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Exploring *in vitro* shoot regeneration studies in pigeon pea (*Cajanus cajan*) *via* organogenesis and somatic embryogenesis

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Abstract
In this study, we explored the *In vitro* shoot regeneration potential of pigeon pea (*Cajanus cajan*) *cv*. BSMR 853 through two distinct pathways: organogenesis and somatic embryogenesis. The use of different plant growth regulators and culture conditions was investigated to optimize shoot regeneration efficiency. For organogenesis, embryos from 5-day-old germinated seeds were employed, and we observed that the highest shoot bud regeneration occurred on media supplemented with 2 mg/L 6-benzylaminopurine (BAP). Subsequent elongation of shoots was achieved by reducing the BAP concentration. In contrast, somatic embryogenesis was initiated from embryo of 5-day-old germinated seeds. Here thidiazuron (TDZ) was used to instead of BAP as a growth regulator. The development of globular embryos was rapid, and the embryos were prolific with TDZ. These globular embryos were then matured using MS media supplemented with abscisic acid (ABA) and subsequently germinated on half-strength MS media supplemented with lower concentrations of BAP. Comparative analysis of the two regeneration pathways in pigeon pea *cv*. BSMR 853 revealed that despite somatic embryogenesis producing a greater number of embryos, organogenesis led to a greater success rate in establishing shoots. This study provides valuable insights into the regeneration processes of pigeon pea, offering a basis for further research and applications in crop improvement and biotechnology.

Keywords: Pigeon pea, organogenesis, somatic embryogenesis, BAP, TDZ, shoot regeneration

1. Introduction
Pigeonpea (*Cajanus cajan*), commonly referred to as red gram, is a crucial grain legume cultivated in the semiarid tropics and belongs to the Fabaceae family. It is the second most important food legume globally, with India contributing to more than 80% of the world's production (Bhardwaj *et al*., 2023) [1]. This versatile crop found in approximately 50 countries across Asia, Africa, and the Americas serves diverse purposes, including food, fodder, fuel wood, etc. (Sinha, 1977) [2].

Pigeonpea stands as the fifth most significant legume on a global scale, accounting for 5% of total pulse production (4.92 million hectares), with India being the primary contributor at more than 70% (3.6 million hectares) and boasting a rich variety of cultivars (Saxena, 2009) [3]. According to estimates by the Food and Agriculture Organization (FAO) in 2020-21, the worldwide annual production of pigeon pea reached 4.3 million metric tons, with India alone contributing 59% of this total (FAO statistics). The cultivation area for pigeon pea spans approximately 5.4 million hectares, with India responsible for 72% of this land, totaling 3.9 million hectares.

One of the distinguishing features of pigeon peas is their protein content, which is approximately 20-22%, with notable levels of sulfur-containing amino acids such as cysteine and methionine, surpassing those found in cereals. This high protein content makes pigeon pea an essential protein source in vegetarian diets in India. Additionally, pigeon peas contain soluble sugars (3-5%), fats (1-2%), crude fiber (3-4%), starch (45-55%), and ash (3-4%) (Singh *et al*., 1990) [4].

However, various biotic and abiotic stress factors are challenging to cultivate in pigeonpea plants, and limited resistance has been found in germplasm accessions worldwide. Traditional breeding methods have struggled to create pigeon pea cultivars resistant to pests

such as the legume pod borer (Helicoverpa armigera) and diseases such as fusarium wilt due to limited genetic variability among germplasm accessions (Minja *et al*., 2000) [5]. These difficulties, combined with breeding incompatibility issues associated with wild species, have led to the exploration of genetic engineering as an alternative approach (Singh *et al*., 2019) [6]. Advances in plant transformation techniques have opened doors to overcoming these challenges, allowing for precision in genetic manipulation. Nevertheless, efficient plant regeneration protocols are crucial for the successful implementation of recombinant technology.

In vitro tissue culture methods that tap into plant cell totipotency provide an avenue for micro-propagating elite plant clones through organogenesis and somatic embryogenesis. Manipulating culture conditions now enables the regeneration of pigeon pea plants from both differentiated and undifferentiated tissues, facilitating genetic transformation. Genetic engineering in plant species typically involves the introduction of foreign genes or the overexpression of native genes without altering nutritional quality.

With this background in mind, the present research aimed to develop a shoot regeneration protocol utilizing organogenesis and somatic embryogenesis in pigeon pea cv. BSMR-853.

2. Materials and Methods

2.1 Plant Material: During the course of this investigation, pigeon pea seeds from the BSMR 853 cultivar were obtained from the Agricultural Research Station (ARS) in Badnapur. BSMR 853 is a high-yielding medium-duration variety that is suitable for mono-cropping and intercropping. It is resistant to Sterility Mosaic Disease.

2.2 Surface sterilization and seed germination

Preparing the seeds for experimentation, a series of sterilization steps were meticulously performed. Initially, the seeds were thoroughly washed in distilled water to eliminate any surface impurities or contaminants. Subsequently, the sections were subjected to a 70% ethanol rinse, and this procedure was repeated twice to ensure effective surface sterilization. Following the ethanol treatment, the seeds were carefully treated with a 0.1% $HgCl_2$ solution, followed by another washing step in double distilled water, which lasted for 5 minutes. This procedure was used to meticulously remove any residual traces of the surface sterilant $HgCl_2$. Once the seeds were sterilized, they were placed within ½ MS seed germination medium supplemented with growth regulator (BAP 2.5 mg/l + IAA 0.2 mg/l), and some seeds were placed on only ½ MS media without growth harmones where they were allowed to germinate and grow over a period of 3-5 days. This thorough sterilization process was vital for maintaining a contamination-free environment and ensuring the reliability of the results.

2.3 Explant preparation

Five-day-old germinated seeds were used to obtain embryos as explants by cutting them with a sharp razor blade.

2.4 Organogenesis

To stimulate shoot bud formation through organogenesis, embryo (from 5-day-old germinated seeds) explants were cultivated on MS basal media supplemented with either BAP (6-benzylaminopurine) or in combination with BAP, NAA (naphthaleneacetic acid), and TDZ (thidiazuron), as outlined in Table 2.1, and 3% sucrose was used as a carbon source and as a gelling agent for agar powder at a concentration of 0.8%. The pH of the media was adjusted to 5.6-5.8. All the media were kept and observed for a minimum period of three days for any contamination before they could be cultured. The excised embryos were inoculated on prepared media supplemented with various combinations and concentrations of auxin and cytokinins at a rate of four embryos per petriplate. All the laboratory experiments were directed under well-defined conditions in the culture room maintained at a temperature of 25±2 °C and a 16-hour photoperiod.

2.4.1 Shoot Bud Elongation

After a 4-week incubation period, the explants were observed to assess the induction of shoot bud formation. Subsequently, the regenerated shoot buds underwent an elongation phase on the same MS Basal Medium supplemented with lower concentrations of 6-benzylaminopurine (BAP).

2.5 Somatic embryogenesis

The zygotic embryo explants were cultured on MS basal media supplemented with various growth hormones, including 6-benzylaminopurine (BAP), in combination with thidiazuron (TDZ) and naphthaleneacetic acid (NAA), as detailed in (Table 2). To ensure optimal conditions, the pH of the media was adjusted to 5.6-5.8 before autoclaving at 15 psi for 20 minutes. Subsequently, the cultures were incubated for four weeks at a temperature of 25±2 °C with a 16-hour photoperiod. After the initial 4-5 weeks of incubation, some shoot growth and globular embryos were observed. These globular embroys were subjected to maturation using MS media supplemented with abscisic acid (ABA). Following maturation, the embryos were germinated on half-strength MS media supplemented with lower concentrations of BAP.

3. Results and Discussion

In our research study, we conducted experiments involving the surface sterilization of pigeon pea seeds. These sterilized seeds were subsequently introduced to half-strength MS (Murashige and Skoog, 1962) [7] media supplemented with the plant growth hormones BAP (6-benzylaminopurine) and IAA (indole-3-acetic acid). We also included control plates without hormone supplementation. Remarkably, we observed early germination within just two days in MS media supplemented with BAP and IAA, for an impressive germination rate of 98%. This was in contrast to the plain MS medium, where 75% of the seeds had germinated within the same time frame. Further investigations of the germination process revealed that after 5 days, both treatments resulted in robust germination, ranging between 95% and 98%.

3.1 Organogenesis

3.1.1 Effect of Growth Regulator Concentrations on Shoot Development

Investigations have explored the effects of diverse growth regulator concentrations on shoot development in the context of plant tissue culture. The incubation of zygotic

embryos under different growth regulator conditions revealed significant variations in shoot bud development.

Concurrently, this investigation examined the impact of varying concentrations of growth regulators on shoot development within a plant tissue culture system. In Treatment T_1, where 2 mg/l BAP was applied, a noteworthy increase in shoot production was observed, averaging 7.3±0.88 shoots per replicate (Table 3). These findings align with earlier reports by Geetha *et al*. (1998) [8] and Nalluri and Karri (2019) [9]. Nevertheless, the challenges associated with this treatment included the formation of multiple shoots and the yellowing/falling of leaves (Fig. 1 d).

Mehta and Moham Ram (1980) [10] reported that higher concentrations of BAP induced direct shoot regeneration from the cotyledonary surface. However, in our study, in T_2 and T_3, the use of higher concentrations of BAP (2.5 mg/l and 3 mg/l, respectively) resulted in a slightly lower average shoot number 3.3±0.883 and 3±0.66 (Table 3) (Fig.1 e, f). Than that in T_1. These treatments were characterized by thick shoot development, larger leaves, and overall reduced shoot growth, indicative of varied responses of different explants to concentrations of plant growth regulators.

TDZ, a compound with diverse roles in physiological systems (Murty *et al*. 1995) [28], has been employed to induce multiple shoot formations in various plant species (Malik and Saxena 1992) [18]. In Treatments T_4, T_5, and T_6, TDZ was introduced in combination with BAP. T_4 and T_5 produced 5±1.52 and 3±0.00shoots, respectively (Table 3.1) (Fig. 1 g, h, i). Similar results were reported by Eapen *et al*. (1998) [11] for a high regeneration frequency of shoots from leaf explants with TDZ and by Krishna *et al*. (2010) [12] for 2.5 mg/L BAP and 0.5 mg/L TDZ. Unfortunately, these treatments were associated with adverse effects such as leaf yellowing, shoot death (T_4), and the production of small, thick shoots (T_5) (Fig. 1 g, h). T_6, which utilized 3 mg/l BAP and 0.5 mg/l TDZ, presented the lowest average shoot number (1.6) and development of large leaves (Fig. 1, i). Moreover, the observed phenomenon was attributed to the inability of the explants to degrade, resulting in the accumulation of TDZ, as explained by Mok and Mok (2001) [20].

Treatments T_7, T_8, and T_9 involved the addition of NAA in combination with BAP. T_7 presented a greater average shoot number (5.3±1.20) and rapid growth. (Fig. 1, j). T_8 plants exhibited slower growth, smaller, thicker shoots, and some shoot death, resulting in an average of 2±0.57 shoots per replicate (Fig.1 k). T_9 yielded an average of 3.4±0.33 shoots, with leaves forming from the bottom and increased shoot height (Fig. l). Similar results were obtained by Nalluri and Karri (2019) [9] for the ICPL 87 variety of pigeon pea using leaf petiole explants. However, the shoots produced in this treatment were slender.

3.2 Somatic embryogenesis

Due to the remarkable proliferation observed in embryonic calli and the development of somatic embryos from individual cells, the preferred method for genetic transformation was considered to involve the pathway of embryogenesis (Hansen and Wright, 1999) [13]. A standardized, genotype-independent protocol for this approach was established using pigeon pea cv. BSMR 853. Previous studies have documented the induction of somatic embryogenesis in pigeon pea on EC6 basal media (Patel *et al*., 1994; Mohan and Krishnamurthy, 2002) [14, 15] and MS basal media (Nalini *et al*., 1996; Sreenivasu *et al*., 1998; Singh *et al*., 2003) [16, 29, 4] for various cultivars of pigeon pea.

Embryonal explants of pigeon pea (cv BSMR-853) developed into compact, nodular calli within 4 weeks on MS media supplemented with different combinations and concentrations of plant growth regulators; the details are given in Table (2). After 4-5 weeks, globular embryos were observed on the calli (Fig 2, d) and these embryos were subsequently transferred to MS media supplemented with the hormone ABA for maturation (Fig 2, e). All the combinations promoted the growth of calli, but the highest percentage of the calli showing embryogenesis was found in the 2 mg/L BAP + 0.5 mg/L TDZ treatment and 2 mg/L BAP + 0.5 mg/L NAA treatment groups. The remaining calli turned brown and did not regenerate. Further development of embryos occurred when the mature embryogenic calli were transferred to half-strength MS media supplemented with lower concentrations of BAP. The detailed results are shown in Table 3 and Fig 2.

In pigeon pea, somatic embryo formation has been observed on calli derived from cotyledon and leaf explants (Sreenivasu *et al*. 1998) [29] and from suspension cultures of leaf explants. TDZ has been used to elicit multiple shoot formations in a broad range of plant species (Malik and Saxena 1992) [18] and somatic embryogenesis in a few crop species. Somatic embryogenesis has been reported on media supplemented with NAA and BAP (Nalini *et al*. 1996) [16]. The maturation of somatic embryos on ABA media was reported for pigeon pea (Mohan and Krishnamurthy 2002) [15], *Phaseolus* sp. (Malik and Saxena 1992a) [18], and chickpea (Suhasini *et al*. 1994). Krishna *et al*. 2011 [15] reported the germination of somatic embryos on half-strength media supplemented with lower concentrations of BAP.

In our investigation, zygotic embryos were inoculated, and the conditions for callus induction and embryo development were established. The development of globular embryos was rapid, and the plants were prolific after treatment with BAP and TDZ. However, the differences in shoot development among these embryos were not significant. The shoot development of the embryos grown with BAP and NAA was significant (Fig 2 g).

After four weeks, well-developed shoots regenerated via organogenesis and via somatic embryogenesis were separated from the shoot clumps and inoculated on shoot elongation media supplemented with 1.0 mg/L BAP every two weeks. The shoots were subsequently sub-cultured on fresh MS media supplemented with a similar hormonal combination, after which the number of well-elongated shoots was counted regularly. Further research work such as shoot.

Table 1: Details of Treatment's used for organogenesis of pigeon pea

Sr. No.	Treatments	Harmone	Concentration (mg/L)
1	T_1	BAP	2
2	T_2	BAP	2.5
3	T_3	BAP	3
4	T_4	BAP + TDZ	2 + 0.5
5	T_5	BAP + TDZ	2.5+ 0.5
6	T_6	BAP + TDZ	3+ 0.5
7	T_7	BAP + NAA	2+ 0.5
8	T_8	BAP + NAA	2.5+ 0.5
9	T_9	BAP + NAA	3+ 0.5

Table 2: Details of Treatment's used for somatic embryogenesis of pigeon pea

Sr. No.	Treatment	Hormone
1.	T_1	2 mg/l BAP
2.	T_2	2 mg/l BAP+0.5mg/l TDZ
3.	T_3	2.5 mg/l BAP+0.5mg/l TDZ
4.	T_4	3 mg/l BAP+0.5 mg/l TDZ
5.	T_5	2 mg/l BAP+0.5mg/l NAA
6.	T_6	2.5 mg/l BAP+0.5mg/l NAA

Table 3: Growth Response of explants on growth regulators for shoot development via organogenesis

Sr. No.	Treatment (Conc. In mg/L)	R_1	R_2	R_3	Total no. of shoots	Mean ± SE	Remarks
T_1	2 mg/L BAP	7	6	9	22	7.3±0.88	Multiple shoot formation, yellowing/falling of leaf
T_2	2.5 mg/L BAP	2	3	5	10	3.3±0.88	Early shoot growth, thick shoot
T_3	3 mg/L BAP	2	4	4	10	3.3±0.66	Thick shoot, large leaf, small growth of shoot.
T_4	2 mg/L BAP + 0.5 mg/L TDZ	2	7	6	15	5±1.52	Yellowing of leaf and shoot became dead
T_5	2.5 mg/L BAP+ 0.5 mg/L TDZ	3	3	3	9	3±0.00	Medium growth small and thick shoot, leaf formation
T_6	3 mg/L BAP+ 0.5 mg/L TDZ	1	2	2	5	1.6±0.33	Hick and small shoot and large leaf
T_7	2 mg/L BAP+ 0.5 mg/L NAA	3	7	6	16	5.3±1.20	Multiple shoots, fast growth, thin shoot.
T_8	2.5 mg/L BAP+ 0.5 mg/L NAA	1	2	3	6	2±0.57	Slow growth, small and thick shoot, some shoot became dead.
T_9	3 mg/L BAP+ 0.5 mg/L NAA	3	4	4	11	3.4±0.33	Leaf from the bottom, high shoot Height.

Table 4: Response of explant on growth regulators for shoot development via somatic embryogenesis

Sr. No.	Treatments	Harmones	R1	R2	R3	Total No. of Shoots	Mean ± SE	Remarks
1.	T_1	2 mg/l BAP	0	0	0	0	0±0.00	Green and brown callus formation. No embryogenesis
2.	T_2	2 mg/l BAP +0.5 mg/l TDZ	6	7	8	21	7±0.57	Green and brown callus formation. Globular embroys were seen
3.	T_3	2.5 mg/l BAP +0.5 mg/l TDZ	0	0	0	0	0±0.00	White callus formation, and small shoot growth. Not showing embryogenesis.
4.	T_4	3 mg/l BAP+0.5 mg/l TDZ	0	0	0	0	0±0.00	Brown callus formation, small shoot growth seen and shoot become dead. Development of embryos were not seen.
5.	T_5	2 mg/l BAP+0.5 mg/l NAA	9	6	7	22	7.3±0.88	Brown and white callus formation and very small leaf growth seen. Embryogenesis were seen
6.	T_6	2.5 mg/l BAP+0.5 mg/l NAA	0	0	0	0	0±0.00	Brown callus formation. Growth of embryos were seen.

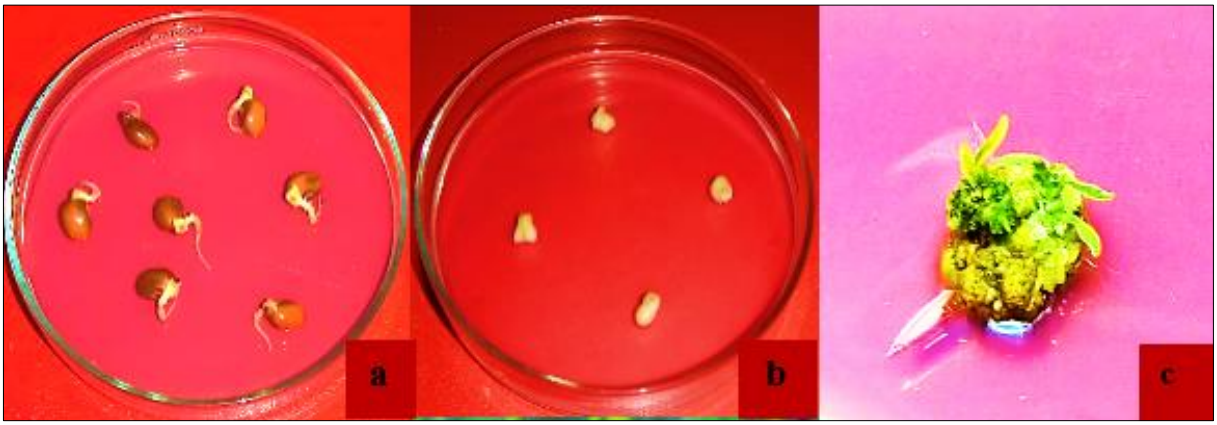
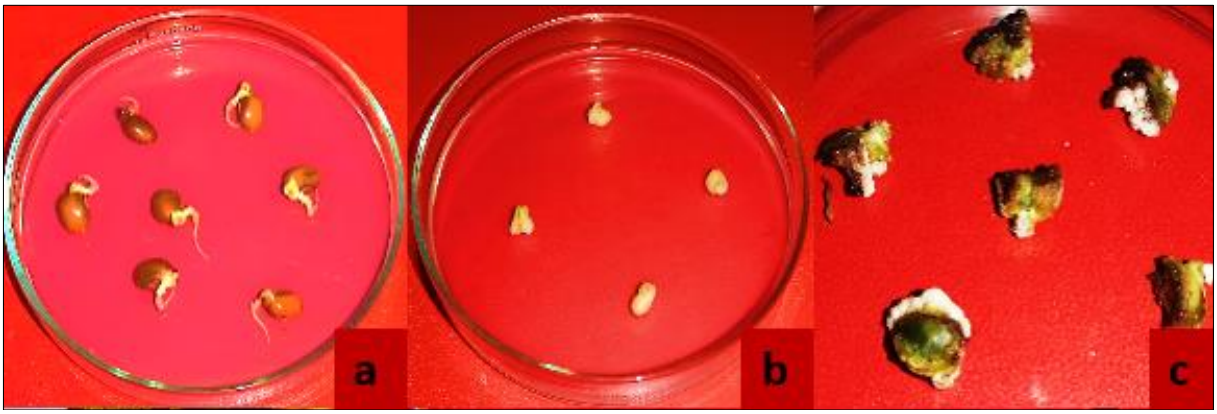


Fig 1: Shoot Development via Organogenesis a. 5 days old germinated seedlings. b. Embyos inoculated on MS media with auxins and cytokinins. c. Shoot bud initiation d-l Shoot elongation with MS medium containing lower concentration of BAP



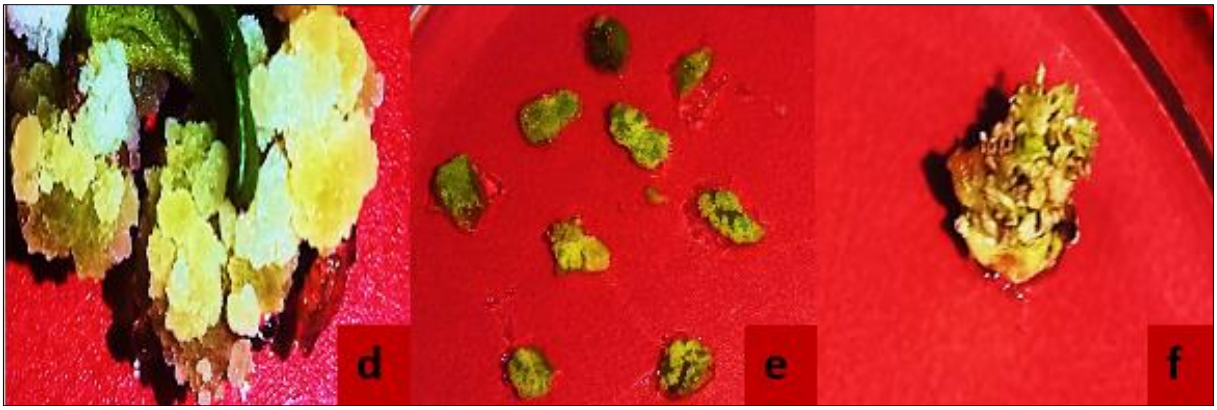


Fig 2: Shoot Development via Somatic Embryogenesis a. 5 days old germinated seedlings. b. Embyos inoculated on MS media with auxins and cytokinins. c. Callus induction d. Development of somatic embryos e. Maturation of somatic embryos f. Germination of somatic embryos

4. Conclusion

To the best of our knowledge, our study examined the first report on the potential for *In vitro* shoot regeneration in pigeon pea (*Cajanus cajan*) cv. BSMR 853 through organogenesis and somatic embryogenesis. We found that using embryos and a medium containing 2 mg/L of 6-benzylaminopurine (BAP) resulted in the greatest shoot bud regeneration through organogenesis. On the other hand, somatic embryogenesis initiated by thidiazuron (TDZ) led to rapid and prolific development of globular embryos. While somatic embryogenesis produced a greater embryo count, organogenesis demonstrated a greater success rate in establishing shoots. These findings provide valuable insights for further research and potential applications in crop improvement and biotechnology for pigeon pea cultivation**.**

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