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# *In Vitro* Regeneration of Pigeonpea [*Cajanus cajan* (L.) Millsp.] Genotype GT 101 using Cotyledonary Node

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## Abstract

**Objectives:** A protocol was developed for plant regeneration using cotyledonary node of high-yielding indigenous pigeonpea [*Cajanus cajan* (L.) Millsp.] genotype GT 101. **Methods:** Induction of multiple shoots directly was completed from cotyledonary node as an explants on Murashige and Skoog's (MS) medium supplemented with various concentrations and combination of 6-benzylaminopurine (BAP), kinetin and α-naphthalene acetic acid (NAA). Elongation of multiplied shoots was performed on MS medium supplemented with different combination of BAP and $GA_3$. These well elongated plantlets were further transferred on MS medium supplemented with various concentrations of indole-3-butyric acid (IBA) for root induction. Regenerated plants were transferred to cocopeat:soil:vermiculite (2:1:2) for acclimation. **Findings:** The frequency of multiplication and number of multiple shoots from cotyledonary node explant were influenced on various types and concentrations of cytokinin. For multiple shoots induction, 3.0 mg/L BAP with 0.5 mg/L NAA was superior as compared to other combinations. The elongation of multiplied shoots was carried out on MS medium supplemented with 0.5 mg/L BAP and 0.5 mg/L $GA_3$. The developed shoots were advanced to rooting on the medium supplemented with 0.5 mg/L IBA. They were subsequently grown in pots with 80% survival rate and these plants produced viable seeds. **Improvement:** The protocol for the production of *in vitro* multiple shoots with high frequency and their successive conversion to whole plants agreements potential for use in the improvement of protocol for development of transgenic in pigeonpea.

**Keywords:** *Cajanus cajan* (L.), Cotyledonary Node, GT101, *In vitro* regeneration, Pigeonpea

## 1. Introduction

Food legumes or pulses play major role in human diet and animal feed bestowing the chief source of protein for vegetarian. Besides an economical source of protein, they also provide carbohydrate, minerals and vitamins, mainly in vegetarian diet[1]. Among the various pulses, pigeonpea is an essential multipurpose grain legume and also a great source of protein for populations living in the semi-arid tropics which is cultivated under rain-fed agricultural condition. It contains about 20-22 per cent protein, chiefly sulphur containing amino

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acids, *viz*., cysteine and methionine[2], which is almost three times than cereals. Also contains 45-55% starch, 3-5% soluble sugar, 1-2% fats, 3-4% crude fiber and 3-4% ash[3].

Pigeonpea is susceptible to various diseases like, *Fusarium* wilt, *Phytophthora* stem blight, sterility mosaic virus as well as insect pests namely, *Helicoverpa armigera*, pod fly and storage grain pests causes major yield losses[4]. Attempts to deal with these problems through conventional breeding methods have met with partial achievements due to limited genetic variability and cross-incompatibility wild species/relatives[5].

As an important crop plant, pigeonpea have been put through the efforts to improve desired characters comprising *in vitro* culture response. Since pigeonpea is infamously obstinate to regenerate by tissue culture, much work has been devoted to develop and optimize effective *in vitro* regeneration procedure which is essential for the successful development of transgenic plant[6].

Genetic modification offers exclusive opportunities to integrate genes from various eukaryote as well as prokaryote sources into pigeonpea[7]. An effective tissue culture or *in vitro* regeneration protocol is a must for transgenic development. Hence, various protocols has been established for tissue culture of pigeonpea[4, 8-11]. Among all the *in vitro* regeneration protocols, direct organogenesis evidenced to be most favourable for the development of transgenics in pigeonpea. Moreover, *in vitro* regeneration from various explants have been attempted in pigeonpea, which include leaf[12-14], cotyledonary node[6, 8-10, 15], apical meristem[4, 16] and epicotyle[6, 17].

The present study was conducted for the development of an efficient protocol for *in vitro* regeneration of pigeonpea [*Cajanus cajan* (L.) Millsp.] cv. GT 101 which can be used for further genetic transformation and/or transgenic plants development.

# 2. Materials and Methods

## 2.1 Plant Material and Explant Preparation

The experimental material of pigeonpea genotype GT 101 was collected from Pulses Research Station, S. D. Agricultural University, Sardarkrushinagar, India. The seeds were surface sterilized with 70 per cent (v/v) ethanol for 30 seconds, followed by 0.1 per cent (w/v) aqueous mercuric chloride ($HgCl_2$) containing one to two drops of Tween-20 solution for 7-8 min and then rinsed five times with sterile double distilled water and germinated aseptically on sterilized MS basal medium[18]. Cotyledonary node excised from 12 days old seedling was used as an explant for *in vitro* regeneration. Explants were placed in glass bottles containing 30-35 ml of culture medium. pH of a medium was adjusted to 5.7 prior to autoclaving. All the bottles inoculated with explants were incubated at 25±0.5˚C with 16 hr photoperiod.

## 2.2 Culture Medium and Condition

The MS medium (solid) supplemented with various mixtures of Kinetin, BAP and NAA was tested for the induction of multiple shoots (The details of treatments are given in Table 1). After multiple shoot induction, various growth regulators *viz*., cytokinin and $GA_3$ were used to standardize the most appropriate medium for shoot elongation (The information is given in Table 2). Properly elongated (3 to 4 cm length) plantlets were transferred on the medium designed for root induction. These elongated shoots were dipped into 5.0 mg/L IBA for 2 min as a pulse treatment, before culture on root induction medium (RIM) which was composed of MS medium with half nutrient strength, 0.7% agar and IBA in different concentrations (The details treatments are given in Table 3).

**Table 1.** Effect of different concentrations of BAP, Kinetin and NAA on multiple shoot regeneration through cotyledonary node

| Growth hormone (mg/L) | | | Days to multiple shoot initiation | Multiplication (%) | Mean average number of shoot/ explant |
|---|---|---|---|---|---|
| BAP | Kinetin | NAA | | | |
| 1.0 | - | 0.1 | 18.6±0.66 | 46.70 | 06.0±0.58 |
| 2.0 | - | 0.2 | 17.3±0.86 | 60.00 | 08.9±0.12 |
| 3.0 | - | 0.5 | 13.7±0.25 | 73.33 | 13.7±0.17 |
| 4.0 | - | 1.0 | 16.5±0.45 | 33.33 | 03.2±0.48 |
| 1.0 | 0.1 | - | 16.0±1.05 | 26.70 | 02.2±0.85 |
| 2.0 | 0.2 | - | 13.5±0.95 | 40.00 | 04.7±0.66 |
| 3.0 | 0.5 | - | 17.5±0.84 | 53.33 | 05.2±0.97 |
| 4.0 | 1.0 | - | 18.2±0.78 | 40.00 | 05.7±0.57 |

Means of three replicate cultures ± SE;
BAP: 6-Benzylaminopurine; NAA: α-Naphthalene acetic acid

**Table 2.** Effect of different concentrations of BAP and $GA_3$ on shoot elongation

| Growth Hormone (mg/L) | | Shoot Length (cm) |
|---|---|---|
| BAP | GA3 | |
| 0.2 | 0.2 | 2.3±0.09 |
| 0.5 | 0.5 | 4.2±0.28 |
| 1.0 | 0.2 | 2.0±0.15 |
| 2.0 | 0.5 | 2.2±0.12 |

Means of three replicate cultures ± SE;
BAP: 6-Benzylaminopurine; $GA_3$: Gibberellic acid

**Table 3.** Effect of different concentrations of IBA and strength of MS medium on in vitro root induction in shoots

| Growth hormone (mg/L) | | Days to root initiation | Rooting (%) |
|---|---|---|---|
| MS Basal | IBA | | |
| ½ MS | 0.5 | 18.5±0.14 | 80.0 |
| ½ MS | 1.0 | 19.3±0.29 | 65.0 |
| Full MS | 0.5 | 21.6±0.45 | 68.7 |
| Full MS | 1.0 | 22.5±0.95 | 62.5 |

Means of three replicate cultures ± SE;
IBA: Indole-3-butyric acid; MS: Murashige and Skoogs medium

Plantlets with sufficient roots were carefully uprooted from the medium in culture bottles followed by washing of roots under running tap water to remove any sticked medium before transplanting into plastic bag containing mixture of cocopeat: soil: vermiculite (2:1:2) with 0.1% carbendazim (fungicide) for 15 to 20 days. The pots were enclosed with perforated polythene bags with small holes for air circulation for 10 days and watered with ¼ MS salt twice a day while being maintained in the green house. After 10 days, the polythene bags were removed and the plants were exposed to the natural environment.

## 3. Results and Discussion

After surface sterilization of seeds, they were cultured on solid MS basal medium excluded growth regulators exhibited 80-95% germination rate after 3-5 days. Further, 10-12 days old seedlings were considered as suitable explants. The cotyledonary node from seedling plant was used as an explant for *in vitro* regeneration.

Various combinations and concentrations of plant growth hormones were used for shoot regeneration and multiple shoot initiation from cotyledonary node, out of which treatment 3.0 mg/L BAP + 0.5 mg/L NAA combined with MS basal medium was proven superior among all the treatments applied for days to multiple shoot initiation, multiplication frequency and number of shoots per explant which is presented in Table 1 and represented in figure 1 (A and B). However, early multiple shoot initiation was observed at lower concentration of BAP and Kinetin whereas multiplication frequency and number of shoots per explant were found poor as compared to other treatments. As the existence of high amount of endogenous auxin in pulses, regeneration of multiple shoots is possible even an auxin supplementation is not provided however, exogenous supply can be used to balance auxin-cytokinin ratio responsible for efficient regeneration[19]. According to previous reports, use of BAP together with NAA was helpful for regeneration of multiple shoots[12,13,19,20]. The present study indicated that multiple shoot regeneration depends on the composition of the medium.

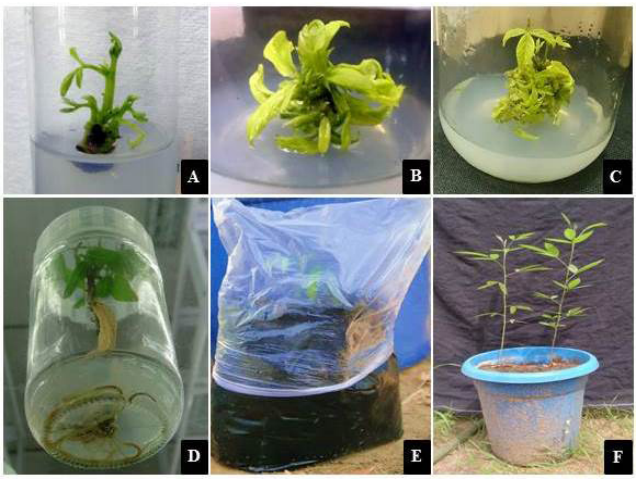


**Figure 1.** *In vitro* plant regeneration from cotyledonary node explants of pigeonpea genotype GT 101.
(A) Multiple shoot initiation on MS medium containing 3.0 mg/L BAP and 0.5 mg/L NAA; (B and C) Shoot elongation on MS medium containing 0.5 mg/L BAP and 0.5 mg/L $GA_3$; (D) Response of regenerated shoots on ½ MS medium containing 0.5 mg/L IBA for root induction; (E) Acclimatization of plantlets in plastic bag containing cocopeat: soil: vermiculite (2:1:2); (F) Acclimatization of plantlets in earthen pot.

By subculturing shoot-initials on the same shoot induction medium, we found difficulty in shoot elongation. Even on prolonged culture of the shoot-initials, they did not grow up in length. However, some leaves showed

expansion but they turned yellow and later dropped. As the amount of BAP was reduced up to 0.5 mg/L and addition of 0.5 mg/L $GA_3$ in medium, shoots elongated rapidly and gave rise to healthy green leaves, indicating that higher cytokinin (BAP) suppresses shoot elongation. Results of shoot elongation are shown in table 2 and figure 1 (C). The successful elongation of regenerated shoots have been reported by some workers in the recent past by the treatment of $GA_3$ alone or in combination with BAP[10-11, 20-22]. In the current study, initially single shoot was developed with unifoliate and bifoliate leaves which lastly reached the actual trifoliate leaves after 4 to 5 weeks of inoculation.

Properly elongated shoots of approximately 3-4 cm length were further inoculated to root induction medium (RIM). Elongated shoots were dipped in 5.0 mg/L IBA for 2 min as a pulse treatment, prior to culture on RIM. Half strength MS medium with 0.5 mg/L IBA induced best response with early root initiation (Table 3 and Figure 1 (D)). This finding was found to be similar to the findings of past works on direct rooting[4, 11-12, 20-23].

For acclimation of plantlets, well rooted plants were transplanted to mixture of cocopeat: soil: vermiculite (2:1:2) with 0.1% carbendazime (fungicide) in plastic bag then covered with polythene bags having small holes for air circulation and placed in green house for 10 days and gradually brought in to uncontrolled then finally to open environmental conditions in earthen pots (Figure 1 (E and F)). The per cent survival during hardening of plantlets was 70 to 80%. The survived plantlets generate new sprouting within 10 days after transplanting in mixture of cocopeat: soil: vermiculite.

## 4. Conclusion

In the current study; a simple, efficient as well as reproducible protocol for *in vitro* regeneration of pigeonpea indigenous variety GT 101 by using 12 days old cotyledonary node acquired from *in vitro* grown septic seedling. This protocol can be appropriate for the development of protocol for genetic transformation of pigeonpea for useful characters such as resistance/tolerance to biotic/abiotic stresses.

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