Successful Propagation of Pigeonpea (*Cajanus cajan L.*) through *In Vitro* Shoot Regeneration and *Ex Vitro* Rooting

Atul Dev [1], Ajinder Kaur[2]
School of Agricultural Biotechnology,
Punjab Agricultural University, Ludhiana, 141004, India

Abstract

A reproducible in vitro shoot regeneration protocol was developed for pigeonpea variety ICPL 87 through proliferation of axillary buds from cotyledonary nodes. The seedlings were cultured on MS + Kin (2 mgL^{-1}) + BAP (5 mgL^{-1}) medium and de-capitated on 7th day of culturing, which resulted in the induction of multiple shoots from cotyledonary node region after 15 days of culturing. Miniature shoots were elongated on MS + adenine suphate (30 mgL^{-1}) medium; the elongated shoots were rooted ex vitro using a rooting powder ROOTEX^{TM}.

Keywords — *Callus, Regeneration, Direct regeneration, Multiple shoots.*

I. INTRODUCTION

Pigeonpea (*Cajanus cajan* L. Millsp., 2n = 22, Family: Fabaceae), also known as arhar, is a short-lived perennial shrub. It is one of the major grain legume (pulse) crops grown in the semi-arid tropics, and is rich in protein. During 2016, global production of pigeonpea was 5.3 mt [1]; at the national level, area, production and productivity of pigeonpea during 2013-14 was 3.90 mha, 3.17 mt and 813 kgha^{-1}, respectively (ICAR-Indian Institute of Pulses Research, Kanpur). India accounts for about 70% of the total global pigeon pea production, followed by Myanmar, Malawi, Kenya and Tanzania [1]. From food security point of view, legumes provide a balanced nutritional diet as compared to cereals commonly grown in semi-arid regions. Due to biotic and abiotic stresses, and due to cultivation of pigeonpea in poor environments, a big gap exists between the possible yield (2500 kgha^{-1}) and actual yield attained on fields (866 and 736 kgha^{-1} in Asia and Africa, respectively) [2].

Inspite of tremendous improvement made over the last few years through breeding and other approaches to reduce the crop duration, mend seed quality, and increase stress tolerance, pigeonpea has remained an orphan crop [3]. Attempts to obtain stress resistant genotypes by conventional breeding methods were not successful because of limited variability in genomes of cultivated accessions and their sexual incompatibility with wild relatives [4]. In this regard, genetic transformation of crops serves as an alternate approach to introgress gene(s) for desirable traits. *In vitro* regeneration, a pre-requisite for carrying out genetic transformation, occurs either directly from the explant or indirectly through callus induced on the explant. Although, a number of regeneration protocols [5]-[11] have been worked out for pigeonpea, it has been found that *in vitro* regeneration in this crop is genotype specific and sluggish. In the present study, a direct and quick regeneration protocol based on organogenesis from cotyledonary nodes of pigeonpea is reported.

II. MATERIALS AND METHODS

A. Plant Material and Sterilization

Pigeonpea variety ICPL 87 was used as experimental material. Seeds of this variety were procured from ICRISAT (International Crops Research Institute for the Semi-Arid-Tropics), Patancheru, Hyderabad, and grown in the field area of Pulses Section, Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana.

For surface-sterilization, seeds were first rinsed in Tween 20 for 5 min, and washed under running tap water till the detergent was completely removed. Thereafter, seeds were treated with 0.1% mercuric chloride (Hi-Media, Mumbai) for 6 min with continuous shaking in a laminar air flow cabinet, washed thrice with autoclaved distilled water, and soaked overnight in sterile water.

B. Callus Induction and Regeneration

Surface-sterilized seeds were de-coated and cultured on basal MS [12] medium for 24 h to obtain embryos and cotyledons; epicotyls were obtained from 3 day-old seedlings; embryonal axes and leaves were excised from 7 day-old seedlings. These explants (leaves, cotyledons, embryos, embryonal axes and epicotyls) were cultured on different media for callus induction *viz*., MS + thidiazuron, TDZ (1, 2, 3 mgL^{-1}), MS + indole-3-acetic acid, IAA (0.2 mgL^{-1}) + benzyl amino purine, BAP (0.4, 0.6, 0.8, 1 mgL^{-1}). The cultures were incubated in dark at a temperature of 25 ± 2ºC. Calli induced from all the explants were sub-cultured on fresh media of same composition and incubated under same conditions as for callus induction.

For regeneration, calli obtained from different explants were cultured separately on different regeneration media *viz*., MS + agar (0.8, 1, 1.2%), Half strength (½) MS + agar (0.8, 1, 1.2%), MS + BAP (1, 2, 3, 4, 5 mgL^{-1}) + IAA (0.2 mgL^{-1}) + agar (0.8, 1, 1.2%), MS + BAP (1, 2, 3, 4, 5 mgL^{-1}) + NAA (0.2 mgL^{-1}) + agar (0.8, 1, 1.2%), B5 + BAP (1, 2, 3, 4, 5 mgL^{-1}) + IAA (0.2 mgL^{-1}) + agar (0.8, 1, 1.2%), and incubated under 16/8 :: light/dark at 25 ± 2ºC with 80% relative humidity.

C. *Direct Regeneration*

De-coated seeds were cultured (two seeds per test tube) in different shoot induction media *viz*., MS + BAP (3, 4, 5, 6 mgL^{-1}), MS + BAP (3, 4, 5, 6 mgL^{-1}) + Kin (2, 3 mgL^{-1}). A portion of the main shoot of 7 day-old seedlings was cut aseptically and remaining part was cultured on fresh medium of same composition. The cultures were maintained at 16/8 :: light/dark, 25 ± 2ºC and 80% relative humidity.

For obtaining shoot elongation, the de-capitated seedlings bearing multiple shoots were cultured on different media *viz*., MS + gibberellic acid, GA_3 (1, 2, 3 mgL^{-1}), MS + adenine sulphate, Ads (10, 20, 30 mgL^{-1}), MS + BAP (1, 2, 3 mgL^{-1}).

For root induction, the elongated shoots were cut near the nodal region and cultured on different media *viz*., MS and ½ MS + sucrose (1, 3, 7%) + agar (0.6, 0.8, 1%), MS and ½ MS + indole-3-butyric acid, IBA (1, 2, 3, 4, 5 mgL^{-1}) + IAA (1, 2, 3, 4, 5 mgL^{-1}) + sucrose (1, 3, 7%) + charcoal (0.2%) + agar (0.8%). The cultures were maintained at 25 ± 2ºC with 80% relative humidity. *Ex-vitro* rooting was also tried by applying ROOTEX™ powder (Agrica Agrovet, Varanasi) at the basal nodal region of the excised shoots. Treated shoots were grown in test tubes containing sand, the tubes were loosely plugged with cotton for air circulation and maintained at 25 ± 2ºC with 90% relative humidity. The test tubes were kept out in sunlight for 2-3 h daily in the morning and watered after every 8 h using a dropper. The healthy plants were transplanted into earthen pots.

D. *Statistical Analysis*

Each experiment was replicated thrice to minimize the experimental error. All data were analyzed using analysis of variance. The statistical assessment was carried out at 5% level of significance.

III. RESULTS AND DISCUSSION

A. *Establishment of Callus Cultures and Regeneration*

Plant growth regulators in the culture medium change the natural growth pattern of plants. With a belief that different explants would have varied endogenous hormonal levels, and callus grown from these would exhibit different response to regeneration, different seedling explants of pigeonpea variety ICPL 87 *viz*., leaves, cotyledons, embryos, embryonal axes and epicotyls were cultured on media having different combinations and concentrations of growth regulators to induce callus.

1) *Effect of TDZ, IAA and BAP on Callus Induction:*

The five different explants were cultured on MS + TDZ (1, 2, 3 mgL^{-1}) media. It was observed that on increasing the concentration of TDZ in the culture medium, percentage of callus induction also increased significantly in all the explants (Table I). Most of the explants exhibited maximum callus induction on MS + TDZ (3 mgL^{-1}) medium (Fig. 1). The embryonal axes showed

TABLE I EFFECT OF DIFFERENT CONCENTRATIONS OF TDZ ON CALLUS INDUCTION IN ICPL 87

S. No.	Type of Tissue	Callus Induction (%) on MS + TDZ (1-3 mgL^{-1})			Mean
		1	2	3	
1	Leaf	31.11	56.67	81.11	56.30
2	Cotyledon	25.00	46.11	78.89	50.00
3	Embryo	41.67	92.22	93.33	75.74
4	Em. Axis	45.00	96.11	96.11	79.08
5	Epicotyl	37.22	68.89	83.89	63.33
Mean		36.00	72.00	86.67	64.89

CD (5%): A (Type of tissue) = 1.26, B (TDZ concentration) = 1.62, AB = 2.81

maximum callus induction (96.11%, Table I, Fig. 1D) on MS + TDZ (2 mgL^{-1}) medium; further increase in TDZ concentration did not increase percent callus induction from this explant. It has been reported that 1-2 mgL^{-1} concentration of TDZ induced somatic embryos from each explant of peanut (Murthy et al. 1995). In the present study, percentage of callus induction was influenced both by type of tissue used as explant and concentration of TDZ in MS medium.

The different explants were also cultured on MS + IAA (0.2 mgL^{-1}) + BAP (0.4, 0.6, 0.8, 1 mgL^{-1}) media for callus induction. As BAP concentration increased, callus induction also increased with a degree of variability among different explants (Table II). Percent callus induction from cotyledons almost got doubled after increasing BAP concentration from 0.4 to 1 mgL^{-1}. Among different explants, embryonal axes showed significantly higher callus induction (93.33%) as compared to other explants on MS + IAA (0.2 mgL^{-1}) + BAP (1.0 mgL^{-1}) medium (Table II).

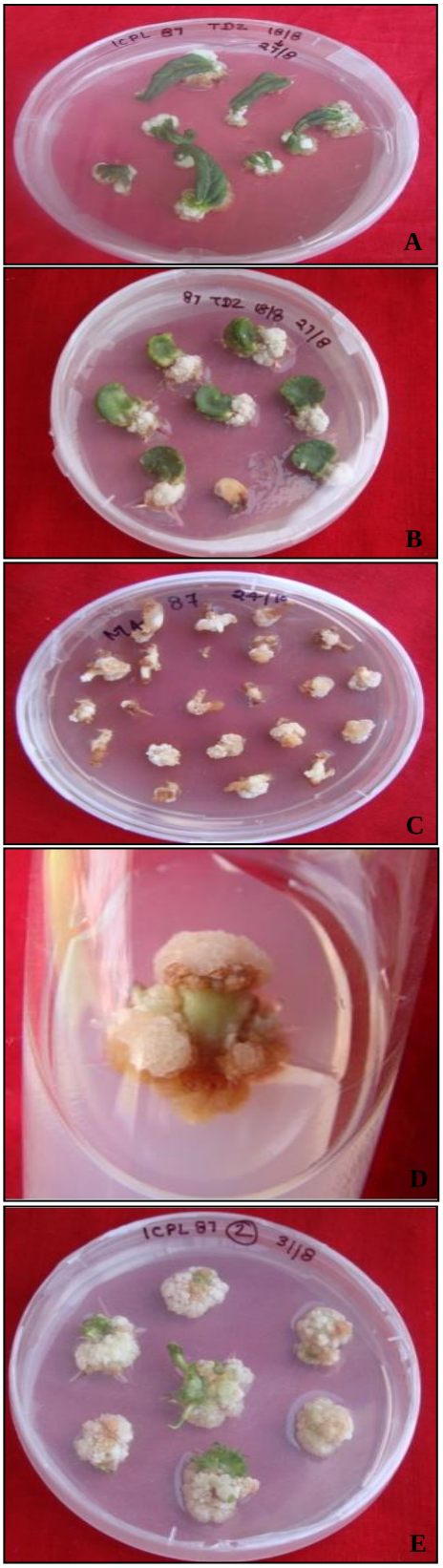

Fig. 1 Callus Induction on MS + TDZ Medium from (A) Leaves, (B) Cotyledons, (C) Embryos, (D) Embryonal axis, and (E) Epicotyls of Pigeonpea Variety ICPL 87

TABLE II EFFECT OF IAA AND DIFFERENT CONCENTRATIONS OF BAP ON CALLUS INDUCTION IN ICPL 87

S. No.	Type of Tissue	Callus Induction (%) on MS + IAA (0.2 mgL^{-1}) + BAP (0.4-1 mgL^{-1})				Mean
		0.4	0.6	0.8	1	
1	Leaf	63.89	70.00	75.56	78.89	72.08
2	Cotyledon	37.78	57.78	70.00	72.22	59.44
3	Embryo	65.56	72.78	80.56	90.00	77.22
4	Em. axis	66.11	73.33	81.11	93.33	78.47
5	Epicotyl	63.89	72.22	77.78	80.56	73.61
Mean		59.44	69.22	77.00	83.00	72.17

CD (5%): A (Type of tissue) = 1.76, B (BAP concentration) = 1.97, AB = 3.94

In general, TDZ proved to be a better growth regulator than IAA and BAP for effecting callus induction. TDZ is widely used in pigeon pea cultures for inducing embryogenesis and somatic embryo formation [10] as it displays both auxin and cytokinin-like activity [13], [14].

2) ***Recalcitrancy of Callus to Regeneration:***

Callus cultures obtained from five different explants were cultured separately on various regeneration media, however no regeneration was observed on any medium. Initially, callus showed change in colour from white to green within 20 days of culturing on different regeneration media, but later turned black without induction of a single shoot bud. Some black calli showed re-induction of callus on regeneration medium, e.g. calli induced from cotyledons.

B. Direct Regeneration

Direct organogenesis is considered as an alternate approach to regeneration in plants which are flagrantly recalcitrant to regeneration via callus formation. In the present study, effect of different plant growth regulators and their combinations was studied on direct shoot induction and elongation from pigeonpea explants.

1) ***Effect of BAP and Kin on Shoot Induction:***

Surface-sterilized and de-coated seeds were cultured on MS medium having different concentrations of BAP. A large number of de-capitated seedlings (> 63%) exhibited multiple shoot induction on MS + BAP (5, 6 mgL^{-1}) medium (Table III) at cotyledonary node region after 15 days of culturing. Pigeonpea cotyledonary nodes cultured on high BAP concentration, started to swell after 5^{th} day of culturing, while multiple shoot bud formation started after 10^{th} day [15]. In other studies on pigeonpea, high BAP concentration resulted in direct shoot regeneration from the cotyledonary node region [16], low concentration induced shoot development from pre-existing meristem [17] and low BAP concentration along with an auxin favoured regeneration through organogenesis and embryogenesis [5].

The number of seedlings forming multiple shoots either decreased or remained the same, upon further increasing BAP concentration to 7 mgL^{-1} in the culture medium. At low BAP concentrations (3, 4 mgL^{-1}), a few de-capitated seedlings (24-46%) showed multiple shoot induction (Table III). The frequency of multiple shoots and percentage of seedlings exhibiting multiple shoots was quite low when main shoot axis was not excised, but once a portion of the seedling main shoot was excised, number of seedlings exhibiting multiple shoot induction increased significantly. This may be due to loss of apical dominance after removal of main shoot axis. To reduce apical dominance and increase the frequency of axillary buds in shoot cultures of plants with broad leaves, culture medium is supplemented with cytokinins [18]. Same strategy was applied in the present study and found to be effective in increasing the frequency of proliferating axillary buds from cotyledonary nodes and number of responding explants.

To further increase the number of seedlings showing multiple shoot induction from cotyledonary node region, a combination of two cytokinins *viz*., BAP and Kin was used in the MS medium. As concentration of Kin was increased with BAP concentration, frequency of seedlings showing multiple shoot induction increased significantly; best result was obtained on MS + Kin (2 mgL^{-1}) + BAP (5 mgL^{-1}) as highest number of seedlings (67.66%) showed multiple shoot induction (Table IV, Fig. 2A) vs. 63.15% on MS + BAP (5 mgL^{-1}) alone (Table III). Further increase in Kin or BAP concentration decreased the number of seedlings showing multiple shoot induction. Direct shoot induction from cotyledonary node explants resulted in efficient multiple shoot regeneration in Glycine max [19], Phaseolus vulgaris [20], Pisum sativum [21], Cajanus cajan ([5], [17]), Cicer arietinum [22] and Vicia faba [23].

Table III Number of Icpl 87 Seedlings Exhibiting Multiple Shoot Induction on MS + BAP

MS + BAP (3-6 mgL^{-1})				Mean
3	4	5	6	
24.42	46.30	63.15	63.49	49.34

CD (5%): A (BAP concentration) = 1.39

Table IV Number of Icpl 87 Seedlings Exhibiting Multiple Shoot Induction on MS + KIN + BAP

MS + Kin (1-3 mgL^{-1}) + BAP (3-6 mgL^{-1})					Mean
Kin (mgL^{-1})	BAP (mgL^{-1})				
	3	4	5	6	
1	22.66	44.66	63.33	58.00	47.16
2	26.66	46.66	67.66	63.00	51.06
3	27.33	47.33	66.66	62.33	50.91
Mean	25.55	46.22	65.88	61.11	49.69

CD (5%): A (BAP concentration) = 0.61, B (Kin concentration) = 0.71, AB = 1.22

2) ***Effect of GA_3, Adenine Sulphate and BAP on Shoot Elongation:***

The miniature shoots were cultured on MS + GA_3 (1, 2, 3 mgL^{-1}) media to obtain elongation. The shoot elongation started after 8 days of culturing; however the elongated shoots were very lean due to abnormal increase in inter-nodal length. To avoid the abnormal pattern of shoot elongation, shoots were cultured on MS + BAP (1, 2, 3 mgL^{-1}) media. At these concentrations of BAP, shoots elongated normally, however callus induction started at the excised shoot ends, consequently very few shoots showed elongation after 4 weeks of culturing. To avoid callus phase, BAP was omitted and a cytokinin derivative, adenine sulphate (Ads) was supplemented in the culture medium at different concentrations (10, 20, 30 mgL^{-1}). At low concentrations of Ads (10, 20 mgL^{-1}), some amount of callus formation at the cut shoot ends was observed, however, at 30 mgL^{-1} Ads, callus phase disappeared totally and 5-6 miniature shoots showed elongation at a time within 2 weeks of culturing (Fig. 2B).

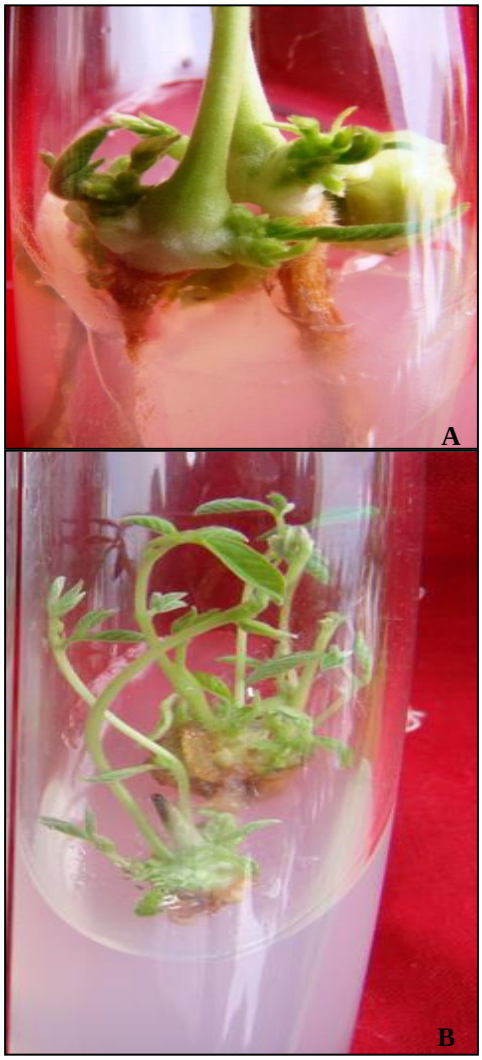

Fig. 2 (A) Multiple Shoot Induction from Cotyledonary Nodes of ICPL 87 Seedlings Cultured on MS + Kin (2 mgL^{-1}) + BAP (5 mgL^{-1}) Medium, (B) Shoot Elongation on MS + Ads (30 mgL^{-1}) Medium

The elongated shoots were excised and the remaining explant was again sub-cultured on MS + Ads (30 mgL^{-1}) medium, subsequently the remaining under-developed shoots exhibited elongation. In this way, fifteen-twenty elongated shoots were obtained from a single explant, after 2-3 times of sub-culturing. Adenine sulphate has been reported to be a precursor for natural cytokinin production in plants that are more active in causing functional response as compared to exogenously supplied cytokinins in the culture medium [24]. Thus, plant uses its own machinery to synthesize its own cytokinins when Ads is added in the culture medium.

*3) **Effect of Sucrose, IBA and IAA on In Vitro Root Formation:***

Elongated shoots were cultured in different rooting media with variable concentration of sugar, IBA, IAA and gelling agent agar, but none of the combination induced rooting in elongated shoots. This might be due to the fact that cultures were exposed to high cytokinin concentration for a long period to obtain shoot induction and elongation. Cytokinins are usually either omitted from culture media or elongated shoots are sub-cultured on a cytokinin-free medium until the level of cytokinin within the tissues is sufficiently reduced [18] so that there is no problem during root induction. In the present study, the culture of elongated shoots on a medium lacking cytokinin resulted in callus induction at shoot ends, and no roots were induced in the elongated shoots on any rooting media.

C. Ex Vitro Root Induction

Ex vitro application of ROOTEX™ powder at the basal end of shoots induced roots in 85-95% shoots after 20 days of transplanting in sand (Fig. 3A). *Ex vitro* rooting of *Passiflora edulis* shoots was obtained by treatment with IBA (200 mgL^{-1}) solution [25]. It has been documented that plants obtained through *ex vitro* rooting endure the environmental stresses in a better way as compared to *in vitro* rooted plants [26]. Rooted and hardened plants so obtained (Fig. 3B) were transferred to earthen pots containing field soil.

IV. CONCLUSIONS

Direct organogenesis proved to be a productive approach for *in vitro* shoot regeneration in pigeonpea variety ICPL 87, that was followed by rooting through *ex vitro* approach. The developed protocol can be used for genetic transformation of pigeonpea using *Agrobacterium* and biolistic gene transfer methods.

ACKNOWLEDGMENT

The authors thank Dr. K.B. Saxena, ICRISAT for providing the seed material of pigeonpea variety ICPL 87.

Fig. 3 (A) Rooting *ex vitro* in Sand (B) Plants Showing Well Developed Roots

REFERENCES

[1] Anonymous, Pigeon Pea Market: Global Industry Trends, Share, Size, Growth, Opportunity and Forecast 2017-2022, Dublin--(Business Wire)--Research and Markets, 2017.

[2] M. G. Mula, and K. B. Saxena, Lifting the Level of Awareness on Pigeonpea—a Global Perspective, International Crops Research Institute for the Semi-Arid Tropics, Patancheru, Hyderabad, 2010.

[3] R. K. Varshney, W. L. Y. Chen, A. K. Bharti et al., "Draft genome sequence of pigeonpea (Cajanus cajan), an orphan legume crop of resource-poor farmers," Nature Biotech., vol. 30, pp. 83–89, Jan. 2012.

[4] Y. L. Nene and V. K. Sheila, Pigeonpea: Geography and Importance, In: Y. L. Nene, S. D. Hall, V. K. Sheila, Eds. CAB International, Wallingford, U.K., 1990.

[5] S. Eapen, and L. George, "Organogenesis and embryogenesis from diverse explants of pigeonpea," Plant Cell Rep., vol. 13, pp. 417–420, Jan. 1994.

[6] A. Das, A. Pattanayak, and G. Bhowmick, "Thidiazuron induced multiple shoot in pigeonpea (Cajanus cajan)," Indian Journal of Hill Frmg., vol. 16, pp. 89–91, 2003.

[7] A. Kaur, R. Devi, and A. Dev, "Efficient in vitro regeneration in pigeonpea from cotyledonary node explants," J. Cell Tissue Res., vol. 12, pp. 3075–3080, 2012.

[8] N. Geetha, P. Venkatachalam, V. Prakash, and G. L. Sita, "High frequency induction of multiple shoots and plant regeneration from seedling explants of pigeonpea (Cajanus cajan L.)," Curr. Sci. vol. 75, pp. 1036–1041, 1998.

[9] N. Mallikarjuna, M. J. T. Reena, D. C. Sastri, and J. P. Moss, "Somatic embryogenesis in pigeonpea (Cajanus cajan L.)," Indian J. Exp. Biol. vol. 34, pp. 282–284, Mar. 1996.

[10] K. Sreenivasu, S. K. Malik, P. A. Kumar, and R. P. Sharma, "Plant regeneration via somatic embryogenesis in pigeonpea (Cajanus cajan L. Millsp)," Plant Cell Rep. vol. 17, pp. 294–297, Feb. 1998.

[11] M. L. Mohan, and K. V. Krishnamurthy, "Somatic embryogenesis and plant regeneration in pigeonpea," Biol. Plant., vol. 45, pp. 19–25, Mar. 2002.

[12] T. Murashige, and F. A. Skoog, "Revised medium for rapid growth and bioassays with tobacco tissue cultures," Physiol. Plant., vol. 15, pp. 473–497, Jul. 1962.

[13] J. C. Thomas, and F. R. Katterman, "Cytokinin activity induced by thidiazuron," Plant Physiol., vol. 81, pp. 681–683, Jun. 1986.

[14] P. K. Saxena, K. A. Malik, and R. Gill, "Induction by thidiazuron of somatic embryogenesis in intact seedlings of peanut," Plant, vol. 187, pp. 421–424, Jan. 1992.
[15] K. K. Sharma, M. Lavanya, and V. Anjaiah, "Agrobacterium-mediated production of transgenic pigeonpea (Cajanus cajan L. Millsp.) expressing the synthetic Bt cry1Ab gene," In Vitro Cell Dev. Biol. Plant, vol. 42, pp. 165–173, Apr. 2006.
[16] U. Mehta, and H. Y. M. Ram, "Regeneration of plantlets from the cotyledons of Cajanus cajan," Indian J. Exp. Biol., vol.18, pp. 800–802, 1980.
[17] S. N. Prakash, D. Pental, and N. Bhalla-Sarin, "Regeneration of pigeonpea (Cajanus cajan L. Millsp.) from cotyledonary node via multiple shoot formation," Plant Cell Rep., vol. 13, pp. 623–627, Aug. 1994.
[18] E. F. George, A. H. Michael and G. D. Klerk, Plant Propagation by Tissue Culture, Exegetics, Basingstoke, U.K., 2008, vol. 1.
[19] M. S. Wright, S. M. Koehler, M. A. Hinchee, and M. G. Carnes, "Plant regeneration by organogenesis in Glycine max (L.) Merr.," Plant Cell Rep., vol. 5, pp.150–154, Apr. 1986.
[20] P. McClean, and K. F. Grafton, "Regeneration of dry bean (Phaseolus vulgaris L.) via organogenesis," Plant Sci., vol. 60, pp. 117–122, Oct. 1989.
[21] J. A. Jackson, and S. L. A. Hobbs, "Rapid multiple shoot production from cotyledonary node explants of pea (Pisum sativum L.)," In Vitro Cell. Dev. Biol.-Plant, vol. 26, pp. 835–838, Aug. 1990.
[22] L. Sharma, and D. V. Alma, "Direct shoot regeneration in chickpea (Cicer arietinum L.)," Indian J. Exp. Biol., vol. 36, pp. 605–609, 1998.
[23] M. M. Khalafalla, and K. Hattori, "A combination of thidiazuron and benzyladenine promotes multiple shoot production from cotyledonary node explants of faba bean (Vicia faba L.)," Plant Growth Regul., vol. 28, pp. 41–47, Mar. 1999.
[24] M. K. Khan, P. Misra1, T. Sharma, P. K. Shukla, and P. W. Ramteke, "Effect of adenine sulphate on in vitro mass propagation of Stevia rebaudiana Bertoni," J. Med. Plants Res., vol. 8, pp. 543–549, Apr. 2014.
[25] M. S. Shekhawat, M. Manokari, and C. P. Ravindran, "An improved micropropagation protocol by ex vitro rooting of Passiflora edulis Sims. f. flavicarpa Deg. through nodal segment culture," Scientifica, vol. 2015, Article ID 578676, 8 pages, 2015. doi:10.1155/2015/578676.
[26] M. S. Shekhawat, N. Kannan, M. Manokari, and C. Ravindran, "Enhanced micropropagation protocol of Morinda citrifolia L. through nodal explants," J. Appl. Res. Med. Aromat. Plants, 2015.