

In Vitro Regeneration of ICP 8863 Pigeon Pea (*Cajanus cajan* (L.) Millsp.) Variety using Leaf Petiole and Cotyledonary Node Explants and Assessment of their Genetic Stability by RAPD Analysis

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Abstract

Objectives: A reliable *in vitro* regeneration protocol by direct organogenesis was developed in ICP 8863 variety of pigeon pea using leaf petiole and cotyledonary node explants. **Methods:** For direct shoot bud induction, leaf petiole explants from seven-day-old *in vitro* grown seedlings and cotyledonary node explants from twelve-day-old were cultured on MS medium supplemented with various combinations and concentrations of BAP, NAA and Kinetin. Induced shoot buds of both the explants were elongated on MS medium fortified with different concentrations of BAP, NAA and GA₃. The well-elongated shoots of both the explants were transferred to MS medium supplemented with various concentrations of IBA. Finally, the regenerated plants were transferred to soil and vermiculate mixture in 1:1 ratio for acclimatization. Further, molecular characterization of the *in vitro* regenerated plants was carried out using eight OPP and OPAZ RAPD primer series. **Findings:** High frequency of shoot bud induction (92 %) was observed in leaf petiole explants with 2.0 mg/L 6-BAP concentration compared to cotyledonary node explants. The induced shoots were kept for elongation and maximum percentage of elongation (93 %) was noticed in leaf petiole explants with 1.0 BAP + 0.1 NAA + 2.0 GA₃ mg/L concentrations compared to cotyledonary node explants. The well-developed shoots of both the explants showed profuse rooting, where high percentage of rooting (95 %) was observed in leaf petiole explants with 0.5 mg/L IBA concentration. The pattern of amplification resulted through RAPD analysis confirmed the genetic stability of *in vitro* regenerated plants. **Improvement:** The regeneration protocol standardized in this study is suitable and reliable to develop transgenic pigeon pea plants by agrobacterium mediated genetic transformation.

Keywords: Auxins, Cytokinins, Gibberellins, Organogenesis, Pigeonpea

1. Introduction

Pigeon pea [*Cajanus cajan* (L.) Millsp.] is one of the most important perennial food legume crops of rain-fed agriculture in the semi-arid tropics, which occupies fifth position in area, compared to the other legume crops like soybean, common bean, peanut and chickpea¹. India contributes to 90 % of the global production, where it is cultivated in 3.8 million hectares^{2,3}. Pigeon pea contains 20-22 % of protein and sulphur containing amino acids,

like cysteine and methionine⁴, whose content is more than three times in cereals. Because of its high protein content, it is used as an important source of protein in Indians vegetarian diet. In addition, it also contains 3-5 % of soluble sugar, 1-2 % fats, 3-4 % crude fiber, 45-55 % starch and 3-4 % ash⁵.

Mostly, pigeon pea is cultivated as an intercrop with other crops like sorghum (*Sorghum bicolor*), pearl millet (*Pennisetum glaucum*), maize (*Zea mays*) or with other legumes like peanuts (*Arachis hypogaea*) to maintain soil

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fertility. It is cultivated in various types of soils like black clay to sandy soil and is sensible to waterlogged conditions. The major constraint in its cultivation is sensitivity to various diseases like, Phytophthora stem blight, sterility mosaic virus, Fusarium wilt and insect pests like *Helicoverpa armigera*, pod fly and storage grain pests, causing major yield loss⁶. Efforts to face these problems through conventional breeding methods were not successful due to limited genetic variation in the cultivated germplasm and incompatibility with wild species⁷. So, these problems can be addressed by introducing genes conferring disease and insect resistance through genetic engineering strategy⁸.

Standardization of an efficient protocol for *in vitro* regeneration is necessary to develop transgenic pigeon pea plants with desired characters. As legumes are reported to be recalcitrant to regeneration, it is crucial to standardize a protocol in pigeon pea.

It depends upon various factors like genotype, source of the explant and combination of various plant growth hormones. Previously, various researchers reported regeneration by using different explants in pigeon pea through organogenesis and somatic embryogenesis⁹⁻¹⁴.

Compared to somatic embryogenesis, regeneration by direct organogenesis was reported as most effective procedure for the development of pigeon pea transgenic plants. Among the various explants used, leaf tissue was used most frequently followed by cotyledon, cotyledonary node, epicotyl, hypocotyl and axillary bud. Type of an explant also plays an important role in regeneration to induce shoot buds. Using leaf explants, regeneration was reported in different pigeon pea varieties like ICPL 161, ICPL 88039 and UPAS 120^{10,15,16}, but these protocols did not show high shoot bud induction through direct regeneration. Thus, there is a need to develop an efficient direct organogenesis protocol, to develop transgenic pigeon pea plants through transformation.

Genetic stability of regenerated plants is another aspect of plant propagation, which is essential for genetic transformation. In tissue culture derived plants, variations are common which may limit the application of *in vitro* regeneration protocol¹⁷. Earlier, genetic analysis of *in vitro* regenerated plants in many species was studied using RAPD¹⁸ and ISSR¹⁹ markers. Earlier, some researchers confirmed genetic stability in pigeon pea plants by RAPD analysis²⁰. Genetic variations may occur in *in vitro* regenerated plants due to various factors like type of explant, genotype, growth regulators, mode of regenera-

tion etc^{21,22}. So, it is required to check the genetic stability of *in vitro* regenerated plants of any plant species before proceeding to genetic transformation studies.

In this study, we intend to develop an *in vitro* regeneration protocol in ICP 8863 variety of pigeon pea using leaf petiole and cotyledonary node explants and to analyze the genetic stability of *in vitro* regenerated plantlets by RAPD analysis.

2. Materials and Methods

2.1 Plant Material

Pigeonpea ICP 8863 variety seeds were selected to carry out this experiment. This is a high yielding and medium duration variety, which is suitable for sole cropping and intercropping.

Seeds were procured from plant breeding Department of International Crops Research Institute for the Semi-Arid Tropics, Patancheru, Telangana, India.

2.2 Seed Treatment and Germination

Mature and uniform seeds were taken and were surface sterilized with 70 % ethanol for 1 min followed by 0.1 % (w/v) mercuric chloride containing 1-2 drops of Tween-20 for 8 min. Later these seeds were washed with sterile double distilled water for four to five times and were soaked for 14 h in sterile double distilled water at room temperature. Then the imbibed seeds were inoculated in sterile test tubes containing solid MS medium²³ containing 0.8 % and 3 % sucrose.

2.3 *In vitro* Shoot Regeneration from Leaf petiole and Cotyledonary Node Explants

Petioles and cotyledonary nodes excised from 7-day-old and 12-day-old aseptically germinated seedlings were used as explants for shoot bud induction. Both the explants were inoculated in culture medium supplemented with various concentrations of cytokinins like 6-Benzylaminopurine (BAP) and Kinetin and auxin like α -naphthaleneacetic acid (NAA) in different combinations for shoot bud induction (The details of the treatments are given in Table 1). Minimum number of 50 explants was taken for each treatment and was repeated thrice. The explants were sub-cultured for every two weeks on fresh MS basal medium with similar combinations of hormones. Data was recorded for various parameters like

Table 1. Effect of various concentrations of NAA, 6-BAP and Kinetin on multiple shoot bud induction from leaf petiole and cotyledonary node explants of *in vitro* germinated seedlings of pigeon pea

Conc. of Plant growth regulators (mg/L)	No. of explants cultured (both explants)	Leaf petiole				Cotyledonary node				
		No. of explants producing shoots	Percentage of explants producing shoots (%)	Avg. no. of shoot buds/explant Mean \pm SE	Avg. no. of shoots / explant Mean \pm SE	No. of explants producing shoots	Percentage of explants producing shoots (%)	Avg. no. of shoot buds/explant Mean \pm SE	Avg. no. of shoots / explant	
1.0 BAP + 0.2 NAA	50	13	26	3.66 \pm 0.34	1.66 \pm 0.33	19	38	3.66 \pm 0.67	1.66 \pm 0.34	
1.0 BAP + 0.4 NAA	50	16	32	4.66 \pm 0.89	1.33 \pm 0.34	21	42	5.0 \pm 0.58	2.0 \pm 0.58	
1.0 BAP + 0.5 NAA	50	20	40	6.33 \pm 0.34	2.66 \pm 0.33	22	44	4.66 \pm 0.67	2.33 \pm 0.34	
2.0 BAP	50	46	92	15.66 \pm 0.34	12.66 \pm 0.34	33	66	10.0 \pm 0.58	6.33 \pm 0.67	
2.0 BAP + 0.2 NAA	50	26	52	9.33 \pm 0.67	4.33 \pm 0.67	31	62	8.33 \pm 0.89	5.0 \pm 0.58	
2.0 BAP + 0.4 NAA	50	35	70	10.33 \pm 0.89	5.66 \pm 0.34	34	68	8.66 \pm 0.34	6.0 \pm 0.58	
2.0 BAP + 0.5 NAA	50	42	84	13.33 \pm 0.67	10.33 \pm 0.34	36	72	10.33 \pm 0.34	7.33 \pm 0.34	
3.0 BAP + 0.2 NAA	50	10	20	2.33 \pm 0.34	0.66 \pm 0.34	38	76	11.33 \pm 0.67	8.0 \pm 0.58	
3.0 BAP + 0.4 NAA	50	08	16	1.33 \pm 0.67	0.00 \pm 0.00	28	56	7.0 \pm 0.58	3.66 \pm 0.67	
3.0 BAP + 0.5 NAA	50	05	10	1.0 \pm 0.58	0.00 \pm 0.00	27	54	6.66 \pm 0.89	3.33 \pm 0.34	
0.5 BAP + 1.0 KIN	50	38	76	11.0 \pm 1.16	6.33 \pm 0.88	26	52	6.33 \pm 0.67	3.0 \pm 1.0	
1.0 BAP + 0.5 KIN	50	30	60	9.66 \pm 0.34	5.66 \pm 0.34	12	24	3.0 \pm 0.58	1.0 \pm 0.58	
1.0 BAP + 1.0 KIN	50	40	80	12.66 \pm 0.34	7.66 \pm 0.67	18	36	3.33 \pm 0.34	1.0 \pm 0.58	
2.0 BAP + 1.0 KIN	50	25	50	8.66 \pm 0.34	4.0 \pm 0.57	24	48	5.33 \pm 0.89	3.66 \pm 0.34	
2.0 KIN	50	32	64	10.0 \pm 0.58	5.0 \pm 0.57	35	70	9.66 \pm 0.67	6.66 \pm 0.34	
1.0 BAP + 2.0 KIN	50	15	30	4.0 \pm 0.58	1.66 \pm 0.34	30	60	7.66 \pm 0.89	4.0 \pm 0.58	
2.0 BAP + 2.0 KIN	50	12	24	3.6 \pm 0.4	1.0 \pm 0.58	25	50	6.0 \pm 0.58	2.66 \pm 0.89	

Table 2. Impact of various concentrations of 6-BAP, NAA and GA₃ on shoot elongation of induced shoot buds from leaf petiole and cotyledonary node explants of pigeon pea

Concentration of Plant growth regulators (mg/L)	Leaf petiole				Cotyledonary node			
	No. of regenerated shoots kept for elongation	No. of shoots elongated	Percentage of shoots elongated (%)	Mean shoot length (cm) Mean ± SE	No. of regenerated shoots kept for elongation	No. of shoots elongated	Percentage of shoots elongated (%)	Mean shoot length (cm) Mean ± SE
1.0 BAP + 0.1 NAA + 0.05 GA ₃	30	13	43	2.5 ± 0.29	27	15	55	2.13 ± 0.14
1.0 BAP + 0.1 NAA + 0.10 GA ₃	32	16	50	2.83 ± 0.17	30	18	60	2.33 ± 0.34
1.0 BAP + 0.1 NAA + 0.25 GA ₃	35	20	57	3.16 ± 0.45	31	20	64	2.53 ± 0.29
1.0 BAP + 0.1 NAA + 0.50 GA ₃	38	27	71	3.33 ± 0.34	33	25	75	2.86 ± 0.14
1.0 BAP + 0.1 NAA + 1.0 GA ₃	40	29	85	3.66 ± 0.34	38	32	84	3.5 ± 0.29
1.0 BAP + 0.1 NAA + 2.0 GA ₃	46	43	93	3.83 ± 0.17	36	26	72	3.26 ± 0.27
1.0 BAP + 0.1 NAA + 3.0 GA ₃	42	28	66	3.0 ± 0.00	34	22	64	2.93 ± 0.07

Table 3. Role of different concentrations of IBA on rooting of well-elongated shoots from leaf petiole and cotyledonary node explants of pigeon pea

Concentration of Plant growth regulators (mg/L)	Leaf petiole				Cotyledonary node			
	No. of elongated shoots kept for rooting	No. of elongated shoots produced roots	Percentage of rooting (%)	No. of elongated shoots kept for rooting	No. of elongated shoots produced roots	Percentage of rooting (%)	No. of elongated shoots kept for rooting	No. of elongated shoots produced roots
0.3 IBA	29	25	86	32	27	84	21	14
0.5 IBA	43	41	95	26	21	80	25	18
0.8 IBA	27	22	81	25	18	72	22	14
1.0 IBA	28	21	75	22	14	63	22	14

number of explants responded and number of shoot buds per explant for interpretation of results.

2.4 *In vitro* Shoot Elongation

After four weeks, well-developed shoots regenerated from both leaf petiole and cotyledonary node explants were separated from the shoot clumps and were inoculated on the shoot elongation medium containing 1.0 mg/L BAP, 0.1 mg/L NAA and various concentrations of Gibberellic Acid (GA₃) (0.05, 0.10, 0.25, 0.50, 1.0, 2.0, 3.0 mg/L) (see Table 2). For every two weeks, they were sub-cultured on fresh MS medium with similar hormonal combination and the number of well-elongated shoots was counted regularly.

2.5 *In vitro* Root Induction and Acclimatization of Plantlets

The well elongated shoots of both the explants were transferred to the root induction medium containing MS medium fortified with various concentrations of Indole-3-butyric Acid (IBA) (0.3, 0.5, 0.8 and 1.0 mg/L). After two to three weeks, the rooted plants with well-differentiated leaves were carefully removed from the test tubes and were gently washed with double distilled water to remove agar from the roots. Then the plantlets were transferred to small plastic glasses containing autoclaved soil and soilrite in 1:1 ratio and were initially hardened in culture room at controlled conditions. After two weeks of hardening, they were transferred finally to small pots for acclimatization in green house.

2.6 Genomic DNA Extraction and Genetic Stability Analysis of Acclimatized Plants by using RAPDs

The genetic stability analysis of *in vitro* regenerated acclimatized plants was carried out using eight RAPD primers from the OPP and OPAZ series (Operon Technologies, Alameda, USA). Genomic DNA was isolated using CTAB method²⁵ from six randomly selected regenerated plants from both the explants (three plants of each explant). Briefly, 2 gm of fresh leaves were grinded to achieve a fine powder using liquid nitrogen and were centrifuged by adding 5 ml of extraction buffer (2 % CTAB, 20 mM EDTA, 2 % PVP) 1.4 M NaCl, 100 mM Tris-HCL pH 8.0 and 1 % β-mercaptoethanol) and were incubated at 65^o C for 1 h. The collected supernatant was treated with RNase

enzyme (100 µg/mL) and incubated at 37^o C for 30 min and was extracted twice with chloroform and isoamylalcohol (24:1 v/v). Afterwards, the DNA was precipitated with ice-cold isopropanol and washed 2-3 times with 70 % ethanol. The DNA pellet was air-dried and eluted with 1X TE buffer (100 µl) and stored at -20^o C. The qualitative and quantitative analysis of DNA was assessed by spectrophotometer and agarose gel electrophoresis. A total of 8 RAPD primers (Operon Technologies Alameda, USA) were used for RAPD analysis.

Total 20 µL of PCR reaction mix was prepared with 2.0 µL of 10x PCR buffer with 15 mM magnesium chloride, 0.5 µL of 10 mM dNTPs, 1 µL forward primer, 1 µL reverse primer, 2 µL of genomic DNA, 0.3 µL of Taq polymerase and sterile milli-Q water. PCR amplification was carried out in a Veriti thermal cycler (Applied Biosystems, USA). The conditions for PCR amplification are as follows: Initial denaturation at 94^o C for 3 min followed by 45 cycles at 94^o C for 1 min, annealing at 37^o C for 1 min, initial extension at 72^o C for 2 min followed by final extension at 72^o C for 7 min. The amplified products were resolved on 1.5 % agarose gel and the DNA band sizes were determined by using 1 kb DNA ladder (Promega, USA).

3. Results and Discussion

The percentage of germination was high (70-80 %) in surface sterilized seeds cultured on MS basal medium (Figure 1 (a) and (b)) compared to sterile wet filter papers (30-40 %). In this experiment, we have selected leaf petiole and cotyledonary node explants, as they are more reproducible and potent to carryout genetic transformation studies. For an efficient shoot bud induction, age of an explant, type of an explant and plant growth hormonal combination plays an important role. In our study, leaf petiole and cotyledonary node explants collected from seven day old and twelve day old *in vitro* germinated seedlings showed high percentage of shoot bud induction and no shoot bud induction was noticed on plain MS medium without any plant growth regulators. Previously in pigeon pea, *in vitro* regeneration was reported from various explants like, cotyledonary node^{11,13,25-28}, leaves^{10,11,13,15,29}, epicotyl¹⁴, shoot apices¹², leaf petiole^{10,30,31}, leaf and apical meristem³² and auxiliary buds³³. For direct shoot bud induction using leaf petiole and cotyledonary node explants, various combinations of growth hormones like BAP, NAA and Kinetin were used in different concentrations. In case of leaf petiole explant, increase in size of the leaf lamina and bulging

at the petiolar end of the leaf was observed in the first week of inoculation (Figure 1c) and after one week, shoot bud induction was initiated from the bulged part of the leaf petiole (Figure 1d).

Among the various combinations and concentrations of BAP, kinetin and NAA tested, MS basal medium fortified with 2.0 mg/L BAP concentration showed highest shoot bud induction (92 %) in leaf petiole explants (Table 1). These results are in correlation to the earlier reports of¹³ and²⁴, where high shoot bud regeneration was observed at 2.0 mg/L BAP concentration using cotyledonary node explants and based on these studies, BAP was noticed to be a potent growth hormone to increase the shoot bud regeneration in pigeon pea^{13,34,35}. In case of cotyledonary node explants, maximum shoot induction percentage (76 %) was observed on MS basal medium supplemented with 3.0 mg/L BAP + 0.2 mg/L NAA concentrations. Recently³⁶ reported direct shoot bud regeneration in ICPL 87 pigeon pea variety at 2.0 mg/L BAP + 0.5 mg/L NAA concentrations. Like wise,²⁶ and³⁷ reported that multiple shoot regeneration of legumes was possible at low concentrations of auxins and reported that higher concentrations of NAA reduces the shoot bud regeneration frequency. Few researchers reported, direct shoot regeneration from the cotyledonary surface at higher concentrations of BAP³⁸. But, in our experiment use of high concentrations of BAP resulted in callus formation, which was similar to the results reported by³⁰, where callus formation was observed in leaf petiole explants cultured with high concentrations of BAP and NAA. ¹⁶also reported shoot regeneration using leaf petiole explants on MS medium supplemented with equal concentrations of BAP and Kinetin, but the percentage of regeneration was less (46 %) compared to our results. ³⁹developed an indirect regeneration protocol in ICP 8863 pigeon pea variety using leaf and epicotyl explants with an intervening callus stage. However, this protocol may have chances of developing phenotypic variations within the same culture and results in genetic instability of the regenerated plants. But, the direct regeneration protocol standardized in the current study enables regeneration through direct shoot organogenesis without any intervening callus stage. There were also some earlier reports on indirect regeneration using leaf explants, but the reported percentage of shoot bud induction was low (36 %)⁴⁰. Even though,⁴¹ reported 81 % of shoot bud induction from cotyledonary explants, the percentage of conversion of shoot buds to fully developed shoots was low.

In vitro regeneration is also reported from leaf explants and apical meristem with attached leaf, where callus formation was noticed in leaf explants using Thidiazuron (TDZ) and the percentage of shoot induction was only 77.8 %³². Based on the studies on whole plant regeneration from axillary buds in different varieties of pigeon pea,³³ stated that regeneration frequency varies among different varieties and the same was earlier reported by other researchers^{13,15,27,41,42-45}. The induced shoots of both the explants were kept for elongation on MS medium containing various concentrations of BAP, NAA and GA₃. According to the previous studies, elongation of induced shoots depends on various factors like substitution of plant growth regulators^{46,47} to alter the medium^{25,48} and change in light conditions. For better elongation of the proliferated shoots, the concentration of BAP was reduced to half of the concentration used for shoot induction and the medium was additionally supplemented with NAA and GA₃. Our results are in correlation with the studies of³⁷ who also reported that use of high concentrations of BAP results in low frequency of shoot elongation. Within two weeks of subculture, the shoots were elongated up to 4 cm and high percentage of shoot elongation (93 %) was noticed in leaf petiole explants cultured on MS medium supplemented with 1.0 mg/L BAP + 0.1 mg/L NAA + 2.0 mg/L GA₃ concentrations (Figure 1 (e) and (f)) (Table 2). In the case of cotyledonary node explants, maximum percentage of elongation was observed with 1.0 mg/L 6-BAP + 0.1 mg/L NAA + 1.0 mg/L GA₃ concentrations (84 %). According to¹⁴, no elongation was observed when the MS medium was supplemented with only BAP or BAP in combination with IAA without GA₃.

Finally, the well-elongated shoots of both the explants were transferred to root induction medium containing various concentrations of IBA. Within two weeks of sub-culture, adventitious roots were initiated and the percentage of rooting was high (95 %) in shoots obtained from leaf petiole explants cultured on 0.5 mg/L IBA compared to cotyledonary node explants (Figure 1 (g) (h)), where only 84 % of rooting was observed with 0.3 mg/L IBA concentration (Table 3). Based on this study, we report that in both the explants, lower concentrations of IBA supported better root proliferation compared to high concentrations.

Our results are in support to the earlier reports of^{27,25,49} and some other researchers, who also reported that the addition of NAA and IBA resulted in 80-85 % of rooting¹⁵. These profusely rooted regenerated plantlets were

transferred to small pots containing autoclaved soil and soilrite mixture in 1:1 ratio and were initially hardened in culture room (Figure 2a) and were finally transferred to green house for acclimatization (Figure 2b). Morphologically the regenerated plants showed normal growth with well-developed leaves. Out of eight RAPD primers from the OPP and OPAZ series tested (Table 4), two primers produced better amplification and resulted in five bands ranging from 520–4000 bp with OPP09 marker (Figure 3) and five bands ranging from 300–2200 bp with OPAZ18 marker (Figure 4) respectively with regenerated plants. The amplification pattern of the *in vitro* regenerated plants was similar to the donor plants of the explants and no apparent phenotypic variations were observed, which confirms their genetic stability. To our knowledge this is the first report of *in vitro* regeneration of ICP 8863 pigeon pea variety through direct organogenesis using leaf petiole and cotyledonary node explants and is suitable for further genetic transformation studies.

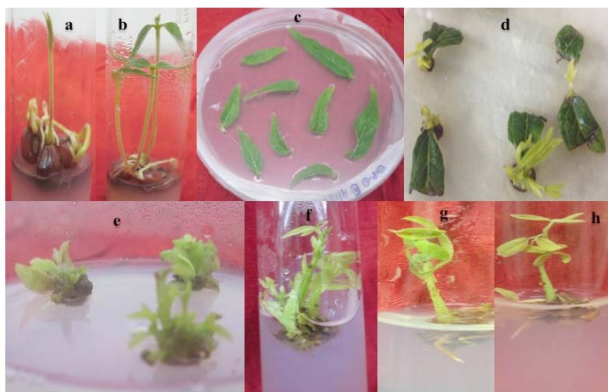


Figure 1. Regeneration of multiple shoots from leaf petiole explants obtained from *in vitro* germinated pigeon pea seedlings. (a) Two day old germinated seedlings on basal MS medium, (b) Six day old germinated seedlings on basal MS medium, (c) Seven day old leaf petiole explants inoculated on shoot induction medium, (d) Induction of multiple shoot buds from the leaf petiolar region inoculated on MS medium fortified with 2.0 mg/l 6- BAP, (e, f) Multiple shoot elongation of proliferated shoots on MS medium containing 1.0 mg/l BAP, 0.1 mg/l NAA and 2 mg/l GA3 and (g, h) Rooting of *in vitro* raised shoots on MS medium supplemented with 1.0 mg/l IBA.

Table 4. RAPD primers tested for genetic stability analysis of the acclimatized *in vitro* regenerated plants from leaf petiole and cotyledonary node explants of ICP 8863 pigeon pea variety

Primer	Primer sequence (5'-3')
OPP-06	GTGGGCTGAC
OPP-07	GTCCATGCCA
OPP-08	ACATCGCCCA
OPP-09	GTGGTCCGCA
OPAZ-16	AGGCGAACTG
OPAZ-17	CACGCAGATG
OPAZ-18	CCGACGTTGA
OPAZ-19	ACACTCTCGG

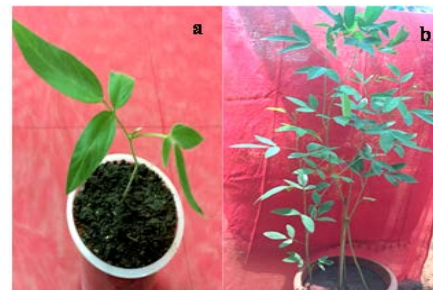


Figure 2. Hardening of *in vitro* regenerated pigeon pea plantlets, (a) Acclimatization of well-rooted plants in 1:1 ratio of soil and soilrite mixture (b) Hardened plant in green house.

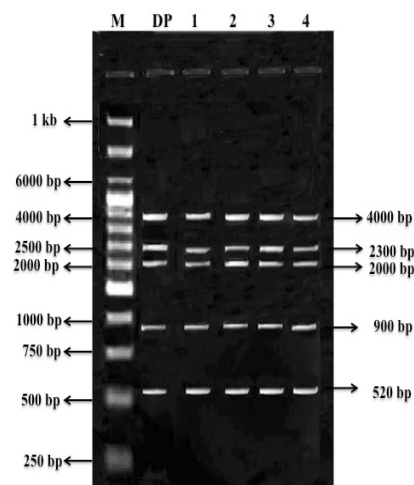


Figure 3. Genetic stability analysis of *in vitro* regenerated plants from leaf petiole explants using OPP09 RAPD primer. M-1kb DNA ladder, DP-Donar Plant, Lanes 1-4 *in vitro* regenerated plants.

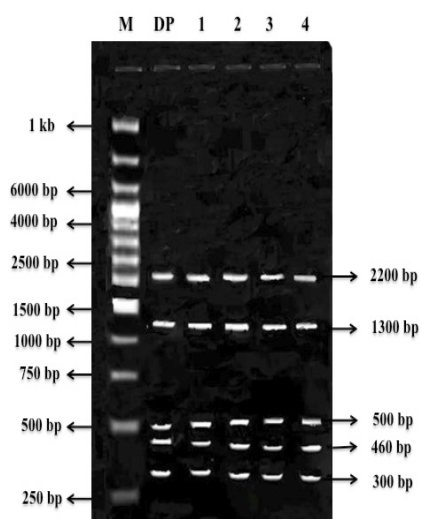


Figure 4. Genetic stability analysis of *in vitro* regenerated plants from cotyledonary node explants using OPAZ18 RAPD primer. M-1kb DNA ladder, DP - Donor Plant, Lanes 1-4 *in vitro* regenerated plant.

4. Conclusion

In the present study, an efficient and reliable protocol through direct organogenesis was developed for *in vitro* regeneration of ICP 8863 variety of pigeon pea using leaf petiole and cotyledonary node explants, which can be used to develop transgenic pigeon pea plants by agrobacterium mediated transformation.

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