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EFFICIENT *IN VITRO* REGENERATION OF PIGEON PEA (*CAJANUS CAJAN*) USING DIFFERENT EXPLANTS

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Abstract
ICPL87 pigeon pea (*Cajanus cajan*) was developed by pedigree selection from the cross ICPX73052 (T21 X JA277), made in 1973. In the present study, more than 50% of the variants showed gains of13.6 to 21.8% in their seed size. The largest seeds (13.3 g/100 seeds) were produced by 'ICPL 99073'. This line also produced high seed yield (2226 kg/ha). Since small seed size is dominant over large seeds, the observed variation for large seeds could be the result of specific mutational events occurring from dominant to recessive form of alleles. The most common seed coat color in pigeon pea is brown, and it is dominant over white seed. Only one or two recessive genes are known to control the expression of white seed. In the present study, 8 (out of the 15 lines evaluated) recorded a change in seed colour from brown to white, emphasizing a change that was induced through point mutations from dominant to recessive form of alleles. It is evident that the dominant genes, governing brown coat colour are prone to such mutagenic forces. Also reported the presence of white seeded mutants in some of the R2 explants.
Key words: ICPL87, L2 medium, DAP.

Introduction
In various Indian trials, ICPL87 pigeon pea (*Cajanus cajan*) was it out yielded the control, UPAS120, by up to 50%, giving 3700-5200 kg/ha. ICPL87 matures 110-130 days after sowing. Plants are smaller than in conventional longer duration types and suitable for high density (330 000 plants/ha) cultivation; they are 80-90 cm tall and semi spreading, with a determinate growth habit. ICPL87 is tolerant of *Fusarium wilt* and compensates strongly during the second flush for damage caused by pod borer (*Heliothisarmigera* (*Helicoverpaarmigera*).
L2 medium is a culture medium used for the regeneration of pigeon pea ((*Cajanus cajan*)) plants. It's one of several media used for legumes, along with MS and BS media. L2 medium used for pigeon pea regeneration

MATERIAL AND METHODS

Plant material and culture medium
The seeds were surface sterilized and inoculated on the MS half strength basal medium containing 0.5 percent sucrose for seed germination. In vitro grown seven-nine days old seedlings were used as a source of hypocotyl and cotyledon explants for plant morphogenesis (Fig. 1a). The age of explants is related with the age of *in vitro* grown aseptic seedlings from where the explants (hypocotyl and cotyledon) were excised. Completely green fully expanded cotyledons and hypocotyl which were greenish in colour and turgid nature from seven to nine days old aseptically grown seedlings were used for efficient shoot regeneration studies. Different

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concentrations and combinations of thidiazuron along with auxins i.e. TDZ alone, TDZ + Adenine, TDZ + NAA, TDZ + IAA were used in the MS basal medium for efficient plant regeneration response. Stock solution of Thidiazuron (TDZ) was prepared by dissolving 10 mg of TDZ in minimum amount of DMSO and the final volume was made to 10 ml by using double distilled water. The pH of the culture medium was adjusted to 5.8 before agar–agar addition to the medium. Then medium was poured in different culture vessels and sterilized at 15 pounds per square inch for 15 min in an autoclave. Aseptic manipulations were carried out in laminar air flow chamber. All the inoculated cultures were grown at 26 ± 2 °C temperature and 70–80% humidity under 16/8 h light/dark photoperiod in the culture room with light intensity of 40 μmol m−2 s−1 using cool white fluorescent lamps.

Explants preparation:

Cotyledon explants, leaf explants, or whole cotyledons are used as explants. Media preparation: L2 medium is supplemented with hormones like 2,4-Dichlorophenoxyacetic acid (2,4-D), benzyl adenine (BA), kinetin, and Gibberellic acid (GA3)

Callus initiation**:**

The explants are cultured on the medium to initiate callus Shoot and root induction: The callus is cultured further to induce shoots and roots.

Murashige and Skoog (MS) medium is used to regenerate pigeon pea plants through direct shoot regeneration. The medium is supplemented with various concentrations of growth hormones to induce shoots, elongate them, and root them.

Steps for regenerating pigeon pea plants using MS medium

1. Explant preparation Use leaf explants from 4–5 day old in vitro seedlings, or cotyledonary node explants from 12 day old seedlings

2. Shoot induction Culture the explants on MS medium with growth hormones like Benzyladenine (BA), kinetin, or Naphthalene Acetic acid (NAA)

3. Shoot elongation Elongate the shoots on MS medium with growth hormones like Gibberellic acid (GA 3).

Cajanus cajan cv. ICPL 87 is used for studying culture response of 11-16 day old immature embryos towards multiple shoot induction andregeneration. The number of immature embryos used varies depending on the germinationpercentage and availability of embryos at the same stage of development. Numbering of days afterpollination (DAP) is taken as the age of immature embryos. The green immature pods collected from fieldgrown plants were surface sterilized with detergent for five minutes, followed by 70% ethanol for 1min, 0.1% HgCl2 for 3 min and then rinsed several times with sterile double distilled water. The greenimmature pods were aseptically dissected to collect embryos and then inoculated on MSmedium [27] and MS medium supplemented with different concentrations of BAP (1, 2, 3, 4 and5mg/l). The cultures were maintained at 25±20C under 16/8hr light /dark photoperiod.

Polyvinylpyrrolidone (PVP, 100 mg/l) was used in the medium to prevent browning of the tissues. Explants with multiple shoots were transferred toculture tubes containing 20ml MS medium supplemented with 0.1mg/l NAA and varyingconcentrations of GA3 for 4 weeks to encourage shoot elongation. The number of respondingexplants and shoots per explants were recorded 30 days after transfer to half MS medium. Mean values were quantified from the data of 50 explants. The experiment was conducted threetimes.Shoots >3cm were transferred to rooting media containing half strength MS basal medium with0.5mg/l NAA. The

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concentration of NAA in rooting media was gradually eliminated over aperiod of 1 month. Well rooted plantlets were transferred to paper cups containing sterilizedmixture of sandy loam soil and vermiculite (1:1) and watered daily with Hoagland's solution [28].Later, they were transferred to pots with sterile soil and maintained in poly-house under cooltemperature for 10days before shifted to open place.

RESULTS:

Explants swelling at the nodal region was the primary response observed within the first five days of culture on all concentrations of BAP added to MS medium and shoot root initiation was observed in explants cultured on MS basal medium (Fig. Ia). Embryos younger to 12 days did not survive on all media. The primary shoot and root exhibited inhibition of growth and induction of shoot buds was observedfrom the axillary buds of the swollen node of 12-16 day old immature embryos in 10 days on allconcentrations of BAP (Fig. Ib). Five to 10 shoot buds were produced per explant in first 20 daysand continued to increase until 40 days. The number of shoots was more on mediasupplemented with 2mg/l and 3mg/l BAP when compared to other concentrations used. There is nomuch variation in the frequency of shoot bud induction from 12 to 16 day immature embryos.The regeneration capacity of 12 day old immature embryos was low when compared to 16 day oldimmature embryos . The number of shoot buds varied from 12 day old to 16 day oldimmature embryos and the number increased from 12th day to 16th day.Multiple shoot buds from 12-16 day old immatureembryos elongated on MS medium fortified with0.1mg/l NAA and 0.3mg/l GA3 (Fig Ic) and shoots longer than 3cm developed roots whentransferred to half strength MS medium supplemented with 0.5mg/l NAA (Fig. Id). Well rooted plants grew well on sterile vermiculate (Fig Ig), polyhouse conditions and flowered in 5-6months in the normal short day flowering season No. of explants: 605 explants in each culture medium bottle16 hrs dark and bottles are kept in light regeneration is 80 percent in of *Cajanus cajan*.

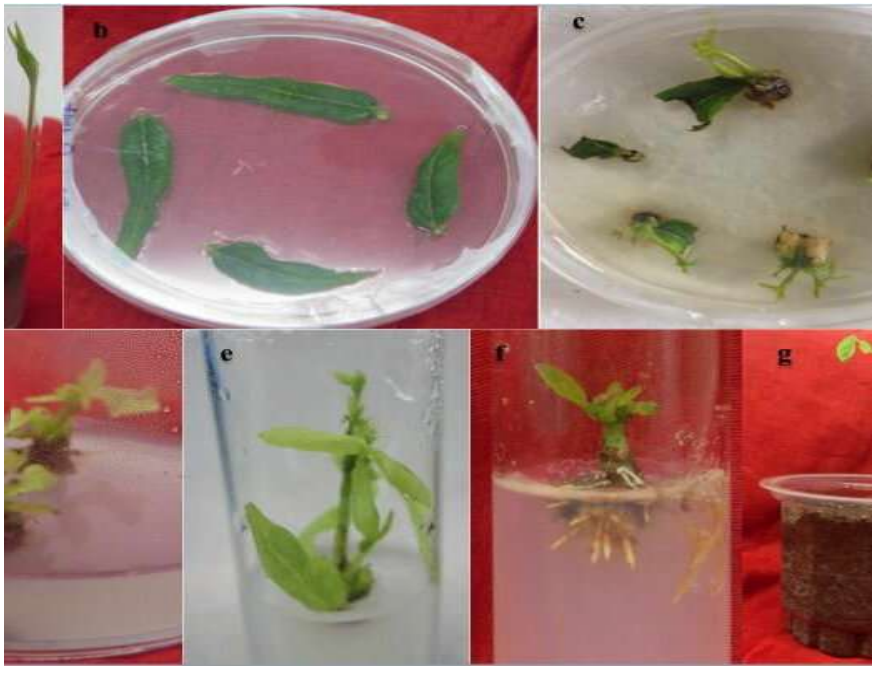

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Fig1: (a-g) Morphogenesis and stages of regeneration in *Cajanus cajan.*

In general, growth habit of the somaclonal variants was found to be more or less similar to that of the parent variety'ICPL 87'. Pooled analysis of the data showed that thedifferences amongst the genotypes over two years weresignificant for days to maturity, plant height, 100-seed weightand seed yield (Table 1). Variation between two years was significant for days to maturity, 100-seed weight and seed yield. The interactions between genotypes × years, however,were found to be significant only for maturity. This suggestedthat in both the years, the performance of genotypes followedmore or less similar trends in respect of seed yield, plant height,seed size and seeds/pod.The genetic variation created through in-vitro culturefor grain yield was significant in both the years (Table 1). In2007, the trial mean yield (1254.5 kg/ha) was low as comparedto that of 2008 (2222.7 kg/ha). On average over the two years, the variant line, 'ICPL 99073' was found to be the best performerwith 2226 kg/ha yield and recorded 25.3% advantage in yieldover the parent variety 'ICPL 87' (1777 kg/ha). This lineproduced maximum yield (2861 kg/ha) in 2008, and was amongthe top performers in 2007. Overall, the genetic variation for days to maturity was large where the parent variety 'ICPL 87'matured in 124 days, while 'ICPL 99068' matured earlier (118days) than the control (Table 2). Since the earliness in *pigeonpea* is controlled by more than one partially dominantgene (Saxena and Sharma 1990), the deviations observedbetween 'ICPL 87' and the somaclonal variants probably represent mutational changes in one or two gene loci, whichcontributed towards earliness in 'ICPL 99068'. Some somaclonal lines such as 'ICPL 99073', 'ICPL 99070' and 'ICPL99066' were taller than the parent variety (Table 2). In pigeonpea, the inheritance of plant height has been reportedto be complex and quantitative in nature. Its expression isoften complicated and masked by thermo- and photo-periodsensitivity (Byth et al. 1981, Wallis et al. 1981), and therefore,no attempt was made to interpret the present results in termsof induced variation at genetic level.

Table 1: Auxin combinations producing callus and somatic embryos

MS Media+ Auxin combinations	**Conc (mg/L)**	**Type of response**	**Degree of callus formed**
2,4D +BAP	1.O+0.5	Callus	+
2,4D+BAP	1.5+1.0	Callus	++
2,4D+BAP	2.5+1.5	Callus	+++
2,4D+BAP	**3.2+2.0**	**callus**	**++**
2,4D+BAP	**4.2+2.5**	**Somatic embryo**	**+ +**

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2,4D+BAP	**5.2+3.0**	**Somatic embryo**	**+ +**

Degree of callus formed was rated using the scale.
+ = Poor
++ =Good
+++ =Very good
+ +=Somatic embryo
+ +=Somatic embryo
PGR: Plant growth regulator.

In pigeon pea, the characters such as seed size and seed colour are very important from marketing point of view. Incomparison to the traditional small brown seeded varieties,large white seeded types are considered to be premium andfetch about 15 - 20% higher price. The popular variety 'ICPL87' has brown seeds with 100-seed weight of 11.1 g.

In the present study, more than 50% of the variants showed gains of13.6 to 21.8% in their seed size (Table 2). The largest seeds(13.3 g/100-seeds) were produced by 'ICPL 99073'. This linealso produced high seed yield (2226 kg/ha). Since small seedsize is dominant over large seeds (Singh and Pandey 1974),the observed variation for large seeds could be the result ofspecific mutational events occurring from dominant torecessive form of alleles. The most common seed coat colour in pigeonpea is brown, and it is dominant over white seed.Only one or two recessive genes are known to control theexpression of white seed (Patil 1970, Singh 1971, Deokar et al.1972). In the present study, 8 (out of the 15 lines evaluated)recorded a change in seed color from brown to white (Fig. 1),emphasizing a change that was induced through pointmutations from dominant to recessive form of alleles. It isevident that the dominant genes, governing brown coat colour are prone to such mutagenic forces. Chintapalli et al. (1997) also reported the presence of white seeded mutants in someof the R2explants and they attributed it to the presence ofsome transposable elements, which were activated during theprocess of tissue culture process. They also postulated the presence of definite genes for white seed colour and high seed mass in the adapted genetic background of 'ICPL 87'.

The present data also showed that the increases in seed size of *pigeon pea* were not restricted to any specific seed colour.Ryan *et al.* (1987) recorded significant gains in seed size inwheat that was induced by somaclonal changes. Morden *etal.* (1989) and Baillie *et al.* (1992), on the contrary, reported nosuch increases among somaclonal variants for kernel weight in barley.Somaclonal induced resistance to Fusarium wilt hasbeen reported in tomato (Shahin and Spivey 1986) andMedicago (Hartman et al. 1984).

DISCUSSION:

Krishnamurthy Studies on 11-16 day old immature embryos ofICPL 87 revealed that there is increase in percentage of germination with age of the embryo.This result is not uncommon because the embryo has the potential to germinate and differentiateinto a plant was after 12 days for ICPL 87 as understood from our studies. But the results of culture of immature embryos of *Cajanus cajan* by different workers appears to be different. Kumar *et al* reported callus development and low level of plant differentiation in 11-14 day old embryos of three varieties and there is direct differentiation into whole plants in 15-19 day old plants with

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small quantity callus appearance.

Our results are in concurrence with Kusum Kanta & Padmanabhan who cultured full embryo and embryos cut into two, three segments on Nitsch's basal medium supplemented with casein hydrolysate, coconut milk, kinetin and 2,4-D.The time window for potential to differentiate into whole plant in *Cajanus cajan* appears to be 12th or14th day in different varieties. Our result appears to be consistent with several varieties studied by Kumar *et al*, with respect to hardening and differentiation which proceeds after 14 days of pollination. The callus induction in Kumar *et al* could be due to use of 2,4 D in both MS and B5 medium and non induction of callus in our studies isbecause of use of MS or MSB where theconditions are not suitable for callus induction. More number of multiple shoot buds wereobserved at node, it can be explained in terms of apical dominance. Franklin *et al* obtained a different result as multiple shoot induction was obtained from apical region when embryonal axes were inoculated on MS medium supplemented with BAP and NAA. Both embryonal axes and nodal explants on MSB1 medium produced callus and shoot, while only shoots were produced on MSB. This is also consistent with well known phenomenon that both cytokinins and auxins are required for callus induction. Higher cytokinin Standardized protocol for media preparation of MS medium, In the present study, a reproducible and highly efficient plant-regeneration protocol in pigeon pea varieties, viz., 'Phule Rajeshwari' and 'Vipula' was developed. Multiple shoot induction was achieved using leaf and apical meristem with attached leaf as explant by use of various concentrations and combinations of growth regulators. Optimum embryogenic callus induction from leaf explant was observed on Murashige and Skoogs medium supplemented with 2.0 mg/l thidiazuron. Apical meristem with attached leaf explants showed early shoot initiation, high frequency of callusing (100%), and shoot regeneration (100%) as compared with leaf explant in both varieties. In vitro rooting (93.33%) was obtained within 10 days on MS medium supplemented with 2.0 mg/l indole butyric acid. The *in vitro* rooted plantlets were successfully established in poly carbonated poly house with 80% survival rate. This optimized regeneration protocol can be efficiently used for genetic transformation in pigeon pea.

In-vitro regeneration by organogenesis of pigeon pea has been attempted using diverse explants like leaf, cotyledons, cotyledonary nodes, embryonal axes, leaf petiole, embryo, epicotyls, embryonal axis attached cotyledons, auxiliary buds and apical meristem with more than fifty diverse cultivars (Krishna et al. 2010, Pawar et al. 2014and Pratap et al. 2018. Several factors like genotype selection, explants tissues, media composition, and plant growth regulators substantially influence the plantlet regeneration via organogenesis in legumes that is amenable to efficient genetic transformation.

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