 1:1 ratio as acclimatization substrate of *Canavalia gladiata*. Zaho *et al.* (2003) acclimatized *in vitro* plants of *Sophora flavescens* in soil and sand 1:1 with 73.6% survival rate. Moharana *et al.* (2018), reported the same composition of soil and sand 1:1 as suitable for acclimatization of *Lawsonia inermis*. Similarly, Sujatha *et al.* (2007) and Singh *et al.* (2012) used sand and soil for acclimatization of *in vitro* regenerated plantlets of *Cicer arietinum*, *Bauhinia variegata* respectively. Soil and sand 1:1 was also acclimatized substrate for *Sophora mollis* (Bhandari *et al.*, 2021).

Conclusion

In this above study, for the first time a reproducible and efficient protocol for *Canavalia gladiata* (Jacq). DC. was developed using *in vivo* node as explants. Though it's a dietary legume, till now it hold the status of an under-utilized legume because of it's anti-nutritional factors. So research should be focused on reduction of toxin level for which our work/protocol can be a step forward. Further, the above protocol may lead to the large scale production of *Canavalia gladiata* (Jacq). DC. as well as its conservation.

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