Application of Somaclonal Variation in Crop Improvement of Pigeonpea (*Cajanus cajan* (L.) Millspaugh)

Prasanna Latha Chintapalli

Thesis presented to the Osmania University for the degree of

DOCTOR OF PHILOSOPHY

In

BOTANY

CERTIFICATE

This is to certify that the thesis entitled "Application of Somaclonal Variation for Crop Improvement in Pigeonpea (*Cajanus cajan* (L.) Millsp.)" **submitted for the degree of Doctor of Philosophy in Botany, Osmania University, is a record of bonafide research carried out by** Ms. Prasanna Latha Chintapalli **under our supervision. Also certified that no part of this thesis has formed the basis for the award of any degree or diploma prior to this date.**

The assistance of all concerned during the course of this investigation and also the source of literature has been duly acknowledged.

11/10/95

J.P. Moss
Research Division Director
Cellular and Molecular Biology Division
ICRISAT

J.K. Bhalla
Professor
Cytogenetics and Tissue Culture Lab.
Dept. of Botany, Osmania University

DECLARATION

The research work embodied in this thesis has been carried out in the Cellular and Molecular Biology Division, ICRISAT Asia Center, Hyderabad and in the Tissue Culture and Cytogenetics Laboratory, Department of Botany, Osmania University, Hyderabad. This work is original and has not been submitted in part or full for any other degree or diploma of any university.

Date:
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Prasanna Latha Chintapalli

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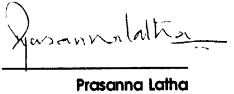

Prasanna Latha

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Chapter 1

1 INTRODUCTION

"Ten thousand years ago some genius reached out and altered our lives forever by planting a seed." - Alvin Toffler. Remarkably enough, the continual endeavours of man to exploit the natural variation present in base population of crop plants to obtain improved varieties was achieved initially by multiplication of best available material. This was followed up by selective cropping and cross hybridization to obtain hybrid crop which eventually led to the perpetuation of desirable germplasm. However, this also led to inbreeding depression.

Breeding for a "plant ideotype" is a postulation in agriculture. Plant improvement is a multidisciplinary activity concerned with the optimization of genetic attributes within the constraints of the environment, and of environmental factors within the constraints of the genetic material (Byth et al., 1980). Conventional breeding exploits the natural variation existing in plant populations to recover elite crops. However, the available genetic variability in gene pools is one of the limits to crop improvement. Conventional breeding in its manifold efforts to produce a plant ideotype exploits this natural variation existing in the base

The evolution of the theoretical aspects of in vitro culture paved the way for the emergence of its practical applications which reached exploding proportions in the past decade or so. Initially used for clonal propagation of plants, it later pioneered several new possibilities like removal of sexual incompatibility by embryo rescue techniques, somatic hybridization through protoplast technology, transgenic plants through genetic engineering, production of haploids via anther culture and most important in the context of the present investigation-- the induction of genetic variability and selection of desirable traits like salt tolerance (Nabors et al., 1980) and disease (Heinz et al., 1977; van den Bulk et al., 1991) and pest resistance (Isenhour et al., 1991). Thus, in vitro culture, no longer sacrosanct, has emerged as a biotechnological tool to widen the germplasm base.

Tissue culture per se is a rich source of variation. The best available germplasm may be subjected to a tissue culture cycle with or without selection pressure and regenerants selected for superiority for one or more traits while retaining all the original characters. Such incremental improvement in desirable traits could therefore lead to the formation of new alleles spontaneously generated in vitro. Thus tissue culture methods leading to somaclonal variation could be capitalized upon to accelerate progress in conventional breeding.

Although there is a high potential and promise for the use of in vitro technique for plant regeneration and selection of useful variants, its successful application for crop improvement by subsequent incorporation of selected variants into plant breeding programmes hinges on the capability to regenerate in high frequency from diverse explants. Among pigeonpea cultivars, ICPL 87 was chosen as the experimental material as it has the desirable traits like fusarium wilt resistance, high yield, short duration and short stature.

Pigeonpea, a grain legume of vast importance in the Indian sub-continent, is believed to have originated in India around 2200-2000 BC, and later, spread to Africa where, a secondary center of diversity developed (van der Maesen, 1980). The phytogeographic data supports its asiatic (Indian) origin (De, 1974 and Smartt, 1980). However, Ladizinsky and Hamel (1980) suggest a polyphyletic origin based on seed protein examination of four species of *Atylosia* and *Cajanus*.

Cajanus cajan grows on a variety of soils like entisols (Indo-gangetic alluvial belt), dry vertisols (black cotton soils) and also on alfisols (red soils of the Deccan) (Reddy and Virmani, 1981). It is one of the most important grain legumes of the tropics and sub-tropics and ranks sixth in area and production when compared to other pulse crops (Nene and Sheila, 1990). Besides being an important source of dietary protein for the vegetarian in the Indian sub-continent, its many uses are as fodder, feed, fuel and also as a host for culturing the lac-producing insect. The stem is used in building huts and the plants are also grown as a hedge.

Tissue culture leading to somaclonal variation could be capitalized on, to accelerate progress in conventional breeding. The best available germplasm may be subjected to a tissue culture cycle and regenerants having all the qualities of the parent while being superior in one or more traits can be selected. This incremental improvement in desirable traits could be due to the new alleles that may have been generated in vitro.

Rapid advances in the field of diagnostics has enabled the detection of polymorphisms at the protein, isozyme or nucleic acid level. Molecular markers have the potential to enhance selection efficiency in plant breeding. The new polymorphism assay (Williams et al., 1990 and Welsh and McClelland, 1990) based on the amplification of DNA sequences by polymerase chain reaction using single oligonucleotide primers of arbitrary sequence is being increasingly

used for identification of randomly amplified polymorphic DNA (RAPD) markers. Michelmore et al. (1991), described a bulked segregant analysis technique which in conjunction with RAPD analysis can help identify molecular markers for a particular gene of interest in a segregating population. Aided by the detection of molecular changes associated with tissue culture by these and other molecular diagnostic tools, it is probable that, the mechanism of somaclonal variation will eventually be unravelled.

In the plant breeders perspective however, the bottom line, remains ultimately that the genetic variability recovered from regenerated plants should result in a phenotype that is agriculturally useful. The present research program thus explored the possibility of exploiting agriculturally useful somaclonal variation for subsequent integration into plant breeding programmes and the following objectives were formulated:

1. Develop tissue culture technology for *Cajanus cajan* (L.) Millsp. cv ICPL 87:
    a. Identify the best explant source.
    b. Optimize reproducible and high frequency regeneration.
    c. Irradiate in vitro cultures with gamma rays for additional variability.
2. Assess for somaclonal variation:
    a. Screen for plants showing new variation coupled with statistical analysis of degree of variation in qualitative and quantitative characters in the R2 and R3 generations.
    b. Identify progenies with increased variation and selection of sources of useful variation with stable inheritance.
3. Diagnostic:
    a. Investigation for somaclonal variation at the molecular level.

2 REVIEW OF LITERATURE:

A recent review on somaclonal variation by various authors in "Somaclonal variation in Crop Improvement I" (Bajaj, 1990) comprehensively covers various aspects like the chromosomal, genetical and molecular variability. Somaclonal Variation in certain cereals, vegetables and fruits and also ornamentals and forage plants is described. The present review briefly focusses on the literature available on (a) the history of somaclonal variation, (b) characterization of somaclonal variation at the molecular level and (c) tissue culture and regeneration studies in legumes in general and pigeonpea in particular.

2.1 SOMACLONAL VARIATION

Sections 2.1 and 2.2 discusses the cytogenetic variation that resulted in haploids, aneuploids and polyploids; in vitro selection for herbicide and salt tolerance and disease and pest resistance; genotypic and phenotypic variation for qualitative and quantitative traits; studies

on culture induced vs mutagen induced variation; and molecular variants at the nuclear and organelle levels in some important crops/plant species.

Genetic variation arising from tissue culture of plants has been termed somaclonal variation (Larkin and Scowcroft, 1981). Variation has been observed in a wide range of species, from plants derived from a variety of explants, using different cultural methods. The addition of growth substances to the culture medium are believed to induce mutations (D'Amato, 1977). In agreement with this all forms of mutational events like changes in ploidy level, chromosome breakage and rearrangements, gene amplification, single gene mutation, variation in quantitative traits and activation of transposable elements have all been reported. This phenomenon stimulated much interest among plant breeders and those researching or using tissue culture, thus contributing to the accumulation of literature. The possibility of changing one or more traits of agronomic importance in an otherwise outstanding cultivar without altering the rest of the genome is a major consideration as an adjunct to conventional breeding. The occurrence and extent of somaclonal variation is influenced by explant source, culture type and age, and genotype (Peschke and Phillips, 1992). Most of this variation has been attributed to aneuploidy (Ahloowalia, 1982), single gene mutations (Evans and Sharp, 1983), and also to transposable elements (Larkin et al., 1984).

2.1.1 CYTOGENETIC VARIATION

Chromosomal variation has been reviewed by Lee and Phillips, (1988) who observe that variation has been recorded using virtually every tool available to the geneticist, from the light microscope to the nucleic acid sequencer. In cereals, Linacera et al. (1992) reported that non-diploid rye plants appeared in R1 and R2 progenies while chromosome mosaicism was restricted to the initial regenerants.

Cytogenetic variation was reported in *Triticum aestivum* (wheat) by Ahloowalia (1982) who explains the presence of aneuploid chromosome numbers in the callus cells and also univalent laggards, chromatin bridges, fragments at Anaphase I, and micronuclei in tetrads of regenerated plants to the occurrence of chromosomal inversions and deletions. The observed variation in morphology, like pollen sterility and reduced seed weight, was thus explained by a loss or gain of a whole chromosome or structural changes in chromosomes. Karp and Maddock (1984) reported 27% of regenerants to be aneuploids. Some of the regenerants were also observed to show chromosomal structural changes, particularly interchanges. Davies et al. (1986) correlated 13 of the 17 regenerants (from a total of 551) having altered ADH1 zymogram with aneuploidy. The rest of the 4 were observed to give rise to euploid progeny, 3 of which were interpreted to possess a 4α isochromosome, a 3BS/4Aα translocation and a 7BS/4Aα translocation respectively. Galiba et al. (1985) observed euploid chromosome number of 2n=6x=42, while in 3 somaclonal families the chromosome number ranged from 41 to 45 with some plants also containing telocentrics.

Regenerated plants with aberrant cytology were also observed in *Avena sativa* (oats) by McCoy et al. (1982) who report an increase in frequency of chromosomal variability from 12-49% to 48-88% with increase in duration of culture. Besides chromosome breakage and loss of chromosome segments, trisomy, monosomy, interchanges and inversions were also observed. Also in oat, Maddock and Semple (1986) observed euploid chromosome number of 42 and aneuploid plants that were shorter and darker green in colour. In about 9% of the cells in progeny of two regenerated oat plants, premature separation of heteromorphic bivalents and lagging during anaphase I were observed by Johnson et al. (1987a). In a later study Johnson et al. (1987b) discussed the possible role of heterochromatin in chromosome breakage induced in oat tissue cultures. They suggest that the presence of proximal and telomeric heterochromatin means that bridges resulting from replication problems in these

regions could result in various alterations including deficiencies, duplications and translocations and can account for heterochromatin amplification.

In *Hordeum vulgare* (barley), Powell et al. (1986) observed 4 of the 10 microspore derived lines to have reduced chiasma frequency, while one line showed evidence of translocation heterozygosity. Although Orton (1980) observed spontaneous polyploidy, aneuploidy and chromosomal rearrangements in callus and suspension cultures of *Hordeum* the regenerated plants showed a complete loss of polyploidy and a decrease in aneuploidy and chromosomal rearrangements. Gaponenko et al. (1988) reported 1% of the regenerated plants to be tetraploid. All the normal diploid and tetraploid plants showed no chromosome aberration. Extensive chromosome breakage was associated with abnormal meiosis in one regenerant from cultured immature embryo of barley (Karp et al., 1987). They also observed unexpected numbers of bivalents, sometimes univalents in metaphase I of pollen mother cells and also occasional fragments and extra pieces of chromatin. Large numbers of micronuclei were observed to be associated with dyads and tetrads.

In *Saccharum officinarum* (sugarcane) Heinz and Mee (1971) observed a cell to cell variation in chromosome number ranging from 2n=94-120 (one exceptional plant showed 2n=17-118) in 36 of 37 plants regenerated from a chromosomal mosaic. 10% of the cells were multinucleate.

In *Zea mays* (maize) callus cultures McCoy and Phillips (1982) observed that with increase in culture age from 4 months to 8 months, the number of abnormal plants increased from 1 to 4 out of 65 regenerants each. Three of the five abnormal plants had both normal and cytogenetically abnormal sectors in the tassels. It was also observed that 97% of the cells after 4 months and 90% of cells after 8 months were normal. Lee and Phillips (1987a) reported 108

chromosomal aberrations in maize regenerants, 96% of which involved structural changes like interchanges (42%), deficiencies (35%) and heteromorphic pairs (19%). They also observed 48% of plants from 8-9 months old cultures to carry a chromosome aberration while all plants from 3-4 month old cultures were cytogenetically normal.

In *Oryza sativa* (rice) Bajaj et al. (1980a) observed aneuploids and polyploids in triploid plants derived from immature and mature embryo callus. In 100 R2 lines screened by Nowick et al. (1988) a low percentage of anaphase bridges were observed. This chromosomal abnormality was non-segregating in the R2 with low variances, although individual plants outside the range of 'Lemont' were seen to be variant among the R2 progenies.

In *Triticale* Jordan and Larter (1985) observed extreme chromosomal instability in one genotype R_{13} with both the rye and wheat univalents at metaphase I.

In *Apium graveolens* (celery), Orton (1983) observed hypodiploid and a mixture of diploid and hypodiploid and also polyploid cells in callus cultures. Some of the plants regenerated via somatic embryogenesis were monosomic. Karyotypic analysis of a clone showed the presence of a large metacentric chromosome.

Cytological instability in *Medicago* was reported by Groose and Bingham (1984) in plants regenerated from callus cultures. 11% of regenerants lost one or more chromosomes thus showing a chromosome number of 28 to 31. Two apparent shifts to octoploids were also observed and approximately 60% of variant regenerants exhibited a change in chromosome number. They attributed the considerable variability in fertility and morphology to changes in chromosome number. Saunders and Bingham (1972) observed 5 of 166 alfalfa plants derived from anthers and 4 of 60 plants derived from ovaries to be octoploid. Hartman et al. (1984a),

in their efforts to select for *Fusarium oxysporium* f. sp. *medicaginis* resistant alfalfa cell lines, observed resistant regenerants to be octoploid and hexaploids in long term cultures only. Johnson et al. (1984) observed only 30% of RS-K1 and 45% of RS-K2 protoclones obtained from mesophyll protoplasts of alfalfa as having the parental chromosome number of 2n=4x=32. Chromosomal changes included increased ploidy, aneuploids and translocations. 58% of RS-K1 and 32% of RS-K2 protoclones were octoploids. Bingham and McCoy, (1986) reviewed most of the cytogenetic variation in alfalfa. Nagarajan and Walton (1987) observed numerical changes in chromosome numbers in all regenerants of *Medicago media*. Heteroploidy was observed for both hypo and hyper aneuploid regenerants. The two cultivars tested produced 64% ('Heinrichs') and 21% ('Reaver') aneuploids while the breeders lines (Br-1 and Le-1) produced an almost identical and lower percent aneuploids (10% and 9.4%) respectively. 'Heinrichs' produced a heteroploid population with 12% being hyperaneuploid i.e., octo and heptaploid. Frequently the heteroploid plants contained cells with anaphase laggards and dicentric bridges. They believe that all these cellular abnormalities indicate heteroploidy that is due to chromosomal elimination (laggards) and unequal distribution during mitotic anaphase resulting in a hypo or hyper aneuploid condition. They also suggest that reduction in fertility in a regenerant population may be due to gross and cryptic chromosomal abnormalities or effects due to 2,4-Dichlorophenoxyacetic acid (2,4-D) or both.

Damiani et al. (1985) observed only 3 of the regenerated *Lotus corniculatus* (birdsfoot trefoil) plants to be octoploid and 6 to be mixoploid.

Chromosomal abnormalities were also studied in many grain legumes. In *Vicia faba*, Zhang et al. (1987) observed that α-Naphthaleneacetic acid (NAA) at 10 ppm and Kinetin (KT) at 2.5 ppm produced haploids while NAA at 30 ppm and KT at 7.5 ppm produced more tetraploids and breakage. They also observed the frequency of tetraploids and breakage to be

correlated with each other and the frequency of total aberration to be linearly correlated with that of micronucleus formation. They also report different frequencies of 1.1 to 13.8% haploids, 22.7 to 77.1% of tetraploids, 0 to 12.5% of breakages and 2.5 to 22.6% of other chromosome aberrations when callus was subjected to different phytohormone concentrations. In *Cajanus cajan* (pigeonpea) Bajaj et al. (1980b) observed anther derived callus to be mixoploid with a wide range of genetic variability. Chromosome number varied from 8 (n=11) to 28 (2n=22). They noted 50.8% of callus to be diploid, 17.4% haploid and 32% aneuploids. Cytogenetic variation was also reported in *Arachis hypogaea* (peanut) by Bajaj et al. (1981b). In cytological studies of anther derived callus of *Cicer arietenum* (chickpea), Gosal and Bajaj (1988) reported cells with varying number of chromosomes ranging from haploids to polyploids. They also observed a frequency of 10-15% haploids, 60% diploids, 18% triploids and 15% tetraploids in *Pisum sativum* (pea) callus.

2.1.2 IN VITRO SELECTION

In vitro selection is practical when there is a correlation between the response of the cell in culture and that of the whole plant, and success in this direction has been rather limited. Even so, heritable resistances to herbicides and pathotoxins have been reported. Chlorsulfuron resistance (Chaleff and Mauvias, 1984), hydroxyurea resistance (Keil and Chaleff, 1983), and picloram (PIC) resistance (Chaleff and Parsons, 1978) in *Nicotiana tabacum* (tobacco), paraquat resistance in *Lycopersicon esculentum* (tomato) by Thomas and Pratt (1982), and imidazolinone resistance in maize (Shaner et al., 1985) have been observed. Wershun et al. (1987) regenerated plants resistant to SYS 67 ME (m-chlorophenoxyacetic acid (MCPA)) at a concentration of 30 mgL^{-1} from cells resistant at the same concentration.

Heritable resistance to non-specific pathotoxins were reported for *Phytophthora infestans* in potato (Behnke, 1980) where plants were regenerated from callus resistant to *Phytophthora infestans* culture filtrate.

Rines and Luke (1985) obtained *Helminthosporium victoriae* insensitive oats and Larkin and Scowcroft (1983) reported *H. sacchari* tolerant sugarcane.

Sacristan (1982) regenerated plants with reduced susceptibility to *Phoma lingam* from callus and embryogenic cultures of haploid rape. The regenerants survived exposure to the toxin produced by the fungus.

Gengenbach et al. (1977) reported selection of somaclonal variation from culture of maize T-cytoplasm resistant to the toxin produced by *Helminthosporium maydis* race 2 and the disease it causes -- southern corn blight. Three of the eight regenerated plants evaluated for in vivo resistance of the pathogen were resistant to the fungus and a high correlation between resistance to culture filtrate and in vivo resistance was observed.

Resistance to *Fusarium* sp. in barley (Chawla and Wenzel, 1987) where callus initiated from 7 to 10 days old immature embryos was subjected to four cycles of selection against fusaric acid, a pathotoxin of *Fusarium*. In the first selection 80% of callus was killed, while in the fourth selection 8 to 11% resistant calli was obtained which retained resistance when maintained on non-toxic medium even after three months of subculture. Some of the plants regenerated from this callus showed resistance at 75% while some at 100% toxin concentration as seen from leaf bioassay tests. Eleven plants from 129 selected calli were selected in vitro against 0.6 mM and 0.8 mM fusaric acid. All showed reduced wilting which was observed clearly at 0.6 mM.

Fifteen plants from 8 lines over 84 hours revealed that damage in 6 lines (12 plants) was less than one arbitrary unit while 2 lines (3 plants) showed intermediate reaction.

In rice Ling et al. (1985) observed resistance to brown spot disease in two plants regenerated from 25% *Helminthosporium oryzae* toxin medium and one plant from toxin free medium. The resistance was observed to be a dominant mutation with the resistant R1 heterozygote segregating for resistance and susceptibility.

In *Medicago sativa* (alfalfa), Hartman et al. (1984a) selected lines with increased resistance to both the culture filtrate and the pathogen *Fusarium oxysporium* f. sp. *medicaginis*. Resistance was observed to be associated with increased chromosome number, but in a second experiment with shorter callus period, resistant plants with unaltered chromosome number were obtained. Arcioni et al. (1987) observed reduced regeneration capability of callus treated with culture filtrate of *Fusarium oxysporium* f. sp. *medicaginis*.

Other reports of in vitro selection are: *Fusarium oxysporium* resistance in tomato (Shahin and Spivey, 1986) where the progeny of the initial regenerants segregated in a ratio of 3 resistant to 1 susceptible. The subsequent generation confirmed that the *Fusarium* resistant plants were either homozygous or heterozygous dominant for the gene conferring resistance.

Cell lines and regenerated plants were also selected for increased concentration of amino acids. Reisch et al. (1981) regenerated 91 plants from 124 diploid alfalfa (HG2) cell lines resistant to growth inhibition due to ethionine (an analog of methionine). High concentration of methionine, cysteine, cystathionine and glutathionine were observed in some of the ethionine resistant callus cultures. One cell line R32 had a ca. tenfold increase in soluble methionine, 43% increase in total free amino acids and 40% increase in amino acids and

protein. They observed 23 of 91 mutagenized cell lines to show morphological differences. Multifoliate leaves, elongated petiolules, compact growth habit, jagged leaf margins, appendage like leaflets were identified. Variation was also observed for length of largest shoot, plant height and number of primary branches after 7 weeks. Few variations were observed among plants regenerated from mutagenized cultures not treated with ethionine. They therefore concluded that the mutagenic effects of ethionine may be responsible for the appearance of a high frequency variation. Among the regenerants 20 tetraploids, 4 aneuploids and 1 hexaploid plants were seen. Aneuploidy (2n=33 and 2n=31) was reported to be concomitant with morphological abnormalities. In all these cases resistant cells have arisen in culture when they have been subjected to positive selection in vitro. In vitro selection increases frequency of resistant cells, thereby increasing the frequency of resistant somaclones in the population. In his review, Wenzel (1985) discussed several reports where resistant mutants were recovered using non-host specific toxins.

Disease and pest resistant plants without any deliberate selection pressure in vitro have been identified on screening the somaclones or their progeny. Shahin and Spivey (1986) besides obtaining tomato plants resistant to *Fusarium oxysporium* f. sp. *lycopersici* race 2 by selection against fusaric acid, a non-specific toxin, also identified resistant plants from cultures of non-challenged cells. Inheritance studies determined the resistant plants to be either homozygous or heterozygous dominant for the gene conferring the resistance. In celery, *Fusarium oxysporium* resistant somaclones were reported by Heath-Pagliuso and Rappaport (1987, 1988). The self pollinated progeny of the fusarium resistant phenotypes were superior to the original celery cultivars 5270 R as measured by vascular discoloration and plant height. Chi-square analysis of the progeny for root and crown decay showed the variation to be heritable and conditioned by more than one locus. Fall armyworm (*Spodoptera frugiperda* (J.E. Smith)) resistant sorghum was reported by Isenhour et al. (1991). They identified two regenerated lines

as having significantly higher level of resistance to fall armyworm feeding as compared to non-regenerated and susceptible parent. From laboratory studies on larval feeding on meredic diet containing dried sorghum foliage, significant reductions in larval weight were detected for two R_3 lines which suggests the involvement of antibiosis. Free choice tests revealed a significant non-preference for the R_3 lines that had shown the greatest adverse effect on fall armyworm growth in the developmental studies. Some of the other reports on increased resistance to specific pathogens by selecting variants on screening populations of regenerated plants are the development of sugarcane varieties with enhanced resistance to eyespot disease, fiji disease and downy mildew (Heinz et al. 1977), and, increased tolerance to *Verticillium albo-atrum* in alfalfa which was associated with increased ploidy level (Latunde-Dada and Lucas, 1983) where, it was hypothesized that increased resistance was due to gene dosage effect.

Selection for salt tolerance however has met with little success. Nabors et al. (1980) identified heritable tolerance that resulted in tobacco plants tolerant of 3.3% sodium chloride (NaCl). McCoy (1987a) observed in vitro NaCl tolerance at 85 mM and 170 mM. However all genotypes exhibiting NaCl tolerance were observed to be NaCl sensitive at the whole plant level. *M. marina* exhibited whole plant NaCl tolerance (0.6% NaCl) but was NaCl sensitive at the in vitro level as the other NaCl sensitive species. A potential NaCl tolerant germplasm source was therefore identified in *M. marina*. In his later study, McCoy (1987b) selected NaCl tolerant cell lines by a step-up selection procedure where cell lines tolerant to 0.5% NaCl were subsequently selected at 1% NaCl, which retained tolerance after transfer to control medium. Extensive somaclonal variation was observed in plants regenerated from these NaCl tolerant cell lines. 44.7% were extreme dwarfs (5 cm); unbalanced polyploid chromosome sets with one variant having 166 chromosomes were observed. The isozyme patterns of plants from NaCl tolerant cell lines were also extensively altered. In vitro NaCl tolerance was maintained

following plant regeneration in 9 of 12 regenerants while whole plant tolerance was expressed in only 2 of 7 regenerated plants tested. However only one plant flowered which was both male and female sterile. Thus although NaCl tolerant cell lines were efficiently selected, the extensive somaclonal variation that occurred as a result of tissue culture was a deterrent to successful recovery of heritable NaCl tolerance. Stavarek and Rains (1984) were unable to regenerate plants from selected cell lines, while Chandler and Vasil (1984) regenerated plants from NaCl cell lines (1.25 to 2%) which did not express NaCl tolerance but were in fact more NaCl sensitive than the plants regenerated from unselected callus.

Conner and Meredith (1983) recovered aluminum resistant *Nicotiana plumbaginifolia* plants from cell cultures treated with 600 µM aluminum. Of the 217 aluminum resistant variants 31% retained aluminum resistance when cells were plated onto the medium containing aluminum; when cell suspension was cultured for 10 days on medium with aluminum and later transferred to standard medium for recovery, 51% were seen to retain resistance. Seedling segregation data indicated a single dominant mutation where the resistant plants were heterozygous for a single dominant mutation.

2.1.3 PHENOTYPIC VARIATION FOR QUALITATIVE AND QUANTITATIVE TRAITS

In a study of over 1000 regenerated plants from 25 different cultivars of wheat, Maddock et al. (1983) recorded differences of up to 40 cm in final height between plants derived from the same as well as from different cultivars. Variation in morphology of heads were also observed. Variation in plant height was also observed by Chen et al. (1987) in winter wheat regenerated from immature embryos. Larkin et al. (1984) observed heritable morphological variation in regenerants derived from immature wheat embryos. The variant characters included alterations in height, awns, tiller numbers, grain colour and glume colour. Ahloowalia and

Sherington (1985) compared the transmission of somaclonal variation in callus derived (SC1 to SC4) and embryo culture derived (E1 to E4) generations in wheat. They noted significantly lower grain yield, reduced kernel weight, and shorter height in the SC generation than the embryo derived generation. Cytogenetic variability was observed in the SC4 progenies of a short, partially sterile SC1 variant which included aberrant plants -- a haploid, few aneuploids, mixoploids, multiploidy, spindle abnormalities, multivalents, bridges and fragments and tillers having supernumerary spikes with branched rachis. Galiba et al. (1985) studied 97 regenerated winter wheat plants and their progenies. Seventeen of the SC2 families showed differences in various morphological traits like plant height, heading date, awns, shoot colour, spike morphology, waxiness, sterility and chromosome number and structure as compared to parental morphology. They observed the degree of somaclonal variation to be genotype dependent. They suggest early variants as well as dwarfs to be promising for practical breeding purposes. In 3 of the somaclonal families the chromosome number ranged from 41 to 45, while some plants also contained telocentrics. Maddock and Semple (1986) observed phenotypic variation in R2 and R3 generations for plant height, loss of awned habit, presence of scurs in the non-awned cultivars and sterility or reduced fertility. The plants that were shorter and darker green in one line RH48 were associated with aneuploidy. Quantitative trait variation was studied in the progeny of regenerated winter wheat by Chen et al. (1987). They observed significant among family variation for heading date, plant height, kernel weight, tiller number, seed yield and fertility. Significant variation within families was also observed for all dependent variables except plant height and kernel weight. Although much of the variation was of negative value, some of the lines were seen to exceed the control for most of the variables examined. Cytological studies indicated 65% of the total population to be cytologically normal. Some of the variants were associated with cytological abnormalities like aneuploids, univalents, chromosome breakage etc. Significant quantitative trait variation was also observed by Ryan et al. (1987) for all characters measured -- plant height, grain number

per spike, kernel weight, yield, total dry weight and harvest index. Variation for both greater than and less than parental controls was observed. Significant variation for the traits was observed for plants derived from both individual as well as more than one embryo. The significant improvements were not maintained in the lines selected for high yield and harvest index. However significant variation was displayed for many of the quality characters like kernel weight, hardness, protein content and a reduction in yellow pigmentation which are all simply inherited. Although the SC4 somaclonal population derived from winter wheat calli showed less frost resistance than control (Galiba and Sutka, 1989), one family possessed significantly higher frost resistance at -13°C with respect to percent survival; 22 of 31 families showed less regenerating capacity; 5 proved to be significantly better. One family was observed to be significantly highly frost resistant by both tests. Earlier Lazar et al. (1988) reported non-significant variation for freezing tolerance in the R2 and R3 generations of tissue cultured winter wheat, although, greater freezing tolerance was observed in the regenerating families.

Bhaskaran et al. (1987) observed variation for plant height, tiller number and seed size in the SC_2 generation of *Sorghum bicolor* plants regenerated from callus derived from seedling explants. While one of the somaclones did not maintain the original differences, the other two showed reduced plant height in both SC_2 and SC_3 generations.

Heinz and Mee (1971) report a greater morphological variation in plants regenerated from the chromosomal mosaic clone H50 7209 (34.8%) as compared to the stable clone H37-1933 (12%) in sugarcane. They noted greater relative frequency of differences for auricle length, presence or absence of hair, sheath colour, diameter and waxiness, and also for plant tops with curving tips or erect blades.

In maize, Zehr et al. (1987) observed qualitative variation in selfed progeny of plants regenerated from immature embryos. They are lethal chlorophyll deficiencies, necrotic leaf tips, ragged leaves, dwarfs, white cob, indeterminate growth habit, brown leaf midribs, wrinkled leaves and albino seedling. They also report quantitative variation for plant height, days to pollen shed and silk emergence relative to pollen shed, decreased ear length, increased kernel rows per ear, and decreased or increased seed weight. McCoy and Phillips (1982) reported defective kernel mutants in the R2 generation for eight R1 families that were previously normal in appearance. Lee et al. (1988) report lower grain yield and moisture in tissue culture derived lines as compared to control lines, although the highest yielding line per se in 3 of 6 trials and top ranking grain moisture lines in 5 of 6 trials were tissue culture derived.

In *Triticale* Jordan and Larter (1985) observed considerable variability in plants regenerated from 15 day old embryos. Variability was greater for spike length (26% shorter than control), fertility and plant height. They also observed the second generation plants to have a significant increase in percent kernel protein relative to its control. They also report that although the electrophoretic pattern for prolamin was the same, the intensity of bands varied.

In rice, Ling et al. (1987) reported 3 of 157 regenerated plants from somatic cell culture to be male sterile. They infer that the male sterile cytoplasm was induced from normal fertile cytoplasm by tissue culture. Nowick et al. (1988) report 9 of the R2 lines sampled to have 75% sterile pollen. One line was observed to segregate for dwarf plants which were also male sterile. Some tall segregating lines with reduced pollen staining were also observed. Anaphase bridges were seen in the pollen mother cells at low percentage. Browning of the leaf was observed but they did not report any association between bridge formation and morphological variations. Sun et al. (1983) observed quantitative trait variation frequencies to

range from 11.5 to 39.5% and a tendency for plant height to become shorter, number of production tillers to increases and grain size to decrease.

In sorghum, Bhaskaran et al. (1987) studied the SC2 generation and observed 3 clones to show significant height reduction and 6 clones to have higher total plant matter production. Seven of the clones had significant increases in number of secondary tillers while all eight had significant decreases in days to flowering. Three clones had significant increase in grain yield and seed number; however all clones had smaller seeds. They also report that in subsequent studies of one somaclone on plant height, tiller number, total shoot weight and days to flower, none of the original differences were maintained; two other somaclones maintained the increased tiller number; one somaclone showed reduced plant height in both SC2 and SC3 generations.

Powell et al. (1984) reported the agronomic performance of 74 spontaneously doubled microspore derived lines (M-lines) of *Hordeum vulgare* cv Sabarlis. Significant differences were observed over the seed derived lines (S-lines) in days to awn emergence, plant height, neck length, number of fertile tillers, number of grains on the main tillers, grain yield on the main tiller and 1000 grain weight. The M-lines tended to be late to awn emergence and taller with larger neck and fewer tillers than S-lines.

Rajasekaran et al. (1986) characterized plants regenerated from embryogenic callus cultures of hybrid triploid napier grass (*Pennisetum americanum* × *P. purpureum*). The field tested tissue culture plants showed significantly greater total biomass yield than the vegetatively propagated plants although no significant differences were seen in the subsequent year. Tissue culture plants had more tillers. 96% of the tissue culture plants were morphologically stable. Twenty three phenotypic variants in a population of 524 tissue culture plants were

observed with most being dwarfs and late flowering. Two of the morphological variants were hexaploids and fertile.

Groose and Bingham (1984, 1986) studied for shifts in traits like anthocyanin pigmentation, multifoliate leaves, ability to regenerate and cytoplasmic male sterility as well as vigor, morphology and ploidy in tetraploid alfalfa regenerated from callus and suspension cultures. Twenty one percent of 116 regenerated plants were considered to be variant for one or more characters. They also reported a white flowered mutant in tissue cultured tetraploid alfalfa with purple flower. The functional allele C2 of a locus required for anthocyanin pigmentation was in a simplex condition C2c2c2c2 in the donor genotype which mutated to an unstable recessive ('mutable') allele c2-m4. Nagarajan and Walton (1987, 1989) reported the polyploids to be more stemmy with long internodes and 'gigas' characteristic for leaf size and thickness of stem. They also reported that the somaclonal population of alfalfa gave dry matter yields about 29% lower than the synthetic population from which they were derived. Similar lower values were also observed for plant height, stem length, number of nodes per stem and internode length. Johnson et al. (1984) observed some alfalfa protoclones regenerated from non-mutagenized mesophyll protoplasts to be more susceptible to winter damage than their parents. They also observed various other phenotypes like deeply serrated leaflets, irregular leaflet margins, narrow or wide leaflets, shortened internodes, stunting, thickened stems and self sterility. Protoclones significantly superior, equal and inferior (P=0.05) in dry weight forage yields were obtained with the latter two classes predominating. One RS-K2 protoclone appeared to exhibit resistance to *Fusarium*. Reisch and Bingham (1981) reported variation for herbage yield.

In *Glycine max* (soybean), Barwale and Widholm (1987, 1990) observed lethal sectorial albinos in the initial regenerants from immature embryos. The selfed generation showed twin seeds,

multiple shoots, dwarfs, abnormal leaf morphology and leaflet number, wrinkled leaves, chlorophyll deficiency and partial and complete sterility. In their later study they report both qualitative and quantitative trait variation in soybean plants regenerated from immature embryos. 40% of the first selfed generation (R1) and 10 of the 16 third selfed generation families (R3) segregated for sterility. 15.6% of the 2nd selfed generation (R2) of a regenerated plant were completely sterile. 32% of R3 generation segregated for wrinkled leaf type; 14% were partially sterile; R2 and R3 generations of one family showed multiple branching; and one of three plants in R3 was a dwarf. Twin seeds, abnormal leaf morphology and leaflet number have been observed as random events. Frequency of qualitative variation per initial regenerant (R0) plant ranged from 0.05 to 1.00. Regenerated plants also showed tolerance to *Phialophora gregata* culture filtrate. Quantitative variation was reported for both early and late maturity. In few somaclones oil content was seen to increase by ca. 25%. Four families showed protein levels 5% greater than respective controls and three families with 3 to 5% lower than control.

Niizeki et al. (1990) suggest that quantitative variation observed in *Lotus corniculatus* could be caused by polygenic changes and also by minor structural alterations in somatic chromosomes. Variances of the traits such as plant height, length of internode, stem diameter, leaflet length and width, dry matter yield and pollen fertility in the protoclonal population were smaller than those of the seed produced population. Mean values of all the traits of protoclonal population shifted to lower values. However plants with less hydrocyanic acid than the initial plant were also obtained.

Mathews et al. (1986) showed 14% of R1 progeny of mung bean to segregate for chlorophyll deficiency and morphological mutations.

In soybean, Greybosch et al. (1987) observed decreased yield in 2 of 19 regenerated families. One family also was taller while 2 showed putative sterility.

In tomato, Buiatti et al. (1985) showed that 17.04% of the progenies of plants regenerated from cotyledons exhibited chlorophyll mutations and morphological abnormalities which segregated in a 3:1 ratio.

2.1.4 GENOTYPIC VARIATION FOR QUALITATIVE TRAITS

The most distinctive feature of somaclonal variation is the occurrence of homozygous mutations in the initial regenerants. Evans and Sharp (1983) recovered 13 single gene mutations (two of which are fruit colour and jointless pedicel mutants) from 230 regenerated tomato plants without any selection pressure. George and Rao (1983) found a yellow seeded mutant in *Brassica juncea*. Dwarf plants in rice (Oono, 1985 and Sun et al., 1983), potato leaf mutant (Gavazzi et al., 1987), and a vigorous growing lettuce seedling (Engler and Grogan, 1984) were also recovered. In wheat, a full awned mutant (Larkin et al., 1984), white grained mutant (Larkin, 1985), and a gliadin mutant (Maddock et al., 1985) were also reported. In mung bean Bhatia and Mathews (1988) reported the recovery of monogenically inherited recessive as well as dominant mutation from tissue cultured plants. The dull seed surface was inherited as monogenic dominant and green cotyledon trait as monogenic recessive. The two true breeding somaclonal variants crossed to their respective phenotypically identical radiation induced mutants did not segregate indicating allelism. Bingham and McCoy (1986) also reviewed qualitative and quantitative variation in alfalfa which included monogenically recessive variants like abnormal flowers, simple leaves and rudimentary flowers. In wheat, Larkin et al. (1984) reported both quantitative and qualitative trait variation. Heritable variation was observed for plant height, presence and absence of awns, tiller number, heading date,

waxiness, glume and grain colour, gliadin proteins and α-amylase regulation. They observed genetic changes from dominance to recessive (awns, grain colour) and from recessive to dominance or co-dominance (glume colour, gliadins). Sun et al. (1983) recovered a dwarf mutant from somatic cell derived plants of rice which was controlled by a single recessive gene. Powell et al. (1986) studied the microspore derived plants of *Hordeum vulgare* after hybridization with *H. bulbosum*. They observed significant departures from the expected 1:1 ratio for 3 of the 5 genes studied-- rachilla hair length, DDT susceptibility, plant height, c-hordein polymorphism and mildew resistance. Shepard et al. (1980) reported a high frequency of variation for several horticultural and disease resistance characters in potato. Stable changes in tuber shape, yield, maturity date, photoperiod requirements for flowering and in plant morphology were noted. The authors suggest that point mutation is a potential mechanism in the context of the culture used ('Russet Burbank') which is truly simplex at key genetic loci especially those of a regulatory nature.

2.1.5 CULTURE INDUCED VS MUTAGEN INDUCED VARIATIONS

Van den Bulk et al. (1990) studied somaclonal variation in tomato comparing tissue culture and methane sulfonic acid ethylester (EMS) induced variation. They report for the mutant types anthocyanin-free, hairless hypocotyl and abscisic acid (ABA) deficiency, an estimated frequency per locus per diploid cell as 0.69×10^{-3} for material obtained from EMS treatment and 0.12×10^{-3} for tissue culture derived material. Studies relating to comparison of frequencies between somaclonal and EMS induced variations were also reported by Gavazzi et al. (1987) in tomato. They recovered the mutants -- seedling lethality, male sterility, resistance to *Verticillium*, short stature, change in number of lateral shoots and leaf shape. In vitro regeneration was quantitatively more efficient and abundant in production of mutants than EMS. Mutant frequencies of 2.9×10^{-2} (male sterility), 11.8×10^{-2} (early ripening), 4.3×10^{-2}

(short stature), and 18.6×10^{-2} (potato leaf character) were observed in regenerated plants while frequencies of only 0.6×10^{-2} for male sterility and 1.7×10^{-2} for early ripening was observed from EMS treatments. From inheritance studies male sterility and short stature appeared to behave as single gene recessive mutants; potato leaf character appeared to be in a homozygous state; and resistance was controlled by a single dominant gene.

Novak et al. (1988) compared gamma ray induced and tissue culture induced mutations in plants derived from zygotic embryos. They observed a greater frequency of variation in tissue culture induced than in irradiated or irradiated followed by in vitro regeneration treatments. Irradiation followed by in vitro regeneration produced chlorophyll variations of 2.07% at 5 Gray units (Gy) and 2.23% at 10 Gy, tissue culture 1.94%, and irradiation 0.22% at 5 Gy and 0.24% at 10 Gy. 14.25% and 9.87% of the plants regenerated in vitro after exposure to 5 Gy and 10 Gy respectively showed morphological variations. Irradiated material gave 3.43% at 5 Gy and 2.77% at 10 Gy, and tissue culture alone gave 5.39% of morphological variants. Early flowering variants were observed at 0.42% and 0.07% frequency when subjected to 5 and 10 Gy of irradiation; 0.5% in tissue cultured plants; 2.89% and 4.14% in regenerated plants after exposure to 5 and 10 Gy respectively. Negruitiu et al., (1984) extensively reviewed mutagenesis of protoplasts and the selection of biochemical mutants.

All reports agree that somaclonal variation has produced an order of magnitude greater numbers of variants than EMS or irradiation, although the two kinds of treatments may or may not generate the same spectrum of mutations.

2.2 MOLECULAR STUDIES OF SOMACLONAL VARIANTS

The use of molecular tools like restriction fragment length polymorphism (RFLP) and random amplification of polymorphic DNA (RAPD) in combination with proper genetic strategies to detect and separate desired from undesired variation will enhance the usefulness of somaclonal variants as gene donors for crop improvement. Whilst karyological aberrations resulting from tissue culture process have been well documented, the more cryptic gene alterations like loss of DNA, rearrangements or transpositions are more difficult to analyze although they alter gene function in some instances. However, the revolution in molecular biology that began in the 1970s has led to greater interest in efforts to unravel the primary reasons for genetic instability of higher plant cells cultured in vitro. It has been observed that much of the phenotypic variation amongst regenerated plants is due to a whole spectrum of genetic changes from chromosome variation to point mutation and sequence amplification. Research conducted in the past decade has accumulated evidence for molecular changes associated with tissue culture.

2.2.1 NUCLEAR DNA VARIATIONS

De Paepe et al. (1982) showed that nuclear DNA of doubled haploids of *Nicotiana sylvestris* resulting from consecutive androgenetic cycles contained on the average an increasing amount of total DNA and an increasing proportion of highly repeated sequences. In an attempt to relate changes in DNA content with reduced agronomic importance, Dhillon et al. (1983) found that nuclear DNA content showed an average 17% reduction in leaf yield per cycle of tobacco anther culture. Amplification of DNA was also reported by Reed and Wernsman (1989) in tobacco cultures.

Landsmann and Uhrig (1985) using a random potato 25S rDNA probe, found a 70% reduction in 25S rRNA genes in 2 of 12 *Solanum tuberosum* plants regenerated from protoplasts. They were regarded as mutants deficient in ribosomal RNA-genes.

The *Triticum aestivum* nuclear rDNA repeat unit has been shown to be heterogeneous in length, due to the non-transcribed spacer region (Gerlach and Bedbrook, 1979 and Appels and Dvorak, 1982). Rode et al., (1987), also observed the rDNA containing the non-transcribed spacer region to display a length polymorphism in gametoclones of wheat. Davies et al. (1986) observed 17 of 551 regenerated wheat plants to have altered ADH1 zymogram. Progeny with altered zymograms in 13 of the regenerants were aneuploid. The remaining 4 regenerants gave rise to euploid progeny with altered ADH1 zymograms. They interpreted three of the somaclonal mutants to possess a 4Aα isochromosome, a 3BS/4Aα translocation and a 7BS/4Aα translocation respectively. Breiman et al. (1987a) analyzed progenies of plants regenerated from scutellar callus of bread wheat (*Triticum aestivum* L.) for the organization of the intergenic spacer of rRNA genes located at the sites of nucleolar organizer regions (*Nor* loci). When the DNA was digested with the restriction endonuclease TaqI and probed with a specific rDNA fragment by southern blot-hybridization, three progeny plants of cultivar 'ND7532' showed reduction in the number of rDNA spacers. The diversity was expressed in qualitative and/or quantitative changes in TaqI fragmented spacer lengths. The regenerated plants had a missing or reduced amounts of the 1.9 kbp TaqI fragment. Another plant showed ratio of 2.7 and 3.0 kbp TaqI fragments different from that of control. One plant showed the loss of the 2.7 kbp TaqI fragment. The variation in the length of the rDNA repeats is probably due to a reduction in the number of 135 bp repeats because the latter repeats occupy most of the DNA sequences between these sites (Appels and Dovarak, 1982). One other plant digested with EcoRI-BamHI showed a loss of the 4.4 kbp EcoRI-BamHI fragment. On screening the progeny of 149 plants regenerated from immature wheat embryos, Ryan and Scowcroft (1987) found

one regenerant which was heterozygous for a variant pattern characterized by the presence of at least 5 new isozyme (β-amylase) bands as well as an increased intensity in existing bands in two more positions and appears to be under the control of a single dominant or co-dominant gene. No phenotypic variation was observed in this variant suggesting that the genetic effect was confined to a small length of chromatin. Progeny of 22 regenerants segregated for variant pattern displaying loss of isozyme bands which is a characteristic of monosomy for chromosome 4A or 4D. Cooper et al. (1986) reported 32 of 5586 selfed progeny of callus regenerated winter wheat to differ agronomically for one or more traits. Four of thirty two varied for gliadin pattern from control of which three were fixed for the presence of mutant protein of 50 relative mobility units (RMU) and the corresponding loss of a parental protein of 26 RMU. The other line segregated for presence/absence of band 50 and loss/retention of band 26. The authors believe that this indicates that either band 50 was coded for by a mutant gene allelic to the gene coded for band 26 or that bands 26 and 50 were coded for by two different structural alleles under the control of a common regulatory locus.

Breiman et al. (1987b), also studied the organization of the rDNA intergenic spacer, the hordein SDS-PAGE pattern, the genomic organization of hordein genes and the mitochondrial DNA arrangement as markers for nuclear and mitochondrial heritable somaclonal variation in *Hordeum spontaneum* plants regenerated from immature embryo derived calli. They observed a loss of 1.8 and 2.5 kb TaqI intergenic rDNA spacer fragments in the TaqI and EcoRI-BamHI hybridization pattern of the intergenic rDNA spacer with P^{32} radiolabelled pHV294. Another plant showed a variant pattern after digestion with EcoRI-BamHI where a 3.2 kb fragment disappeared and a shorter fragment of 3.0 kb was detected. The variation in length is believed to be due to variation in number of 115 bp repeats because these repeats occupy most of the DNA between the TaqI sites. One variant was observed to vary in number and intensity of bands migrating in the B and C hordein regions.

Brettell et al. (1986) analyzed regenerated *Triticale* plants for changes in rRNA genes located at the site of nucleolar organizer regions (the *Nor* loci) in chromosome 1B, 6B and 1R and found reduction in the rDNA units. The reduction in the 1R rDNA spacer sequences was heritable and correlated with reduced c-banding at the position of *Nor*-R1 on chromosome 1R. Lapitan et al. (1988) also reported amplification of DNA in *Triticale*. Both amplification and deamplification of DNA sequences among callus cultures of maize were reported by Brown et al. (1991).

In sugarcane Heinz and Mee (1971) detected metabolic differences which might not affect morphological characteristics on observing the isozyme patterns of esterase, peroxidase, amylase and transaminase. 75% of the 53% of regenerants which showed morphological variations revealed isozyme differences.

In alfalfa Baertlein and McDaniel (1987) observed 9.1% of plants regenerated from callus to be significantly different from the parent and from the other somaclones as judged from the quantitative protein pattern. Somaclones were grouped according to linkage based on protein electrophoresis pattern. Two showed non-overlap with parental grouping indicating significant molecular divergence.

2.2.2 MITOCHONDRIAL DNA VARIATION

Many authors have investigated mitochondrial DNA (mt DNA) variability in tissue cultured plants. Umbeck and Gengenbach (1983) observed the absence of a 6.6 kb mitochondrial DNA fragment in fertile variant lines regenerated from a culture of a Texas male sterile cytoplasm maize. This 6.6 kb fragment is believed to be involved in the male-sterile toxin-susceptible phenotype. McNay et al., (1984) observed minor changes in the mt DNA of maize

when hybridization pattern of regenerated plant and 4 year old suspension cultures were compared by membrane hybridization with *nick*-translated plasmid-like mt DNA probes S1 and S2 obtained from cytoplasmic male sterile maize. Similarly no major molecular alteration in restriction fragments were generated by the endonucleases Bam HI and Hind III. The variation observed was mainly in the stoichiometry of several restriction fragments. They suggest that minor variations in restriction pattern could reflect alterations in frequency of circular mt DNA molecules, perhaps related to nuclear alterations occurring during the extended period of culture. Gengenbach et al. (1981) regenerated *Helminthosporium maydis* resistant maize plants which distinguished them from each other and from the T and N cytoplasm maize. The regenerated maize plants had a distinct mitochondrial DNA organization.

In sorghum cell culture protoclones Kane et al. (1992) observed hypervariability in the mitochondrial DNA. Loss, amplification and also a new set of fragments were observed.

Considerable variation in restriction pattern of mt DNA of rice cell cultures was observed by Chowdhury et al. (1988, 1990). Rearrangements involving repeat sequences and loss or duplication of genomic regions were reported. Saleh et al. (1990) observed substantial changes in the relative abundance of specific DNA sequences in long term suspension cultures of rice. Structural rearrangements in mt DNA of regenerated wheat plants were also observed (Hartman et al., 1989). Ozias-Akins et al. (1984) reported quantitative differences in restriction patterns of pearl millet mt DNA of cell suspension cultures.

Graybum and Bendich (1987) also observed variable abundance of mt DNA fragments in cultured tobacco cells. In *Brassica campestris* Shirzadegan et al. (1989, 1991) detected both stoichiometric changes and the appearance of novel restriction fragments in 2 year old cell suspension cultures and also in a group of protoclones.

2.2.3 CHLOROPLAST DNA VARIATION

Whilst there are many examples of nuclear and mitochondrial DNA variations resulting from tissue culture, there are very few examples of corresponding changes in the chloroplast DNA. Day and Ellis (1984) found upto 80% deletion in the major chloroplast DNA molecules in wheat albino plants regenerated from pollen. Sun et al. (1979) observed albino plants derived from anther cultures of rice to contain little or no 23S and 16S RNA and highly reduced levels of fraction 1 protein.

2.2.4 VARIABILITY IN DNA METHYLATION

The role of DNA methylation in controlling gene expression has been extensively reviewed by Doerfler (1983). Brown and Lörz (1986) observed gross methylation changes in R2 generation of maize plants on digestion with the isoschizomer restriction endonucleases HpaII and MspI. They also observed tissue culture induced changes in gene methylation in both normal as well as variant plants. Brown (1989) observed the changes to persist through several generations of selfing. Phillips et al. (1992) observed a reduction in methylation in 34% of the R1 families studied.

In soybean Quemada et al. (1987) hydrolyzed the 5S gene (CCGG sequences) by both HpaII and MspI. They presumed that DNA tissue cultured plants lacked methylation (CCGG) which is found in the intact plants (CmeCGG). They propose that tissue culture conditions prevent methylation of these sites resulting in susceptibility to HpaII or that a small proportion of cells within the intact plant lack these methyl groups and these cells are selected during cell culture. They also observe that upon prolonged culture some cells again became resistant to HpaII suggesting remethylation of internal cytosine of the CCGG tetranucleotide.

2.3 TISSUE CULTURE AND REGENERATION STUDIES

The regulation of morphogenesis in vitro is largely dependent on three factors: explant choice, medium composition and the control of the physical environment.

Murashige (1974) identified the explant source, age, size and physiological status as factors to be considered in explant selection. George and Sherington (1984) recognized genotype, explant orientation, pretreatment and inoculation density as determinants of organogenic response of a given species. However, genotype is one of the most influential factors in determining the morphogenic response. A continuum from no to high response is observed in different genotypes of the same crop species (Ozias-Akins et al. 1992). Matheson et al., (1990) reported the selection of eleven highly regenerative alfalfa genotypes with frequencies of 4.5% for the cultivar Algonquin and 11.7% for Apica. Two genotypes (Al 93 and Ap 20) were observed to exhibit prolific embryogenesis from hypocotyl and petiole callus and also from suspension cultures. In *Trifolium*, Phillips and Collins (1979a) observed a frequency of 30 to 80 % of genotypes cultured to regenerate from meristem-derived calli. Different red clover cultivars regenerated either on picloram (PIC) or 2,4-D (Redman) or only on PIC (Tensas) or 2,4-D (Redman). Another cultivar regenerated on medium containing 2,4-D and other hormones. Besides they also observed that Atlaswede, Arlington and Redman were highly regenerative, while Kenstar did not regenerate well and Tensas regenerated only to a small extent. Bhojwani et al. (1984) highlight the importance of screening of a large number plants within a cultivar of outbreeding species to achieve reproducible plant regeneration from tissue cultures. Within the 3 species of *Trifolium* studied, they observed considerable differences between individual plants; some plants were completely non-responsive while other showed profuse differentiation of shoots. In soybean, Parrott et al. (1989) observed a large effect of the

genotype on the ability of immature soybean cotyledons to undergo auxin stimulated somatic embryogenesis. Among the 33 lines studied, good regeneration ability was evident in those with one or both of the highly regenerable lines ('Manchu' and 'A.K.Harrow') in their pedigree. The F1 hybrid cotyledons obtained from a cross between 'Manchu' and 'Shiro' (a poorly regenerating line) showed intermediate regenerative capacity. Barwale et al. (1986) induced multiple shoots in vitro from 155 *Glycine max* and 13 *Glycine soja* genotypes of maturity groups '000' to 'VII' when cultured on B5 medium supplemented 1 or 5 μM BA. Genotype and hormone concentration specific responses were observed with number of shoots ranging from 1 to 12 for different genotypes. All these case studies show regeneration to be genotype specific.

2.3.1 TISSUE CULTURE MEDIA

The requirements of plant tissues grown in vitro are similar in general to those of intact plants growing in nature. But the isolated tissues and organs lack the capacity to synthesize their own supply of carbohydrates, most vitamins and plant growth substances for whole plant regeneration. A major effort in developing a plant tissue culture medium was made by Murashige and Skoog (1962) which is the now universally used MS medium. It was initially used to optimize the growth of tobacco pith callus in vitro. While Murashige and Skoog (1962) determined the mineral nutrient requirements, Linsmaer and Skoog (1965) in their LS medium examined the organic growth requirements and found that only thiamine is important. Later media (B5) developed by Gamborg et al. (1968) had substantially higher levels of potassium salts and lower levels of ammonium. Chu (1978) developed the N6 medium for cereal anther culture. The L2 medium of Phillips and Collins (1979) contains the major salts formulation similar to that of MS, but with a reduction in the concentration of ammonium (NH_4^+) and increased phosphate (PO_4^{3-}), potassium (K^+), magnesium (Mg^{2+}), and calcium (Ca^{2+}). The organic

formulation has increased concentrations of thiamine and myoinositol. The inorganic salt compositions of different tissue culture media employed in the present study is listed in Table 1.

The physical form of the cultures i.e., semi-solid or suspensions, the pH, humidity, light and also the type, size and seals of culture vessels play an important role in morphogenic responses (Narayanaswamy, 1977). Moss et al., (1992) reported significant influence of light and temperature on plant regeneration.

2.3.2 REGENERATION STUDIES

A large number of plant species have been regenerated from cell, tissue, and organ cultures. Plant regeneration in vitro has been established for many forage legumes. In spite of the rapid progress in tissue culture systems in some plant species, grain legumes were considered recalcitrant to regeneration in vitro (Mroginski and Kartha, 1985) and many have remained even so until today. Flick et al. (1983) listed more than twenty legume species that have been successfully regenerated although in most cases the regeneration was sporadic and transient due sometimes to recalcitrance problems. Later, tissue culture and regeneration in legumes has been comprehensively reviewed by Parrott et al. (1992) in 'Biotechnology and Crop Improvement in Asia' (ed: J.P. Moss), who observe that despite the widely reported in vitro recalcitrance of legumes, at least 75 species from 25 different genera have been regenerated de novo although the contributions to crop improvement have been limited. They conclude that although some species of legumes are amenable to regeneration by both organogenesis and embryogenesis, some genera and species contain genotypes capable of regeneration via one mode or the other; that regeneration is genotype specific; and that there is a need for further media optimizations for each species. Whole plant regeneration in some legumes is listed in Table 2.

Table 1

Inorganic salt composition of different plant tissue culture media used in the present study.

Ingredients	Concentration in mg L^{-1}		
	L2[a]	B5[b]	MS[c]
Macronutrients			
NH_4NO_3	1000.00	-	1650.00
KNO_3	2100.00	2500.00	1900.00
KH_2PO_4	325.00	-	170.00
$MgSO_4.7H_2O$	435.00	250.00	370.00
$NaH_2PO_4.H_2O$	85.00	150.00	-
$(NH_4)_2SO_4$	-	134.00	-
$CaCl_2.2H_2O$	600.00	150.00	440.00
Micronutrients			
H_3BO_3	5.000	3.000	6.200
$MnSO_4.4H_2O$	15.000	10.000	15.600
$ZnSO_4.7H_2O$	5.000	2.000	8.600
$Na_2MoO_4.2H_2O$	0.400	0.250	0.250
$CuSO_4.5H_2O$	0.100	0.025	0.025
$CoCl_2.6H_2O$	0.100	0.025	0.025
KI	1.000	0.750	0.830
$FeSO_4.7H_2O$ (EDTA)	25.000	-	27.800
Na_2EDTA	-	-	37.300
EDTA Ferric salt	-	43.000	-
Vitamins			
Thiamine.HCl	2.00	10.00	0.50
Pyridoxine.HCl	0.50	1.00	0.50
Nicotinic acid	-	1.00	0.05
Myo-Inositol	250.00	100.00	100.00
Sucrose (g)	25.00	20.00	30.00
pH	5.6	5.5	5.8

a Phillips and Collins (1979)
b Gamborg et al. (1968)
c Murashige and Skoog (1962)

Table 2

A comprehensive list of whole plant regeneration in some important legume species

Species	Type of regeneration	Explant	Reference
Forage and Tree Legumes:			
Lotus corniculatus	Org.	LP, Ca, cc,	Ahuja et al. 1983, Nilzeki and Saito 1986, Orshinsky et al. 1983, Damiani et al. 1985, Swanson et al. 1980
	Emb.	L	Arcioni and Mariotti 1982
Medicago sps.	Org.	A, IN, H, IMO, ST, S, P, L, C, R, Hy, LP, O	Saunders and Bingham 1972, Kao and Michayluk 1981, Walker et al. 1978 & 1979, Santos et al. 1980, Brown and Atanassov 1985, Lupotto 1983 & 1986, Nagarajan et al. 1986, Bingham et al. 1975, Mariotti et al. 1984, Kuchuk et al. 1990, Bianchi et al. 1988, Skotnicki 1986
	Emb.	L, LP, R, H, C, O, P, S, ST	Denchev 1991, Arcioni et al. 1982, Gilmour et al. 1987, Nagarajan and Walton 1987, Hartman et al. 1984, Reisch and Bingham 1980, Yu et al. 1990, Bianchi et al. 1988, Meijer and Brown 1987a, Senaratna et al. 1989, Kao and Michayluk 1981, Latunde-Dada and Lucas 1988, and Johnson et al. 1981
Trifolium sps	Org.	Sd, Sh, HCa, A, H, C, ImC	Phillips and Collins 1979, Oleck and Schieder 1983, Campbell and Tomes 1984, White 1984, Parrott 1991, Barakat 1990, Webb et al. 1987b, Beach and Smith 1979
	Emb.	H, C, H, R, P, A, Ca, SC, ZE	Bhojwani et al. 1984, Yamada and Higuchi 1990, Pederson 1986, Keyes et al. 1980, Collins and Phillips 1982, Maheswaran and Williams 1986b, Phillips and Collins 1980, Bhojwani et al. 1984, McGee et al. 1989
Stylosanthes sps	Org.	H, L, R, LP, C, L	Meijer and Broughton 1981, Meijer and Steinbiss 1983, Godwin et al. 1987, Mroginski and Kartha 1981b, Szabados and Roca, 1986, Manners and Way
1989,			Manners 1988
Tree Legumes:			
Albizia sps	Org.	H, R	Gharyal and Maheshwari 1981 & 1990, Tomar and Gupta 1988
	Emb.	H	Gharyal and Maheshwari 1981, Tomar and Gupta 1988
Acacia sps	Org.		Jones et al. 1990
Prosopis sps	Org.		Nandwani and Ramawat 1992

continued...

Species	Type of regeneration	Explant	Reference
Grain Legumes:			
Arachis sps	Org.	C, CN, L, M, Sd, EA, Ca, Ep, E, A, ZE	Atreya et al. 1984, Bhatia et al. 1985, McClentiy et al. 1985 & 1991, Moss et al. 1992, Diamon and Mii 1991, Mroginski et al. 1981, Pittman et al. 1983, Shyluk et al. 1981, Bajaj et al. 1981, Nalini et al. 1992, Narasimhulu and Reddy 1983 & 1985, Rugman and Cocking 1985
	Emb.	ImZE, ImC, EA, Sd, L, C, Lf, A, CN, S, ZE	Hazra et al. 1989, Ozias-Akins 1989 & 1992, Sellars et al. 1990, Gill and Saxena 1992, Chengalrayan et al. 1994
Cicer sps	Org.	ShCa, CN, ImLf, ST	Sharma et al. 1979, Kahn and Ghosh 1984, Barna and Wakhlu 1994, Shella et al. 1992
	Emb.	L, ST, C	Rao and Chopra 1989, Kumar et al. 1994, Prakash et al. 1992, Sagare et al. 1993
Glycine sps	Org.	CN, L, C, CP, P, H, HP, R	Cheng et al. 1980, Barwale et al. 1986, Wright et al. 1986, Hammat et al. 1987a, b & 1989, Meyers et al. 1989, Newell and Luu 1985, Kameya and Widholm 1981, Hymowitz et al. 1986
	Emb.	ImE, ZE, C, H, IN, O, ImC, Sd	Ferriera et al. 1990, Lazzeri et al. 1985 & 1988, Barwale et al. 1986, Komatsuda and Ohyama 1988, Ranch et al. 1985, Hammat and Davey 1987, Finer and Nagasava 1988, Hammat et al. 1986, Li et al. 1985, Christianson et al. 1983, Ghazi et al. 1986, Parrott et al. 1985 & 1989, Hartweck et al. 1988, Shoemaker and Hammond 1988
Phaseolus sps	Org.	L, LCa, SA, H, ImC, Sd, CN	Crocomo et al. 1976, Kumar et al. 1988b, Martin and Sondhal 1984, Sreedhar and Mehta 1984, Allavena and Rossitti 1986, Angelini and Allavena 1989, Genga and Allavena 1991, Franklin et al 1991, McClean and Grafton 1989, Kartha et al. 1981
	Emb.	L	Kumar et al. 1988b
Pisum sps	Org.	Ca, ImLf, CN, Lf, L	Malmberg 1979, Hussey and Gun 1984, Ezhova et al. 1985a, Mroginski and Kartha 1981, Rubulo et al. 1984, Jackson and Hobbs 1990, Nielsen et al. 1991

continued...

Species	Type of regeneration	Explant	Reference
	Emb.	ImE, LP, ST, ZE, SA, M	Natali and Cavallini 1987, Lehminger-Mertens Jacobson 1989, Kysely et al. 1987, Griga et al. 1986, Kysely and Jacobson 1990
Vicia sps	Org.	ImC, M	Griga et al. 1987, Busse 1986, Galzy and Hamoui 1981, Schulze et al. 1985
	Emb.	LCa, ZE, ImE, ST	Albrecht and Kohlenbach 1989, Taha and Francis 1990, Pickardt and Schieder 1987, Pickardt et al. 1989
Vigna sps	Org.	ST, C, CN, P	Mathews 1987, Gulati and Jaiwal 1990 & 1992, Krishnamurthy et al. 1984
	Emb.	LCa	Kumar et al. 1988

1. Org.=Organogenesis; Emb.=Embryogenesis;

2. A=anther; C=cotyledon; Ca=callus; cc=cell culture; CN=cotyledon node; E=epicotyl; EA=embryo axis; H=hypocotyl; HCa=hypocotyl callus;IN=internode; ImC=immature cotyledon; ImL=immature leaf; ImO=immature ovary; L=leaf; Lf=leaflet; M=meristem; O=ovary; P=petiole; R=root; S=Stem; Sd=seedling; Sh=shoot; ST=shoot tip; ZE=zygotic embryo

Cajanus cajan:

In pigeonpea, Shama Rao and Narayanaswamy (1975), obtained callus from hypocotyl explants derived from seeds exposed to 5 or 10 Kilorad (KR) gamma irradiation. Organogenesis was induced in fifty percent of the callus initiated on MS medium supplemented with 2,4-D and kinetin. Multiple shoot formation was achieved from heart shaped or differentiating immature embryos cultured on MS medium fortified with 2,4-D and casein hydrolysate while globular to early heart shaped embryos developed directly into plantlets (Gosal and Bajaj, 1979). Callus proliferation from anther cultures was also obtained by these authors. Mehta and Mohan Ram (1981), reported regeneration from cotyledonary node cultured on Gamborg's B5 medium supplemented with N6-Benzylaminopurine (BA) and 2% sucrose. However presence of phenolics was a major problem. Seeds cultured on the same medium also formed shoot buds along the margins of cotyledons which were elongated and rooted. However, when cotyledons from soaked seeds were separately explanted very few shoots were obtained. Kumar et al. (1983, 1984), reported adventitious shoot formation from shoot apices, epicotyl and seedling cotyledons explanted on Blaydes medium with BA alone or in combination with α-Naphthaleneacetic acid (NAA), and with KT and Indole-3-acetic acid (IAA). Shoot bud formation was also achieved from leaf and cotyledon callus raised on Blaydes medium supplemented with BA or BA, NAA and gibberellic acid (GA3). Kumar et al. (1984) observe that cotyledons are the best explants for regeneration. Nodal halves developed more shoots when compared to distal halves on MS medium with 2,4-D and BA. Kumar et al. (1985) obtained direct plantlet recovery from immature embryos cultured on MS or B5 fortified with 2,4-D although, B5 was found to be superior. In an effort to develop haploids, Subrahmanyam et al. (1988) obtained callus from anthers cultured on MS with 2,4-D which failed to differentiate on various combination of media. Baldev and Gleba (1991) observed multiple shoot formation from basal parts of embryos and adjacent parts of

cotyledons. Reena et al. (1992) regenerated plants from mature and seedling cotyledons cultured on MS plus BA and NAA. Nodular green compact calli developed on cotyledons formed large numbers of shoot buds on transfer to lower phytohormone concentrations and elongation of shoots was achieved on addition of GA3. Eapen and George (1993) regenerated shoot buds from leaves cultured on MS supplemented with BA and IAA. George and Eapen (1994) obtained organogenesis from mature cotyledon, primary leaves and roots. Also multiple shoots produced from cotyledonary node of whole seeds cultured on BA enriched medium were rooted and transferred to soil. However, somatic embryos developed from immature cotyledons and embryonal axes could not be converted into whole plants. Prakash et al. (1994) obtained plant regeneration via multiple shoot formation from cotyledonary node of seedlings germinated on MS supplemented with BA. Continued shoot initial formation in separated cotyledons with multiple shoots was achieved on topical application of IAA. Shoot initiation was also observed after cutting off surface layers to completely remove pre-existing shoot initials of cotyledonary node. Progress was also made in isolation of protoplasts from pigeonpea leaves. Shohet and Richard (1987) found primary leaves from 9-11 day seedling to be most suitable for protoplast isolation. Ramana Rao et al. (1992) obtained high yields of protoplasts from mesophyll cells of both wild and cultivated pigeonpea leaves. They also observed callus and embryoid formation when these protoplasts were plated on modified B5 medium. In an effort to mechanically transmit the sterility mosaic virus, Nene and Sheila (1992) induced callus in the presence of 2,4-D from sterility mosaic infected leaves, but the presence of the pathogen in the callus was not ascertained.

2.3.3 IRRADIATION STUDIES ASSOCIATED WITH TISSUE CULTURE

Reports on in vitro exposure to physical mutagens like gamma irradiation are limited. Hutabarat (1986), and Douglas (1986) observed increased regeneration frequencies relative

to controls with the use of gamma rays. However, the regenerated plants were normal morphologically. Heil et al., (1978), reported enhanced bud formation in gamma irradiated tissues of tobacco.

From the account of the accumulated literature as described above on tissue culture, the associated somaclonal variation and its characterization, demonstrates the potential and promise and the use of in vitro techniques for plant regeneration and selection of useful variants for subsequent incorporation into breeding programmes.

3. TISSUE CULTURE AND REGENERATION IN PIGEONPEA

3.1 MATERIALS AND METHODS

3.1.1 PLANT MATERIAL

Seed of *Cajanus cajan* (L.) Millsp. cv ICPL 87 used in this investigation was obtained from the seed stock maintained by the Pigeonpea Breeding Unit of ICRISAT. This is a high yielding, short duration, short stature and widely adapted genotype with fusarium wilt resistance. It was released by ICRISAT in 1986 as Pragati in the Indian peninsula. It was developed by pedigree selection from a cross ICPX 73052 (T 21 x JA 277) (ICRISAT Plant Material Description No. 42 (1993) ICRISAT).

3.1.2 TISSUE CULTURE TECHNIQUES

A *Explant sterilization and preparation*

Mature cotyledon and zygotic embryo explants were derived from seed of *Cajanus cajan* (L.) Millsp. cv ICPL 87 which was surface sterilized by treatment with 0.1% (w/v) mercuric chloride for 6 minutes. The traces of the mercuric chloride were removed by six serial washes with sterile distilled water. Following this treatment, seed was soaked in sterile distilled for 4 hours. The seed coat from good imbibed seed was removed aseptically and the two cotyledons separated. The zygotic embryo was detached and each cotyledon was prepared for culturing by totally cutting away the proximal region at the point of attachment of embryo axis.

The 6 or 7-day old seedlings were derived from surface sterilized and imbibed seed germinated on filter paper bridges in sterile 25 x 150 mm borosil test tubes containing 10 mL of hormone-free liquid L2 medium (Phillips and Collins, 1979) with 1% (w/v) sucrose. Kap-uts (Bellco) were used as closures for the tubes. The 6 or 7 day seedling cotyledons were explanted under sterile conditions for experiments on regeneration. The cotyledons along with the cotyledonary node encompassing a small portion of both the epicotyl and hypocotyl were detached from the seedling. The two cotyledons were separated by cutting through the epicotyl-cotyledonary node-hypocotyl. The axillary bud, surrounding meristematic regions and the epicotyl-hypocotyl halves were meticulously removed by cutting at an angle under the axillary bud using a fine scalpel blade (No. 11 Sigma Chemical Co.). This method ensured total removal of the axillary bud to preclude its proliferation. The seedling cotyledons thus prepared were used as start material in regeneration experiments.

Immature cotyledon explants obtained from green pods at different maturity levels were first rinsed thoroughly under tap water followed by a wash with sterile distilled water. An alcohol rinse for 60 seconds was then followed by the same sterilization treatment as described above for seeds, but, with 10 minutes exposure to mercuric chloride. The pods were opened aseptically along the anterior suture and green, immature and filling stage seeds with cotyledon measuring 2, 4, or 6 mm across were identified. A longitudinal incision of the testa along the side away from the hilum was made through which the cotyledons were pushed out on to the petri dish by gentle application of pressure at the hilum. The immature zygotic embryo axis was detached and the cotyledons alone were cultured.

Younger pods with developing ovules were identified for immature zygotic embryo explants. The excised embryos were grouped by the developmental stages: globular, heart to torpedo and cotyledonary stages. An incision along the length of the side opposite the hilum was made and the endosperm exposed by holding down the testa with forceps. The different stages of embryos were dissected from the ovules using a needle.

B *Cultural conditions*

All tissue culture procedures were carried out in a laminar flow cabinet. MS (Murashige and Skoog, 1962) media contained macro- and micro-nutrients, phytohormones, 3% (w/v) sucrose with the pH adjusted to 5.8. L2 (Phillips and Collins, 1979) media contained L2 salts, 2.5% (w/v) sucrose with pH adjusted to 5.6. All media were gelled with 0.7% Difco-Bacto agar and autoclaved for 20 min at 121°C and 15 psi before dispensing into sterile plastic petri dishes or glass test tubes. In the test tubes media was then allowed to solidify as slants. The test tubes were sealed with non-absorbant cotton wrapped in one layer of cheese cloth unless otherwise mentioned. The cotyledon explants were oriented with the abaxial side in contact with the

medium. The zygotic embryos and the stem explants cut longitudinally were cultured with the cut surface in contact with the medium. The cultures were incubated at 24 ± 2°C under a 16 h photoperiod and a light intensity of 80 μE $m^{-2}s^{-1}$. Observations were recorded every 2-3 weeks.

C *Somatic embryogenesis*

Somatic embryogenesis from immature zygotic embryos

The globular, heart to torpedo and cotyledonary stage zygotic embryos were cultured on MS semi-solid medium. Four regeneration media were used supplemented with two different concentrations of 27 or 54 μM α-Napthaleneacetic acid (NAA) or 23 or 45 μM 2,4-Dichlorophenoxyacetic Acid (2,4-D) individually. All explants were cultured in 100 x 15 mm sterile disposable polystyrene petri dishes (Fisher Scientific, Co.) containing 25 mL medium. The plates were sealed with parafilm. Two replicates of fifteen explants each were used.

Somatic embryogenesis from mature zygotic embryos

Each zygotic embryo was cut into four pieces, first longitudinally followed by a transverse cut. The explants were cultured in 12 x 150 mm borosil test tubes with 9 mL L2 medium. Two different concentrations of 2,4-D (27 and 54 μM) and NAA (23 and 45 μM) were tested individually. Three replicates with 12 explants each were used.

Somatic embryogenesis from mature and immature cotyledon

The de-embryonated and mature cotyledons from imbibed seeds and the 2, 4 or 6 mm long immature cotyledons were prepared for explanting as described above. The explants were

cultured on MS semi-solid medium supplemented with p-Phenoxyacetic acid (p-CPA) and 6-Benzylaminopurine (BA), either alone or in combinations. Twenty four different treatment combinations were used. The concentrations of p-CPA tested were 3, 5, 21, 54, 107 and 214 µM and that of BA were 0, 4, 9 and 17 µM. The explants were cultured in 12 x 150 mm borosil test tubes containing 9 mL of medium. The globular structures developing on the cotyledons were separated and inoculated into MS liquid medium containing 0.17 µM BA and 0.3 µM p-CPA at two different sucrose levels of 2% and 3% (w/v) for further development.

D *Organogenesis*

Organogenesis from mature cotyledon

The proximal region containing the rudimentary embryo axis was removed from the mature imbibed cotyledon and the rest of the cotyledon cultured on MS medium with 21.18 µM BA and 5.37 µM NAA (phytohormone combination of Reena et al., 1992). The regenerating cotyledons were transferred to MS media with one-tenth concentration of phytohormones for shoot bud proliferation. Gibberellic Acid (GA_3) at 0.3 or 3.0 µM was incorporated for shoot elongation.

Organogenesis from seedling cotyledons

Seedling cotyledonary explants were obtained from 6-day old seedlings as already described. Initial experiments using L2 medium supplemented with various concentrations of auxins and cytokinins either alone or in combinations indicated that BA alone was the best phytohormone for whole plant regeneration. The effect of different salt formulations -- L2, MS and B5 (Gamborg et al., 1968) -- for high frequency shoot formation was then determined using the

best BA concentration of 8.9 µM. Adventitious shoot buds were induced from 6-day old seedlings in 100 × 15 mm sterile disposable petri dishes containing 25 mL medium. These shoot buds were then subcultured at 3 wk interval on maintenance medium in 12 × 150 mm borosil test tubes containing 9 ml of L2 medium with 0.44 µM BA for proliferation. The cotyledonary explants bearing shoots greater than or equal to 1 cm long were transferred to test tubes containing shoot elongation medium which comprised of L2 salts, 0.44 µM BA, 2% sucrose and 1% activated charcoal.

Gamma irradiation experiments were initiated using 7-day old seedling cotyledons, prepared for culture as before, but pre-cultured for three days on regeneration medium i.e., L2 salts plus 8.9 µM BA. Such cotyledons were exposed to a dose range of 0.5, 1.0, 2.0, 4.0, 8.0 and 12 kilo Rads (KR) γ-irradiation at a dose rate of 2.87 KR min^{-1} from a ^{60}Co unit at ICRISAT. After irradiation, the explants were immediately transferred to fresh regeneration medium to overcome the unknown effects of γ-irradiated medium on cotyledon regeneration. The regeneration protocol was similar to that used for organogenesis from untreated seedling cotyledons.

Organogenesis from seedling stems

Stems from six-day old seedlings were cut 5 mm in length and halved longitudinally. Explants were cultured with the cut surface in contact with the medium. Sixteen different media with BA and NAA either alone or in combinations were tested. BA was tested at 0, 5, 10 and 20 and NAA at 0, 0.1, 1.0 and 2.0 µM.

E *Induction of rooting*

In vitro grown seedlings were used in separate experiments to discover the best medium to be used for rooting in vitro regenerated shoots. To determine this, besides L2 medium fortified with 10.74 µM NAA and 4.44 µM BA, five different treatments-- (1) L2 hormone-free (basal) medium, (2) ½ strength basal at pH 5.0, (3) ¼ strength basal at pH 5.0, (4) ½ strength basal at pH 5.8, and (5) ¼ strength basal at pH 5.8 were tested.

F *Acclimatization*

The regenerated shoots were rooted on hormone-free L2 medium. The rooted shoots were washed under tap water to remove traces of agar. The roots were treated with the fungicide Thiram® and plants potted in 7 x 7 x 7.5 cm size plastic pots containing sterile sand. The plants were covered by a polythene bag to maintain high relative humidity. The plants were then hardened at 90% humidity in an incubator maintained at 16/8 h light/dark regime and 24°C. The plants were regularly nourished with Broughton's nutrient solution. Three weeks later the plants were transferred to larger pots containing 3 sterile soil : 1 farmyard manure : 1 sand in the greenhouse where they flowered and seed was harvested on maturity.

G *Histological studies*

The study material was the responding mature cotyledons with globular structures. The cotyledons were fixed by immersion in formalin acetic alcohol (FAA) (2 formalin (37-40%) : 1 glacial acetic acid : 10 alcohol) for 24 h. Following dehydration in a xylene series, the material was infiltrated and embedded in paraplast (Oxford Labware) in an incubator maintained at 40°C. Microtome sections were cut at 8 µm thick and the ribbons were

attached to glass slides using Haupt's adhesive. The slides were rinsed with xylene to remove paraffin and then stained sequentially in 1% saffranin O in 50% ethanol for 4 h and 0.5% fast green dissolved in 95% ethanol for 30 seconds. Excess stain was removed in clove oil and sections were mounted in DePeX.

3.2 RESULTS

3.2.1. SOMATIC EMBRYOGENESIS

Three weeks after culture all the globular and heart to torpedo stage (immature) zygotic embryos cultured on same concentrations of both 2,4-D (23 or 45 µM) and NAA (27 or 54 µM) and the cotyledonary stage cultured on 45 µM 2,4-D did not show any morphogenic response but gradually necrosed. On an average 4 explants (26.7%) of cotyledonary stage cultured on 54 µM NAA gave rise to embryogenic callus. In the next two weeks one of these four cotyledonary explants (6.7%) completely callused and globular somatic embryos appeared on the surface (FIG 1: for convenience the figure shows hormonal concentration in mg L^{-1}; PLATE 1A). A single globular somatic embryo developed into the next torpedo stage (PLATE 1B). Further development of this somatic embryo was abnormal with the cotyledons remaining fused. On its transfer along with the surrounding callus to MS hormone-free medium, the region distal to the fused cotyledons produced two roots (PLATE 1C). However, further development into a normal plant appeared to be arrested.

In the next experiment with mature zygotic embryos cultured on the same concentrations of either 2,4-D (23 or 54 µM) or NAA (27 or 54 µM), all the explants produced callus within a week. This callus resembled embryogenic callus as the cells had dense cytoplasm with large nuclei and were rich in starch when seen after acetocarmine staining. Different stages of pro-embryoids of single cell origin were observed on medium containing 23 µM 2,4-D or 54 µM NAA (PLATE 2A-D). However after 4 wk on the same medium the callus lost its embryogenic capacity and began to necrose. Similar observations were recorded when transferred to hormone-free medium.

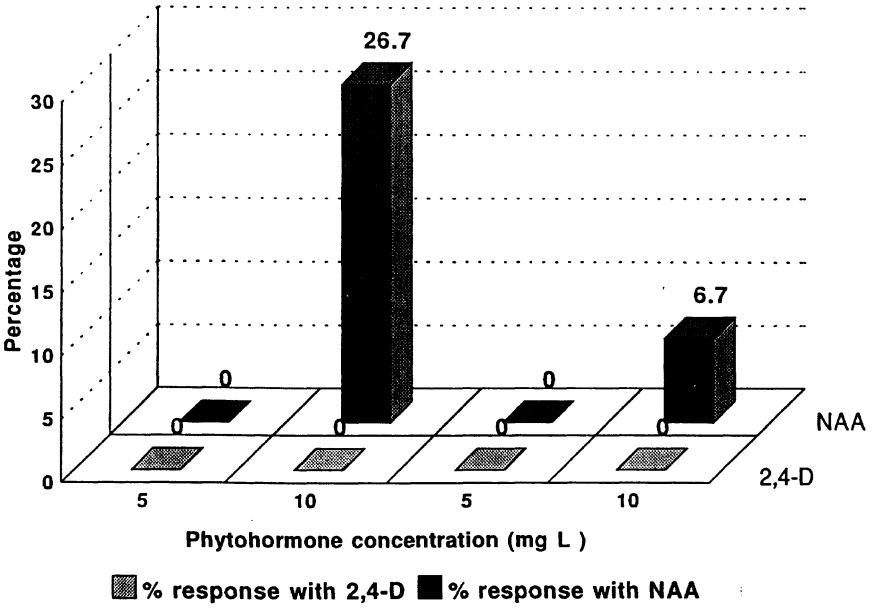


Fig 1. Influence of NAA and 2,4-D on somatic embryo formation from immature zygotic embryo of pigeonpea; First two values on x-axis are for induction of somatic embryos and the later two are for embryo maturation from the responding induction medium; Number of embryos cultured = 15

PLATE 1

1A. Callused cotyledonary stage immature zygotic embryo of *C. cajan* cv ICPL 87 cultured on MS medium supplemented with 54 µM NAA showing globular and torpedo stage somatic embryos.

1B. Abnormal somatic embryo with fused cotyledons on 54 µM NAA.

1C. Precocious germination of the abnormal somatic embryo on hormone-free medium.

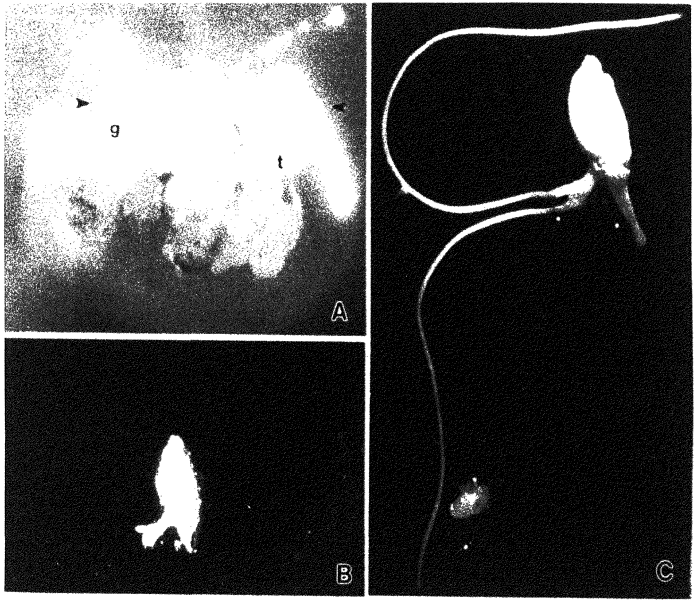


PLATE 1

PLATE 2

2A. Callus squash of mature zygotic embryos of *C. cajan* cv ICPL 87 cultured on MS medium plus 54 µM NAA exhibiting actively dividing embryogenic cells.

2B. 7-8 celled proembryoid in callus of mature zygotic embryo cultured on 54 µM NAA.

2C 8-10 celled proembryoid on same medium.

2D. 16-20 celled proembryoid on same medium.

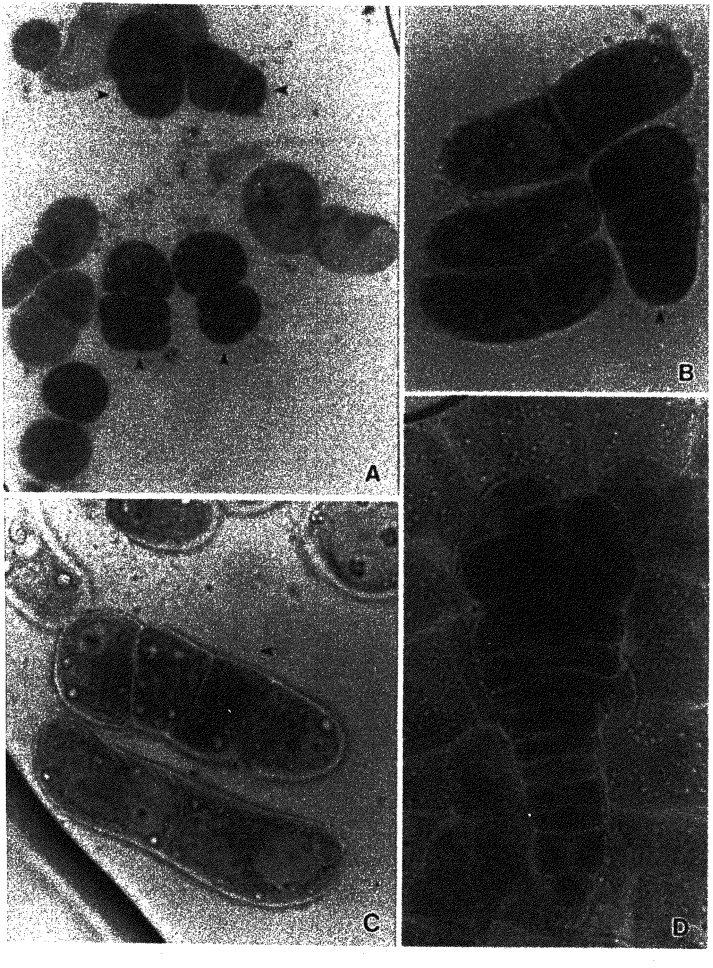


PLATE 2

The mature and immature cotyledons cultured on 24 different combinations of BA and p-CPA showed globular outgrowths on the adaxial surface. Mature cotyledons on phytohormone combinations of 17 µM BA with 3, 21 or 214 µM pCPA and 4 or 9 µM BA with 21 µM pCPA produced globular structures in higher frequencies both in number of cotyledons responding (33 to 67%), as well as the area covered with these structures (TABLE 3; PLATE 3A). The phytohormone combinations of 4 or 9 µM BA with 3, 107 or 214 µM pCPA also gave frequencies ranging from 33-67 %. However, the area covered by these globular structures was lesser. The cotyledons were devoid of these structures in the absence of BA. On transfer of the globular structures to liquid media with lowered phytohormones of 1.67 µM BA plus 0.54 µM pCPA and two different sucrose levels of 4 or 6%, they de-differentiated into callus and showed no further development. Histological observations of these cotyledons (PLATE 3B), showed these structures to have an outer epidermal layer and an independent development of vasculature.

3.2.2 ORGANOGENESIS

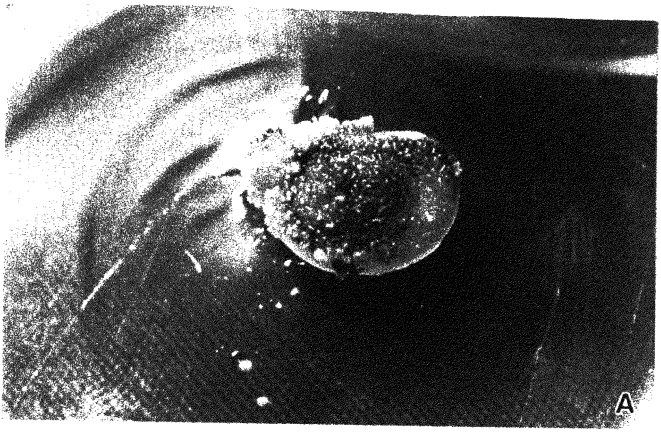
In the initial experiments, mature cotyledons with embryo axis excised were cultured on MS medium supplemented with 21.18 µM BA and 5.37 µM NAA since these concentrations were known to produce morphogenic response (Reena et al., 1992). Green nodular outgrowths were produced on the abaxial surface in contact with the medium (PLATE 4A). On transfer to 2.22 µM BA and 0.54 µM NAA, shoot buds developed from the nodular outgrowths (PLATE 4B). Attempts to elongate these shoots by addition of GA, at 0.3 or 3.0 µM were not successful.

Seedling cotyledons 1, 3, 5 and 7 days old were screened for morphogenic potential and 7-day old seedling cotyledon was selected as the best explant for a reproducible and high

PLATE 3

3A. Mature imbibed cotyledon of *C. cajan* cv ICPL 87 cultured on MS medium plus 17 µM BA and 3 µM p-CPA producing globular somatic embryos.

3B. Histological section of mature cotyledon (from 3A) with globular somatic embryos having distinct epidermal layer and independent vasculature.



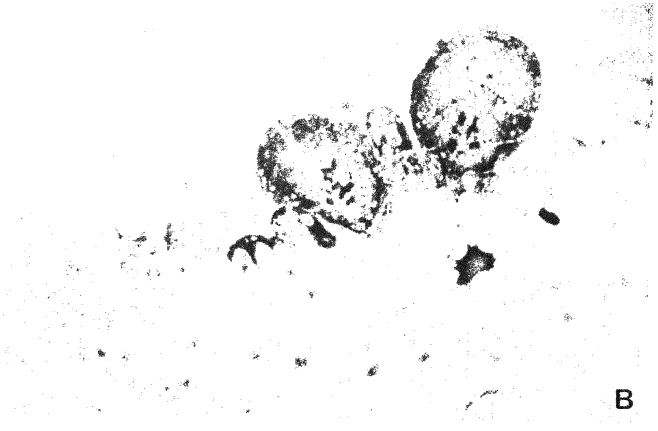


PLATE 3

PLATE 4

4A. Mature cotyledon of *C. cajan* cv ICPL 87 cultured on MS medium supplemented 21.18 µM BA and 5.37 µM NAA producing green nodular outgrowths.

4B. Mature cotyledon of *C. cajan* cv ICPL 87 cultured on 2.22 µM BA and 0.54 µM NAA showing proliferation of shoot buds from the green nodular outgrowths seen in 4A.

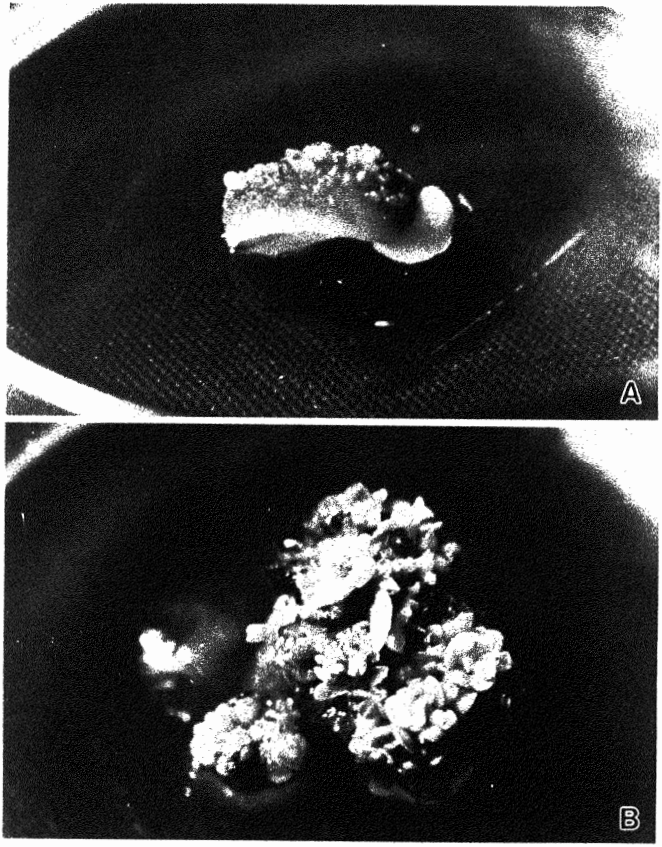


PLATE 4

TABLE 3

Effect of different hormonal combinations on somatic embryogenesis in mature cotyledons of *Cajanus cajan* (L.) Millsp. cv ICPL 87

BA (µM)	0	4	9	17
pCPA (µM)		Response (%)		
3	0	33.0	58.3	66.7
5	0	0.0	0.0	33.0
21	0	45.0	33.0	33.0
54	0	8.3	16.7	0.0
107	0	58.3	45.0	8.3
214	0	66.7	33.0	50.0

Number of explants per treatment = 12
Culture period = 21 days
Medium used was Murashige and Skoog (1962)

TABLE 4

Frequency of shoot regeneration from 6-day old seedling cotyledons of *Cajanus cajan* (L.) Millsp. cv ICPL 87 cultured on different basal media supplemented with 8.9 µM BA

Media	No. cotyledons cultured	No. cotyledons producing shoots	% response
L2	24	24	100
MS	24	19	79
B5	24	14	58

Observations were recorded 3 weeks after culture initiation; L2= Phillips and Collins (1979); MS= Murashige and Skoog (1962); B5= Gamborg et al. (1968).

frequency regeneration. L2 medium supplemented with 8.9 µM BA gave 100% regeneration while 79% regenerated shoot buds on MS medium plus 8.9 µM BA and only 58% of cotyledons cultured on B5 medium with 8.9 µM BA formed shoot buds after 3 wk in culture (TABLE 4). One week after culture initiation all cotyledons on L2 medium produced nodular protuberances at the proximal region. Shoot buds were observed on these protuberances in the next two weeks (PLATE 5A). To optimize whole plant regeneration, these cultures were transferred to maintenance medium with 0.44 µM BA. Extensive proliferation of shoot buds was observed which required subculturing on to the same medium at every 3 wk interval. Elongation of these shoot buds was observed on their transfer to L2 medium supplemented with 0.44 µM BA, 2% sucrose and 1% activated charcoal (PLATE 5B). After 6 weeks in culture 75% of cultures produced 1 to 3 shoots of 2 to 4 cm in height and after 9 weeks 100% of cotyledons cultured had formed shoots (FIG 2). This protocol allowed the harvest of 1 to 3 shoots from each culture at each sub-culture. Thus a total of 10 to 15 shoots were harvested from an initial culture after 6 to 9 weeks. At any given time 2-4 shoots were formed, suppressing other shoots from elongation. Additional shoots elongated on frequent removal of these longer shoots.

Various concentrations of BA and NAA were used to induce shoots on longitudinally cut stem segments obtained from 6 day seedlings (TABLE 5). In all the treatments tested 30 to 100% of cultures gave rise to callus while 20 to 70% gave rise to nodular outgrowths. No morphogenic response was observed. The 10 µM BA and 0.1 µM NAA combination however produced green nodular outgrowths at the periphery of cut surface in contact with the medium in 40% of the cultures. Shoot buds differentiated from these outgrowths which elongated on transfer to hormone-free medium. The shoots were excised and rooted on L2 hormone-free medium (PLATE 5C).

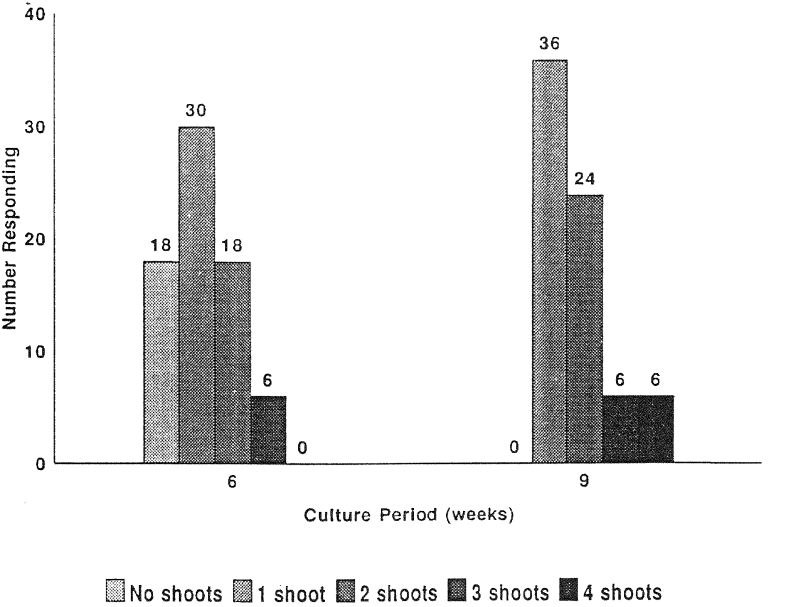


Fig 2. Frequency of 6-day old seedling cotyledons of pigeonpea producing elongated shoots when transferred to L2 medium supplemented with 0.44 µM BA, 1% activated charcoal and 2% sucrose. No. of cotyledons cultured = 72

PLATE 5

5A. Seven day seedling cotyledon of *C. cajan* cv ICPL 87 cultured on L2 medium supplemented with 8.9 µM producing adventitious shoots from nodular protuberances at proximal region after 3 wk in culture.

5B. Cotyledon cultures (from 5A) transferred to 0.4 µM BA, 1% activated charcoal and 2% sucrose showing elongating shoots at 8 to 9 weeks in culture.

5C. An elongated shoot rooted after 2 wk on L2 hormone-free medium.

5D. Acclimatization of a rooted plantlet.

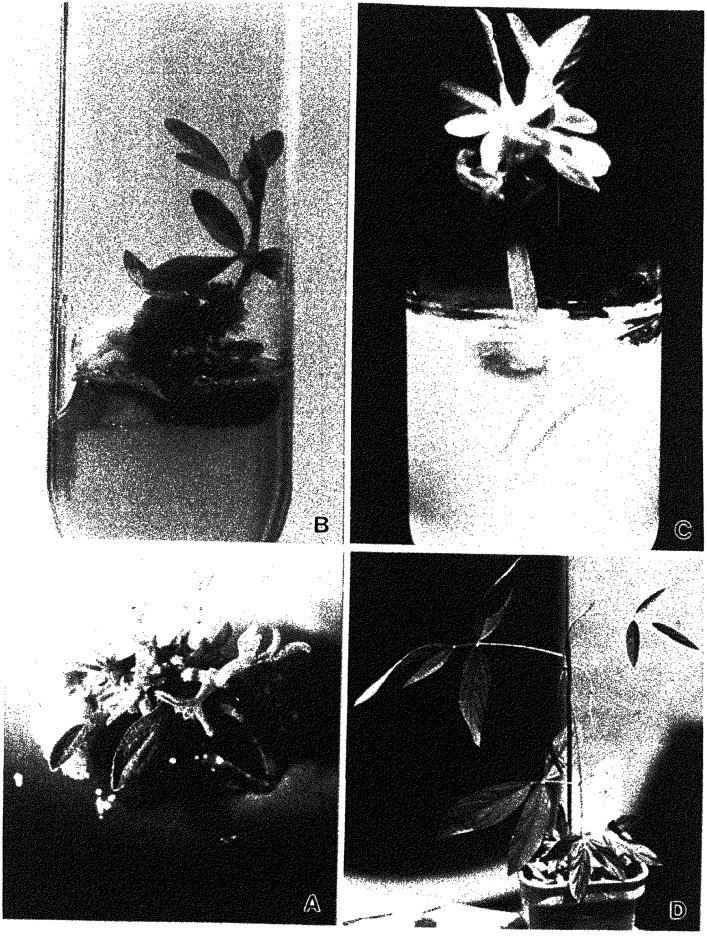


PLATE 5

TABLE 5

Influence of various concentrations of BAP and NAA on shoot morphogenesis in longitudinally cut seedling stem segments of *Cajanus cajan* (L.) Millsp cv ICPL 87

Concentration (μM)		% cultures giving rise to		
BAP	NAA	callus[1]	green nodular outgrowths	nodular outgrowths with shoot buds
0	0.0	100	0	0
0	0.1	100	0	0
0	1.0	100	0	0
0	2.0	100	0	0
5	0.0	30	70	0
5	0.1	80	20	0
5	1.0	100	0	0
5	2.0	100	0	0
10	0.0	70	30	0
10	0.1	60	0	40
10	1.0	80	20	0
10	2.0	80	20	0
20	0.0	80	20	0
20	0.1	50	50	0
20	1.0	100	0	0
20	2.0	100	0	0

[1] 20 explants were used per treatment.

Increasing the doses of γ-irradiation inhibited the regeneration of precultured seedling cotyledons (FIG 3). At 0.5 KR, 94% of cultures regenerated; 78% at 1.0 KR and 11% at 2.0 KR. Explants exposed to 4 KR and above did not show any morphogenesis as they began to necrose very early. In a subsequent experiment to detect the minimum lethal dose, γ-irradiation at 2.0, 2.5, 3.0, 3.5, and 4.0 KR was used. It was observed that while 38% of cultures regenerated at 2.5 KR, and 33% at 2.0 KR all cultures exposed to 3.0, 3.5 and 4.0 KR died. In later experiments plants were regenerated from explants exposed to 1 KR irradiation, the dosage at which 78% regeneration was observed. The regenerated (R1) plants however, showed no morphological variations.

3.2.3 ROOTING

Rooting medium was optimized by using in vitro shoots for the initial experiments. Shoots excised from in vitro grown seedlings were cultured on L2 basal medium fortified with 10.74 μM NAA and 4.44 μM BA, and on half-strength and quarter-strength hormone-free L2 medium with pH adjusted to 5.0 or 5.8. The frequency of root formation is summarized in FIG 4. Full strength hormone-free L2 medium was best with 100% response as to number of shoots forming roots and an average number of 3 roots per shoot. At quarter-strength and half-strength basal medium with pH adjusted to 5.0 only 50% of shoots produced roots with an average number of 1 root per shoot. All treatments adjusted to pH 5.8 showed 67-100% rooting with 1 to 3 roots per shoot. In the presence of hormones, roots tended to callus (result not shown in Table). The tissue culture generated shoots were rooted on the best rooting medium i.e., hormone-free L2 medium.

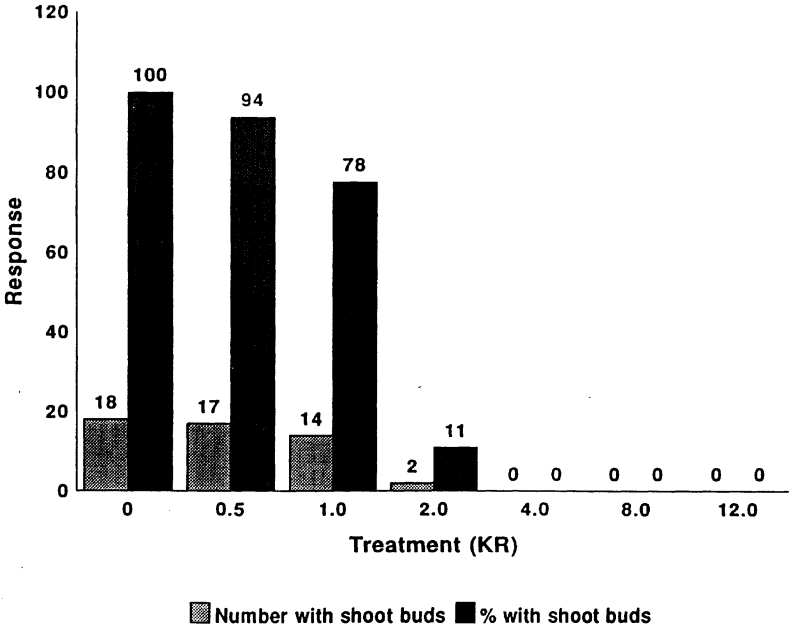


Fig 3. Regeneration frequency of 6-day old seedling cotyledons of pigeonpea cv ICPL 87 exposed to different doses of gamma-irradiation; Number of cotyledons cultured per treatment = 18

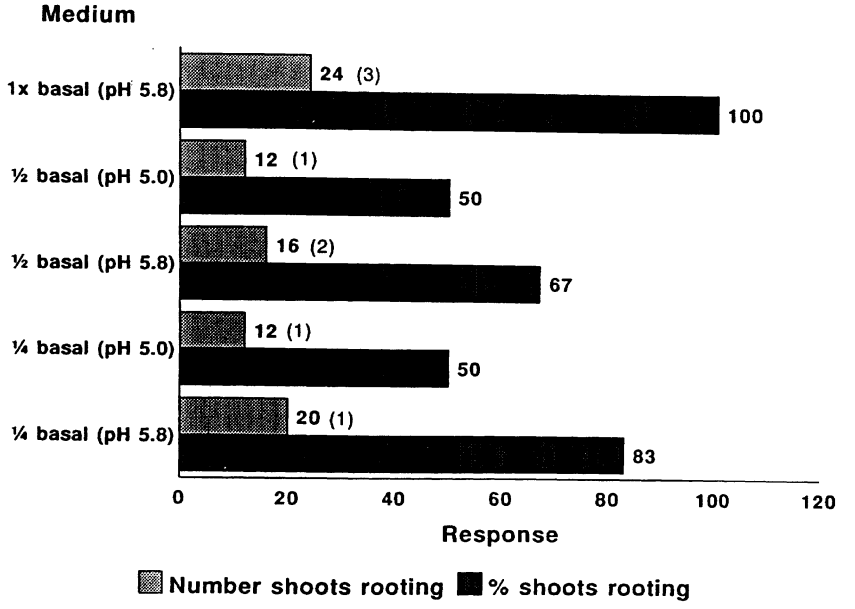


Fig 4. Frequency of root formation on shoots from in vitro grown seedlings of pigeonpea cv ICPL 87
Number of shoots per treatment = 24
Values in parenthesis are average number of roots per shoot

3.2.4 HISTOLOGY

Microtome sections of mature cotyledons with globular structures (PLATE 3B) showed that these structures were independent with a prominent outer epidermal layer. A rudimentary vasculature unconnected with the cotyledon's vasculature was observed.

3.3 DISCUSSION

3.3.1 SOMATIC EMBRYOGENESIS

Auxins

In the present study embryogenic determination of the cells was observed in all the explants--immature zygotic embryos, mature embryo axes and also immature and mature cotyledons. The first two explants displayed a better induction of embryogenesis in the presence of NAA rather than 2,4-D when the two auxins were compared at similar phytohormone concentrations of NAA 27 µM and 2,4-D 23 µM and also NAA 54 µM and 2,4-D 45 µM. The development of the single somatic embryo originating from the cotyledonary stage immature zygotic embryo into a normal plant was arrested. Therefore the requirement for a specific and sequential media for conversion of somatic embryos into a complete plant is evident. When mature zygotic embryo axes were cultured, embryogenic determination was observed in the presence of 23 µM 2,4-D or 54 µM NAA. This was conspicuous from the internal divisions of single cells having dense cytoplasm, prominent nuclei and thick cell walls. However the embryogenic potential was lost on prolonged culture on the same or on hormone-free medium. Frequent subculturing on to fresh medium was thus necessary to maintain the embryogenic potential which is an essential requirement for application of selection pressure besides maintenance of elite germplasm, for example cytoplasmic male sterile lines. The globular structures formed on the immature and mature cotyledons also appear to require definite media regulations for their further development. Somatic embryogenesis in pigeonpea was reported by George and Eapen (1994) using immature cotyledons and embryo axes. They observed immature cotyledons to produce highest frequencies of responding cultures (36.7%) and a greater mean number of somatic embryos (7.1) per responding culture when

picloram (5 mg L^{-1}) was added to L6 salts with B5 vitamins, 1% sorbitol. 0.1% activated charcoal and 6% sucrose. They also noted that when embryo axes were cultured in the presence of 2,4-D, they showed the highest frequency of responding cultures (90.9%) and a high mean number of embryos (4.7) as compared to PIC or NAA. When transferred to MS medium containing 0.1 mg L^{-1} zeatin, 1 mg L^{-1} GA_3, 0.25 mg L^{-1} ABA, 10 mg L^{-1} silver nitrate and mannitol, the cotyledons produced 5 normal mature somatic embryos of which one formed a small plant that was not capable of transfer to field.

Parrott et al. (1990) observe in their review that the auxin type, concentration and exposure time have proven important for somatic embryogenesis in most legumes. Lazzeri et al. (1987) reported somatic embryogenesis from immature soybean embryos where MS medium containing NAA (100-150 µM) improved the embryogenic capacity. Also, NAA-induced somatic embryos closely resembled zygotic embryos than did the 2,4-D-induced embryos. They also found ABA to be useful in altering the morphology of the 2,4-D induced embryos. Griga et al. (1987) cultured immature cotyledons of *Vicia faba* on L2 medium supplemented with 2,4-D and observed different embryogenic calluses from which globular to late torpedo stages were found when transferred to basal medium and higher (2.5%) sucrose levels. In peanut, Baker and Wetzstein (1994, 1995) using immature cotyledons found that auxin at high concentrations in the induction medium (MS plus B5 vitamins) promoted high induction levels but only 58% embryogenesis, while lower levels produced over 90% embryogenesis. 2,4-D in the induction medium resulted in a greater percent embryogenesis and mean number of embryos. The presence of converting embryo types with well defined axes and apices were rarely observed in cultures induced with NAA. On continual exposure to 2,4-D at 20 mg L^{-1} the immature cotyledons showed repetitive (41% to 46%) embryogenesis. Hazra et al. (1989) also reported 2,4-D to be superior to NAA for inducing somatic embryogenesis from embryonic axes of peanut. Chickpea was regenerated via somatic embryogenesis from mature embryo

axes when cultured on MS medium supplemented with (2,4,5-Trichloroacetic acid) 2,4,5-T (Suhasini et al., 1994).

Besides NAA or 2,4-D, several workers also reported somatic embryo induction in the presence of synthetic auxins like picloram. Eapen et al. (1993) studied the influence of growth regulators on somatic embryogenesis in immature embryo axes and cotyledons of peanut and reported 2,4-D as the best auxin to effect highest frequency of responding cultures and highest number of somatic embryos per responding culture as compared with dicamba, (picloram) PIC, NAA, 2,4,5-T and (6-Naphthoxyacetic acid) NOA. In chickpea, 2,4-D, Picloram and Dicamba were all superior to NAA for somatic embryogenesis from immature cotyledons (Eapen and George, 1994). Kysely and Jacobson (1990) cultured immature zygotic embryos of peanut in MS medium and observed higher frequencies of somatic embryogenesis in the presence of picloram or 2,4-D but not with NAA. Also in peanut Ozias-Akins (1989) observed the number of immature embryo cotyledons (50 to 60%) responding on B5 medium with 0.5 to 2.0 mg L^{-1} picloram was the same as with other auxins like NAA (5 to 30 mg L^{-1}) and 2,4-D (2 to 10 mg L^{-1}). However the quantity and quality of recognizable somatic embryos were much greater with picloram. Sellars et al. (1990) reported somatic embryogenesis in peanut to be most effective on L2 medium where 73 to 100 % shoot germination was observed. They also found 53% of cultured immature zygotic embryos to form adventitious somatic embryogenesis.

Cytokinins

In the present study the mature cotyledons produced globular structures on the adaxial surface which on histological studies proved to be globular somatic embryos. It appears that the cytokinin (BA) complement is essential for somatic embryogenesis where 4, 9 or 17 µM BA in combination with 3, 21, 107 or 214 µM pCPA gave a good response. In *Trifolium* Collins and

Phillips (1982) observe that somatic embryogenesis proceeded in the presence of low levels of 2,4-D (0.1 mg L^{-1}) and a weakly active cytokinin adenine (2.0 mg L^{-1}). Also in *Geranium*, Visser et al. (1992), showed that both auxins and cytokinins were obligatory for somatic embryogenesis.

Maheshwaran and Williams (1984) reported embryoid formation from immature zygotic embryos of *Trifolium repens* when cultured on a cytokinin (BA 0.1 to 2.0 mg L^{-1}) containing medium. In *Vigna radiata*, Patel et al. (1991) found BA (2.2 to 4.4 µM) and an incubation for 7 days in dark to be essential for formation of somatic embryoids. Malik and Saxena (1992) observed both shoot and somatic embryos when cultured on BA alone.

Gill and Saxena (1992) observed that while the application of BA in a MS medium induced organogenesis, thidiazuron (TDZ), a substituted phenylurea resulted in stimulation of somatic embryos from cotyledon and leaf explants of peanut. Kanyan et al. (1994) found higher concentrations of TDZ (0.5 to 30 mg L^{-1} to produce higher numbers of shoot primordia from various explants derived from seedlings obtained from zygotic embryos of peanut. Murthy et al. (1995) reported TDZ to induce somatic embryos in a range of concentrations between 0.5 to 10 µM. Young seedlings with cotyledons intact responded better to TDZ. They believed that TDZ induced somatic embryos in peanut by influencing the endogenous levels of both auxins and cytokinins.

There are very few instances where cytokinins are reported to promote somatic embryogenesis. This prompted Parrott et al. (1992) to maintain that auxin is all that is necessary. Thus depending on the explant type auxins alone or auxins plus cytokinins induced somatic embryogenesis.

Explant

In the present investigation the induction of somatic embryogenesis was observed in cotyledonary stage zygotic embryo, embryo axis, immature and mature cotyledons. Eapen and George (1994) were able to induce somatic embryoids from both embryo axis and cotyledons of pigeonpea. In peanut the explant age appears less significant (Sellars et al., 1989, Ozias-Akins 1989, Hazra et al., 1989, George and Eapen 1993). In soybean, Hammat and Davey (1986) cultured immature zygotic embryos at different developmental stages on medium consisting of B5 salts and vitamins, 2% (w/v) sucrose, 0.1 mg L^{-1} IBA, 10% (v/v) coconut milk and found heart stage embryos being most responsive. They also observed enhanced germination of mature embryoids on dessication. Ranch et al. (1985), Barwale et al. (1986), Lazzeri et al. (1987), Ozias-Akins (1989), Sellars et al. (1990), Gill and Saxena (1992) and Chengalrayan et al. (1994) concluded that immature zygotic embryo was a good source of somatic embryogenesis. Thus it is perceptible from literature that induction of somatic embryogenesis from most legumes has been achieved from immature embryos alone.

3.3.2 ORGANOGENESIS

Mature cotyledons cultured on MS medium supplemented with BA and NAA produced shoot buds from the nodular outgrowths, which, however could not be elongated even on addition of GA_3. The reasons for this are not clear as Reena et al. (1992) obtained shoot elongation with this protocol although the frequency was low. Gibberellic acid (GA_3) was efficient in shoot elongation in other species too. Addition of GA_3 to lowered BA concentrations enhanced shoot proliferation and elongation in *Camellia* (Offord et al., 1992). Diamon and Mii (1991) also elongated multiple shoots obtained from cotyledonary nodes of peanut on their transfer to B5 medium with zeatin and GA_3. More recently, Naidu et al., (1995) incorporated GA_3 into shoot

elongation medium for elongation of shoots derived from embryo axes and intact seeds of pigeonpea.

Basic medium

The most effective basal media for organogenesis and shoot formation in pigeonpea was L2, followed by MS and B5. In the present investigation polyphenols were produced excessively in MS and B5 media. However polyphenol production was relatively lower in L2 medium. Mehta and Mohan Ram (1980) using Gamborg's B5 medium and Kumar et al. (1984) using Blaydes medium also encountered this problem and attempts to alleviate it by the use of polyvinylpyrrolidone, an anti-oxidant, were not successful. Despite the recurring problem of polyphenols, several workers regenerated pigeonpea plants using different basal media. Shama and Narayanaswamy (1975), Gosal and Bajaj (1979), Kumar et al. (1984), Subrahmanyam et al. (1988), Reena et al. (1992), Eapen and George (1993, 1994) and Prakash et al. (1994) used MS medium in regenerating pigeonpea. Gamborg's B5 medium was used by Mehta and Mohan Ram (1980) and Kumar et al. (1985) while Blaydes medium was employed by Kumar et al. (1984).

Explant

In a few studies where seedlings were the source of explants like cotyledonary nodes, it was essential for the inclusion of BA in the germination medium as well. In pigeonpea, George and Eapen (1994) germinated seeds on MS medium containing BA at 15 mg L^{-1} while Prakash et al. (1994) used MS medium with 2 mg L^{-1} BA. Wright et al. (1986) and Hinchee et al. (1988) obtained cotyledonary nodes from seedlings grown on 5 µM BA. McClean and Grafton (1989) also used 5 µM BA in MS medium with B5 vitamins to germinate *Phaseolus vulgaris* seeds to

obtain cotyledonary node tissue of seedlings. Cotyledonary nodes derived from soybean seedlings grown on ½MS medium (Wright et al., 1984) or full-strength MS medium (Hinchee et al., 1988) supplemented with 5 µM BA proved effective in obtaining plant regeneration via organogenesis. Malik and Saxena (1992) used 5 µM BA in the germinating medium. Cheng et al. (1980) in soybean and Franklin et al. (1991) in *Phaseolus* also included BA in their regeneration medium. In the present investigation BA was not included in the germination medium. Similarly Jackson and Hobbs (1990) did not require BA as cotyledonary node explants responded well for organogenic system of regeneration from seedlings grown on MS basic medium.

Organogenesis in grain legumes has been achieved from a variety of explants like cotyledons, leaves, petioles, hypocotyls, stems, roots and anthers (see Table 2 in Review of Literature). In most grain legumes besides leaves, cotyledon explants have been consistently shown to have a high potential for adventitious shoot formation. Such results were observed in *Glycine max* from immature cotyledons (Chen et al., 1980; Saka et al., 1980; Lazzeri et al., 1985) and from cotyledon protoplasts (Barwale et al., 1986; Wei and Zu 1988; Wright et al., 1986; Hinchee et al., 1988; Dhir et al., 1991). In *Arachis hypogaea*, Atreya et al. (1984), Bhatia et al. (1985), McKently et al. (1990), Diamon and Mii (1991) and Cheng et al. (1992) also used cotyledon explants to regenerate plants. Mathews (1987), and Gulati and Jaiwal (1990, 1994) from *Vigna radiata*, Angellini and Allavena (1989), McClean and Grafton (1989) and Franklin et al. (1991) from *Phaseolus*, Selva et al. (1989) from *Vicia faba*, Chandra et al. (1993) with *Cicer arietenum* and Jackson and Hobbs (1990) from *Pisum* were the other workers who also obtained successful regeneration using cotyledons. As with other grain legumes in pigeonpea too cotyledon tissues are quite amenable to organogenic system of regeneration. Among the different tissues tested -- leaves, shoot tips, stem, hypocotyl, epicotyl, root, mature and seedling cotyledons -- seedling cotyledons were efficient for a high frequency regeneration.

Mehta and Mohan Ram (1980), Kumar et al. (1983), Kumar et al. (1984a, b), Reena et al. (1992), Eapen and George (1994) and Naidu et al. (1994) all reported regeneration from cotyledon explants. Prakash et al. (1994) regenerated pigeonpea plants from cotyledonary nodes explanted on MS plus 2 mg L^{-1} BA and a topical application of IAA.

Cytokinin

Parrott et al. (1990) observe that there has been a widespread use of BA as the cytokinin for induction of organogenesis in legumes with most studies using BA exclusively. In the present investigation too, use of BA alone was observed to induce complete plants. George and Eapen (1994) also observed in pigeonpea the beneficial use of BA and not kinetin, zeatin or 2,i-P for organogenesis from mature cotyledons. This was also true for regeneration of mung bean from shoot tips (Gulati and Jaiwal, 1992). Although multiple shoots were produced in 100% of cultures using BA, kinetin or zeatin at 0.005 µM each, the number of shoots per explant (9) was highest in the presence of BA. In pea, (Mroginski and Kartha, 1981a) BA was more effective than either kinetin or 2,i-P. Rubluo et al. (1984) also reported BA to be more efficient than zeatin or 2,i-P in induction of regeneration of pea. Hammatt et al. (1987) observed that when BA was replaced by zeatin, 2,i-P or kinetin in a B5 medium, shoot bud formation occurred at lower frequencies.

In peanut, McKently et al. (1990, 1991), obtained shoot formation from cotyledon on using very high concentrations of BA (25 mg L^{-1}). They also studied the effect of 4 different BA concentrations of 1, 3, 5 and 10 mg L^{-1} in organogenic regeneration from leaf and found BA at 5 mg L^{-1} to produce highest bud regeneration. Barwale et al. (1986) observed that regeneration of soybean via organogenesis requires high concentrations of BA (9.9 or 13.3 µM) while lower (3.3 and 6.6 µM) concentrations produced low numbers of organogenic cultures.

Barna and Wakhlu (1994) observed high concentrations of BA (20 µM) to decrease shoot formation in immature leaflet derived callus cultures of *Cicer arietinum* while in *Vicia faba*, Selva et al. (1989) noted BA at 17 µM to be very effective in promoting high rates of shoot development from cotyledonary node explants. Similarly, Diamon and Mii (1991) observed multiple shoots from cotyledonary nodes of peanut cultured on B5 medium supplemented with BA alone at 10 or 50 mg L^{-1}. However, Atreya et al. (1984) cultured segmented cotyledons of peanut and found that BA alone at only 2 mg L^{-1} showed greater shoot bud induction than in combination with NAA. Although 1 mg L^{-1} NAA with varying levels of BA resulted in regeneration of whole plants, the rate of regeneration was lower compared with regeneration figures on medium containing BA alone. In soybean however, 0.5 mg L^{-1} 2,4,5-T in a B5 medium was essential for regeneration while addition of BA (5 µM) enhanced regeneration (Wright et al., 1987). The response of pigeonpea to lower concentrations of BA in the present study is corroborated by other reports on pigeonpea regeneration from Eapen and George (1994) and Prakash et al. (1994).

The in vitro activation of morphogenic potential of BA is also reported in *Arachis hypogaea* (Diamon and Mii, 1991), *Glycine max* (Cheng et al., 1980, Saka et al., 1980, Wright et al., 1986), *Vigna radiata* (Mathews, 1987 and Gulati and Jaiwal, 1994), *Phaseolus vulgaris* (McClean and Grafton, 1989), and *Vicia faba* (Selva et al., 1989) and *Pisum sativum* (Jackson and Hobbs, 1990).

Wright et al. (1986) and Hinchee et al. (1988) obtained shoot formation from cotyledonary nodes of seedlings grown on BA (5 µM) containing medium. The axillary buds were then removed by scraping the axis and the surrounding region prior to culture on medium of the same BA complement. It was proposed that the excision or wounding may be responsible for meristem initiation. In the present study, shoot formation was obtained when 'seedling

cotyledon' with the axillary bud totally removed was cultured as a whole on L2 medium supplemented with 8.9 µM BA. Proliferation of shoots was observed on the same medium with 0.44 µM BA and elongation was achieved with the same proliferation medium but with only 2% sucrose and the incorporation of 1% activated charcoal. Most of the earlier reports on pigeonpea regeneration described morphogenesis from mature cotyledon or seedling cotyledonary node (George and Eapen, 1994), cotyledonary nodes (Prakash et al., 1994) and callus derived from seedling cotyledons (Kumar et al., 1983). The seedling cotyledons were derived from seeds grown on BA enriched medium and all emphasized the requirement of an auxin for shoot bud regeneration. In addition, Prakash et al. (1994) reported regeneration from 'cotyledonary nodal' explants after removing the surface layers, when cultured on BA alone. Mehta and Mohan Ram (1981) reported regeneration from cotyledonary node and along the margins of cotyledons cultured on MS medium supplemented with BA. The regeneration system summarized in the present study is distinct in that direct regeneration of shoots associated with some callus was obtained from proximal end of 'seedling cotyledons' cultured as a whole. The cotyledons were explanted from seedlings grown on one-tenth L2 basal medium and 1% sucrose. There was also no requirement of auxins for shoot regeneration.

Naidu et al. (1995) elongated shoots obtained from embryo axes and intact seeds cultured on 2.32 µM kinetin, 22.2 µM BA only on transfer to MS medium supplemented with 0.46 µM kinetin, 0.53 µM NAA and 0.29 µM GA_3. In peanut too, Diamon and Mii (1991) elongated the multiple shoots obtained form cotyledonary nodes cultured on B5 medium with BA only when transferred to B5 medium plus zeatin and GA_3 each at 1 mg L^{-1}. The protocol described in this study for organogenic regeneration of whole plants in pigeonpea had no requirement of GA_3 for shoot elongation. This was effectively achieved in the presence of activated charcoal. The effectiveness of activated charcoal in efficient regeneration of whole plants in pigeonpea is a useful finding. The beneficial use of activated charcoal has also been observed in other

species producing polyphenols in culture (Lambardi et al., 1993). Selva et al. (1989) reported low temperatures (14 to 18°C) to be suitable for in vitro culture limiting the formation of phenolics in plant material and making activated charcoal supplement unnecessary. The other feature highlighted in this system is the rooting on hormone-free medium which is necessary to avoid callused roots which are a hinderance to further plantlet establishment. All previous workers on pigeonpea regeneration required auxin for rooting shoots in vitro.

Stem segments cut longitudinally and explanted on various media combinations of BA and NAA produced 40% regeneration on BA 10 µM and NAA 0.1 µM, while on all other combinations either callus or callus with nodular outgrowths was observed. Eapen and George (1994) obtained regeneration from explants like leaf, cotyledon and roots but were unsuccessful with immature cotyledons and epicotyl segments when cultured on MS medium supplemented with BA (1 or 5 mg L^{-1}) and IAA (0.1 mg L^{-1}). Kumar et al. (1984) cultured epicotyl explants on Blaydes medium supplemented with kinetin (2 mg L^{-1}) and IAA (1 mg L^{-1}) or BA (2.5 mg L^{-1}) and observed 2 to 5 shoots in 38% of cultures. Naidu et al. (1995) recovered 2 to 4 shoots from epicotyl explants of different genotypes cultured on MS medium supplemented with various phytohormones.

3.3.3 IN VITRO IRRADIATION

For additional variability 6-day old seedling cotyledons precultured on 8.9 µM BA were subjected to a range of γ-irradiation. Increasing the dose of γ-irradiation from 0.5 to 3.0 KR decreased the shoot regeneration capacity while doses above 3.0 KR did not regenerate plants. A gradual decrease in regeneration capacity was also observed in *Echinocereus* (Cactaceae) (Hutabarat, 1986), and in poplar stem explants (Douglas, 1986). No morphological variations were observed in these regenerated plants just as in the present

study with pigeonpea. Since LD 50 produces less desirable features like chimeras it is preferable to use a lower dose like LD 30 (Pinet-Leblay et al., 1992) for which reason, in the present investigation plants regenerated after exposure to 1 KR which gives a regeneration equivalent to LD35 were screened for additional variability. In *Nicotiana* (Heil et al., 1978), initial enhancement of bud formation in stem explants exposed to γ-irradiation was followed by a gradual decrease with increasing radiation. Their results indicate that it is possible to arrive at a particular optimum dosage to obtain enhanced regeneration with the use of gamma irradiation. They also opine that recovery of dominant mutations at LD 50 can prove to be difficult probably due to higher rates of cell death as well as domination of chimeras in the regenerated plants. In the present study γ-irradiation did not enhance regeneration capacity of pigeonpea.

4 ASSESSMENT OF SOMACLONAL VARIATION

4.1. MATERIALS AND METHODS

4.1.1 PLANT MATERIAL

The extent of somaclonal variation and its stable inheritance was studied in two generations of selfing of the putative pigeonpea somaclones. Plants were regenerated in vitro (as described in chapter 3) from seedling cotyledon explants of *Cajanus cajan* (L.) Millsp. cv ICPL 87 . The initial regenerants are termed R1 plants since these are equivalent to F1 plants resulting from sexual crossing. R2 plants were grown from seed produced by the selfing of R1 plants. The first study was conducted on these R2 plants which were field sown in the rainy season (Kharif) of 1993 at ICRISAT Asia Center. The subsequent selfing of R2 plants gave rise to R3 plants. The second study included the R3 plants derived from thirteen selected putative

somaclones. These were sown in the rainy season of 1994. This planting also included the R2 plants derived from gamma-irradiated explants. Non tissue cultured parent lines obtained from original ICPL 87 seed stock used for regeneration studies were designated as control (C) plants.

4.1.2 ASSESSMENT OF FIELD PERFORMANCE OF R2 AND R3 GENERATIONS

Design of experiment

a *R2 Generation*: All R2 seed collected was planted in an alfisol field in 61 different plots. Since the numbers of seed varied for each R1 plant, randomizations were not attempted. Each plot comprised of all R2 plants derived from a single R1 plant.Thus each plot consisted of 2 to 18 rows. Plant to plant distance was maintained at 20 cm and rows were spaced 150 cm apart.

b *R3 Generation*: The first experiment in this field trial was a randomized complete block design (RCBD) with three replications. The blocks represented the progeny of eleven putative somaclones selected for various agronomic traits and the control ICPL 87 lines, each being assigned to different plots. Each plot consisted of three rows of 20 to 25 plants spaced at 20 or 35 cm apart. Row to row spacing was maintained at 75 cm.

The other experiment on the incidence of *Helicoverpa* damage had three replications in a split plot design. Thus each block had both the unsprayed and sprayed treatments. The field specification are the same as described above for RCB. Two somaclonal lines each of low *Helicoverpa* incidence and high *Helicoverpa* incidence and the control ICPL 87 lines were all designated separate plots in each treatment.

Measurements of qualitative characters

a *R1 Generation*: The R1 plants grown to maturity in the glasshouse were only scored for morphological variations that could be rapidly and easily assessed on single plants in pots. Quantitative characters e.g., yield per se, were not assessed.

b *R2 Generation*: Observations were recorded for qualitative traits like flower colour, seed coat colour and pattern, seed shape, presence or absence of strophiole, colour around hilum, leaf shape and flowering habit. The frequency of these variant phenotypes was calculated as total number of variant phenotypes seen in all R1 families originating from a single explant divided by the total number of R1 families or plants from that particular explant. The variants of a similar phenotype occurring in more than one progeny line of the same regenerated plant were considered as arising from a single mutational event and were thus counted only once.

c *R3 Generation*: Data on inheritance and segregation of the selected putative variants were collected for the altered traits like seed coat colour, presence of strophiole and flowering habit.

Measurement of quantitative characters

a *R1 Generation*: Quantitative traits were not scored in the R1 generation. Not all cultures regenerated at the same time, so the plants were transferred to the glasshouse over a period of months, and the plant height differed drastically with the seasonal variation. Variation for pod bearing length was noted but measurements were not taken.

b *R2 Generation*: Data on R2 plants were collected separately for each individual progeny plant of each R1 plant (border plants were excluded for statistical analysis) to enable single plant selections to be made. The data was scored for the following parameters:

Plant height: Measured at maturity.

Raceme number: Total number of racemes on each plant.

Flower number per raceme: Mean number of flowers as calculated from three racemes randomly chosen on the plant.

Raceme length: Mean length of raceme from three racemes chosen randomly on the plant.

Pod bearing length per branch: Mean distance between lowest and topmost pod on three different randomly chosen branches.

Pod bearing length per plant: Distance between lowest and topmost pod on the plant.

Helicoverpa damaged pod number: Number of pod borer damaged pods harvested from the plant.

Undamaged pod number: Number of undamaged pods harvested from the plant.

Leaf Stem Weight: Weight of whole plant cut at the base after harvest.

Helicoverpa damaged pod weight: Weight of damaged pods.

Undamaged pod weight: Weight of undamaged pods.

Seed yield from damaged pods: Number of undamaged seed collected from *Helicoverpa* damaged pods.

Seed yield from undamaged pods: Number of undamaged seed harvested from undamaged pods.

The following derived data were calculated.

Number of damaged pods (%): Number of *Helicoverpa* damaged pods/total number of pods harvested × 100.

Seed number per pod: Total number of seeds from a single plant/total number of pods from that plant.

100 seed mass: Weight of all good seed from a single plant/total number of good seed collected from that plant × 100.

Biomass: Total weight of damaged and undamaged pods plus weight of freshly harvested plant (visual observations indicated that the leaf fall was not significantly different for each plant and hence was not included in the biomass calculations; also it was not possible to include measurements on root growth).

Shelling percentage: Ratio of weight of mature seed to total weight of pods expressed as percentage.

Harvest index: Ratio of seed yield to biomass expressed as percentage.

c *R3 Generation*: Data was scored for the particular selected trait in the progeny lines of the somaclones selected for that trait. Initial calculations were carried out as in R2 generation.

Statistical analysis

a *R2 Generation*:

Qualitative traits: Chi-square tests for goodness of fit to Mendelian ratios were performed using GENSTAT version 5 (Genstat 5-committee, 1987).

Quantitative traits: The summary statistics like mean, standard deviation and coefficient of variation were calculated in SAS (SAS Institute Inc., USA) for the traits-- plant height, raceme number per plant, flower number per raceme, pod bearing length per branch and per plant,

damaged pod number (%), seed number per pod, 100 seed mass, biomass, shelling percentage and harvest index. Cluster analysis was performed on the mean values of these traits to group somaclones similar for the traits under study. This enabled the selection of the best performing somaclones from different clusters varying for one or more traits. Analysis of variance (ANOVA) was conducted using SAS to determine the genetical variances between and within the somaclones/somaclonal progeny and, that due to the explants giving rise to the somaclones. The square of correlation coefficient was employed to assess the source and proportion of variation induced through tissue culture in the two independent variables (explants and somaclones).

b *R3 Generation*:

Qualitative and Quantitative traits: All statistical analyses were carried out as described for R2 data analysis. General linear model was used for analysis of variance. In addition to cluster analysis Duncan's multiple range test was used to identify somaclones differing significantly.

4.2 RESULTS

4.2.1 SOMACLONAL VARIATION IN R1 GENERATION

Phenotypic variation for qualitative traits : The R1 plants exhibited a complete spectrum of floral alterations with supernumerary wing and standard petals associated with gross variations in shape (PLATE 6A-C). Anthers protruding from out of buds (PLATE 6D), flowers arising from within a flower (PLATE 6E), petaloid sepals (PLATE 6F), and petaloid stamen (PLATE 6G) were also observed. Some plants continuously produced a few flowers with 2 or 3 gynoecia which formed twin or triple pods (PLATE 6H-K). These floral variations however were not observed in the R2 generation. Although the control plant population had inherent variation for flower colour, mostly having yellow flowers but also some yellow flowers with few to medium numbers of red streaks, some of the R1 plants had yellow flowers with dense red streaks. The R1 plants with yellow flowers and medium to dense red streaks produced R2 population which segregated for purple colour flowers.

4.2.2 SOMACLONAL VARIATION IN R2 GENERATION

Phenotypic variation for qualitative traits: A single R2 plant from a putative somaclone flowered profusely but failed to set pods. The frequency of pollen fertility as measured by stainability with acetocarmine ranged from 38% to 64%. Also the anthers generally contained fewer pollen grains. Pod set was also not observed in crosses with the parent ICPL 87 pollen. Another putative somaclone (SC 7) segregated for flowering habit where 6 plants (4.3 % of the progeny) were indeterminate (PLATE 7A). A single R2 plant from a different somaclone SC 24, (1.9 % of the progeny) was semi-determinate (PLATE 7B). Leaf shape variants (obtusely-

PLATE 6

Morphological variations observed in R1 generation of pigeonpea (*Cajanus cajan* (L.) Millsp.) in the greenhouse.

6A-C Variation in number of wing and standard petals.
6F Flower with a petaloid sepal.
6E An abnormal flower giving rise to two additional flowers.
6D Stamens protruding out of a flower bud.
6G Petaloid stamens.
6H A dissected flower with 2 gynoecia.
6I The two gynoecia producing twin pods.
6J The three gynoecia producing triple pods.
6K Flower with three gynoecia.
6L A normal regenerated plant.

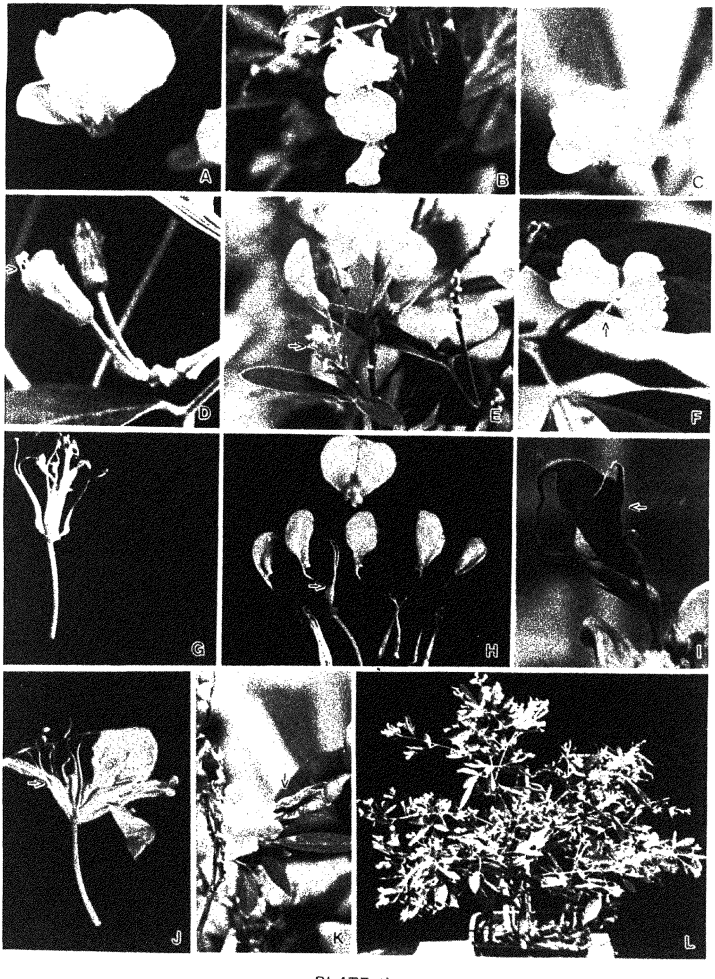


PLATE 6

PLATE 7

Phenotypic variation in the field grown R2 generation of pigeonpea (*Cajanus cajan* (L.) Millsp.).

7A An indeterminate inflorescence.

7B A semi-determinate inflorescence.

7D Variation in seed coat colour, pattern and presence of strophiole.

7C Leaf shape variant (normal leaf shape in (Plate 6L).

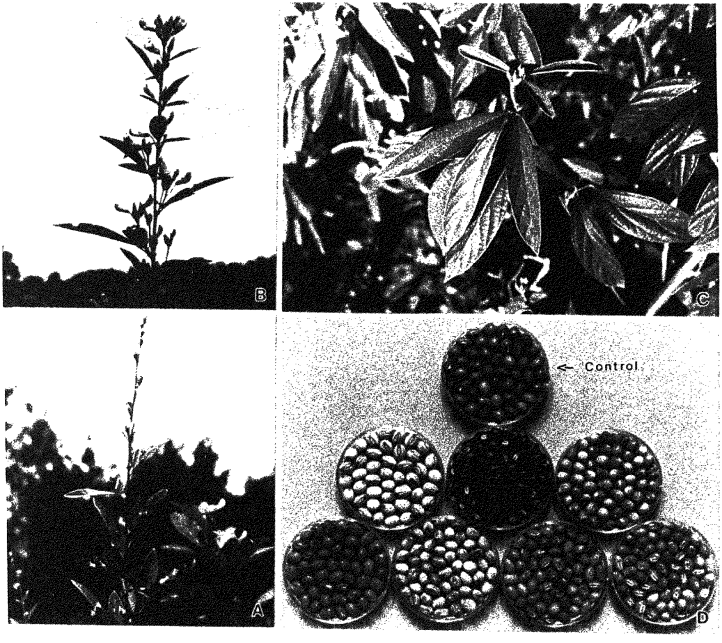


PLATE 7

lanceolate) were observed in certain R2 plants (PLATE 7C). The R2 plants also segregated for purple flower colour, reduced plant height, white seeds, seed coat pattern, colour around hilum, seed shape and presence of strophiole (PLATE 7D). These traits are not present in the parental population.

The possible frequencies of the above mentioned variant phenotypes arising in a given family of R1 plants derived from different cotyledonary explants are seen to range from 0.25 to 4.00 per regenerated plant per explant (TABLE 6). Frequencies of 1.00 to 4.00 were observed in cotyledonary explants with a single R1 progeny plant surviving to maturity. A frequency range 0.25 to 1.00 was observed in those explants with two or more R1 plants. The frequencies of variant R1 plants for the various types in the somaclonal population ranged from 0.016 to 0.41 (TABLE 7).

Genotypic variation for qualitative traits: The genotypic constitutions of the phenotypic variants for colour around hilum and presence of strophiole varied in different progenies of R1 plants which was evident from chi-square tests for goodness of fit to known Mendelian ratios using GENSTAT. From TABLE 8, it is seen that for both the characters all clonally related R1 plants segregated either as dominant or recessive traits. In addition, for a particular trait one set of clonally related plants segregated as dominant while the other set segregated as recessive.

Phenotypic variation for quantitative traits: Differences were recorded between the progeny populations of different R1 plants for plant height, seed mass, biomass, harvest index, *Helicoverpa* damage, raceme number and length, flowers per raceme and pod bearing length per branch and per plant. The size of R2 populations ranged from 2 to 413 plants, but populations of less than 10 R2 plants were excluded from analysis of quantitative traits. On the basis of Ward's minimum variance dendrogram fifty two somaclones originating from thirty one

Table 6

Frequency of phenotypic variation seen in families of R1 plants regenerated from different cotyledonary explants[a] of *Cajanus cajan* (L.) Millsp. cv ICPL 87.

Explant number	Number of R1 plants	Number of variant phenotypes[b]	Frequency of variant phenotypes[c]
1	8	5	0.63
2	6	5	0.83
3	7	7	1.00
4	3	1	0.33
5	4	1	0.25
6	8	4	0.50
7	2	2	1.00
8	2	1	0.50
9-12	1	1	1.00
13	1	4	4.00
14	1	3	3.00
15,16	1	1	1.00
17	1	2	2.00
18	1	3	3.00
19	1	2	2.00
20,21	1	1	1.00
22	1	3	3.00
23	1	2	2.00
24	1	3	3.00

a. The culture time prior to regeneration from the different cotyledonary explants ranged from 9-50 weeks; b. The variant phenotypes recorded for calculating the frequency are the qualitative characters: presence of hilum, seed shape, seed coat colour and pattern, colour around hilum, flower colour, and flowering habit; c. Total number of variant phenotypes seen in all the R1 families originating from a single explant divided by the total number of R1 plants from that particular explant.

Table 7

Comparison of frequency of variant phenotypes in R2 progenies of putative somaclones of *Cajanus cajan* (L.) Millsp. cv ICPL 87

Trait	Explant number	Number of R1 plants regenerated	Number of R1 plants with variant phenotype	Frequency of variant phenotype[a]	Frequency of variant R1 plant[b]
Colour around hilum	2	6	3	0.5	0.18
	3	7	7	1.0	
	26	1	1	1.0	
Seed coat colour	1	8	3	0.38	0.16
	2	6	3	0.50	
	3	7	1	0.14	
	10	1	1	1.00	
	15	1	1	1.00	
	20	1	1	1.00	
Seed coat pattern	1	8	6	0.32	0.41
	2	6	5	0.83	
	3	7	7	1.00	
	14	1	1	1.00	
	15	1	1	1.00	
	16	1	1	1.00	
	20	1	1	1.00	
	21	1	1	1.00	
	24	1	1	1.00	
	26	1	1	1.00	
Seed shape	1	8	3	0.38	0.11
	3	7	1	0.14	
	14	1	1	1.00	
	19	1	1	1.00	
	25	1	1	1.00	
Presence of strophiole	1	8	2	0.25	0.31
	2	6	5	0.83	
	3	7	7	1.00	
	6	8	1	0.13	
	7	2	1	0.50	
	8	2	1	0.50	
	14	1	1	1.00	
	26	1	1	1.00	
Semi-determinate	6	8	1	0.13	0.02
Indeterminate	3	7	1	0.14	0.02

The control seed derived plants did not exhibit these variant phenotypes.

a Number of variant and clonally related plants divided by total number of clonally related plants

b Total number of variant R1 plants divided by total number of regenerated plants irrespective of their origin

Table 8

Genotypic variation in qualitative traits in progeny of tissue cultured *Cajanus cajan* (L.) Millsp. cv ICPL 87

Variant phenotype	Explant number[b]	R1 plant identity	R2 plants segregating[a] for the variant phenotype		
			Absent	Present	Ratio
Colour around hilum	2	8	10	199	1 : 15
		14	19	120	3 : 13
	3	7	79	54	9 : 7
		22	61	55	9 : 7
		23	72	39	9 : 7
Presence of strophiole	2	6	33	112	1 : 3
		8	43	166	1 : 3
		14	25	114	3 : 13
	3	7	101	32	3 : 1
		22	88	28	3 : 1
		23	85	29	3 : 1

These two variant phenotype are not observed in seed derived plants. a. segregating ratios of only those R1 plants with large progeny number are shown. Both these traits are absent in the control ICPL 87 plants.
b. explant number from Table 3

different cotyledonary explants were grouped into six clusters (FIG 5). Cluster I formed the largest group with 28 putative somaclones, followed by clusters III, VI, II, V, and IV, with 8, 6, 4, and 2 somaclones respectively (TABLE 9). The somaclones originating from cotyledons with two or more regenerants are seen to be distributed in different clusters. The rest of the somaclones which are single regenerants from different cotyledons are seen to be largely distributed in cluster I, followed by clusters VI and III. The cluster analysis following Wards minimum variance linkage grouped these putative somaclones into six clusters. From the cluster means (TABLE 10), it is clear that cluster V is furthest from cluster I and contains plants with reduced plant height (68 to 72 cm) and the associated traits like low biomass (64 to 83 g) and high harvest index (20 to 28). However this group also had R2 plants with low *Helicoverpa* damage (20%). Cluster I included R2 plants which were tall (66 to 98 cm) with high biomass (124 to 212 g), seed mass (8 to 12 g), raceme number and *Helicoverpa* damage (87%); and low harvest index (4 to 21) and shelling percentage (25 to 68%). The R1 plants originating from cotyledonary explant 1 were distributed in clusters I, II and V, from explant 2 in clusters II, III, IV and VI, from explant 3 in II, III and V, from explant 4 in I, II and III and from explant 6 in I, III and IV. The R1 plants originating from the other explants, most of which were single regenerants, only occurred in one cluster, mostly cluster I which was the largest.

Somaclones for contrasting characters are seen to be distributed in different clusters. From the means (TABLE 11), the best performing R1 plant of the individual clusters were selected. Somaclone (SC 40) 31, segregating for high raceme number per plant is grouped in cluster I and SC 49 for low raceme number in cluster III. Cluster I includes SC 3 with high biomass and high harvest index, and, cluster IV includes SC 42 with low biomass and high harvest index. SC 20 with low helicoverpa damage is placed in cluster V, while, SC 40 with high helicoverpa damage and high seed mass is placed in cluster I.

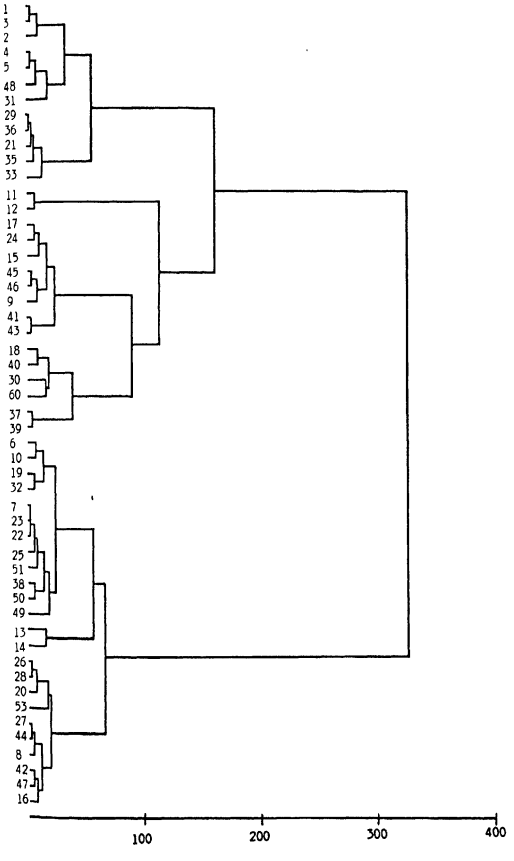


Figure:1 Ward's minimum variance dendogram showing the clustering of seed progeny of selfed tissue cultured R1 plants of Cajanus cajan (L.) Millsp. cv ICPL 87 for yield parameters.

Table 9

Cluster information based on Ward's minimum variance dendrogram of the somaclones originating from different cotyledonary explants.

Cluster No.	No. of R1 plants	Somaclone	origin [a]	Cluster No.	No. of R1 plants	Somaclone	Origin [a]
1	29	4	1	3	8	38	2
		17	1			7	3
		18	1			22	3
		41	4			23	3
		11	5			51	4
		12	5			50	6
		34	5			25	17
		43	5			49	26
		15	6				
		21	6	4	2	14	2
		29	6			13	6
		35	6				
		36	6				
		48	6	5	4	20	1
		30	7			26	1
		46	7			28	1
		37	8			53	3
		1	10				
		2	11				
		3	12	6	6	8	2
		5	13			27	2
		9	14			16	15
		24	16			42	22
		31	18			44	24
		33	19			47	25
		40	20				
		39	21				
		45	23				
		60	30				
2	4	19	1				
		6	2				
		32	3				
		10	4				

[a] The cotyledonary explant from which the somaclone originated

Table 10

Cluster means for the yield parameters on seed progeny of tissue cultured R1 plants of *Cajanus cajan* (L.) Millsp. cv ICPL 87.

Trait	Cluster[a] means					
	I	II	III	IV	V	VI
Plant height (cm)	83.94	82.24	79.55	79.68	69.46	69.76
Biomass (g)	140.21	114.34	115.21	79.74	73.34	83.36
Helicoverpa damage (%)	55.42	28.87	35.91	41.66	35.57	41.00
100 seed mass (g)	9.31	8.66	8.25	8.66	8.16	9.17
Shelling %	47.86	58.89	56.58	58.84	56.36	58.20
Harvest index	13.70	20.81	19.48	21.78	21.80	20.49
Raceme number	102.96	87.54	77.18	85.22	51.95	72.17
Raceme length (cm)	9.11	8.26	7.52	15.34	6.89	7.35
Flowers per raceme	14.98	16.57	13.68	9.61	12.77	14.24
PBL per branch (cm)	24.17	22.84	22.40	22.25	18.23	20.38
PBL per plant (cm)	62.63	51.11	63.51	58.49	53.88	47.73

a. Ward's minimum variance was used for cluster analysis; PBL=Pod bearing length.

Table 11

The average values of various yield parameters for the different putative somaclones obtained from different cotyledonary explants of pigeonpea.

No.	Plant height	*Helicoverpa* Damage(%)	100 seed weight	Shelling Percent	Biomass	Harvest Index	Seed No. per pod
1	92.43 ± 0.54	25.86 ± 0.96	8.55 ± 0.05	67.95 ± 0.55	160.16 ± 3.99	19.97 ± 0.31	3.11 ± 0.04
2	94.85 ± 0.64	40.55 ± 1.15	8.30 ± 0.04	63.55 ± 0.63	171.12 ± 4.24	18.99 ± 0.32	2.59 ± 0.04
3	97.53 ± 0.91	32.15 ± 1.59	7.90 ± 0.20	62.43 ± 1.43	141.01 ± 5.61	21.08 ± 0.55	2.95 ± 0.06
4	86.37 ± 0.82	42.05 ± 1.35	10.12 ± 0.09	58.11 ± 0.77	123.64 ± 4.73	17.29 ± 0.49	2.42 ± 0.05
5	90.60 ± 0.90	49.23 ± 1.86	9.49 ± 0.07	55.33 ± 1.18	137.50 ± 5.80	15.67 ± 0.55	2.19 ± 0.06
6	78.84 ± 1.13	33.89 ± 1.49	9.77 ± 0.10	67.04 ± 0.52	115.99 ± 6.66	22.53 ± 0.78	3.01 ± 0.05
7	77.51 ± 1.41	35.79 ± 1.63	7.46 ± 0.08	60.83 ± 0.96	103.24 ± 5.71	22.89 ± 0.90	2.63 ± 0.06
8	74.96 ± 0.82	37.52 ± 1.42	9.77 ± 0.07	59.62 ± 0.81	78.24 ± 3.55	19.92 ± 0.44	2.48 ± 0.05
9	74.78 ± 1.23	74.20 ± 1.35	8.45 ± 0.12	45.68 ± 1.21	91.17 ± 5.65	14.46 ± 0.99	1.48 ± 0.06
10	93.19 ± 1.46	40.64 ± 1.84	8.79 ± 0.06	54.43 ± 0.96	142.94 ± 8.34	17.13 ± 0.54	2.21 ± 0.06
11	94.24 ± 1.32	70.70 ± 3.53	11.85 ± 1.16	41.67 ± 2.79	178.77 ± 13.98	10.50 ± 1.25	1.41 ± 0.12
12	93.40 ± 1.72	59.60 ± 2.78	11.03 ± 0.13	46.70 ± 1.51	189.92 ± 17.39	13.15 ± 1.01	1.69 ± 0.08
13	85.61 ± 1.13	46.52 ± 2.04	7.86 ± 0.17	59.04 ± 2.18	87.76 ± 6.28	23.50 ± 0.76	2.60 ± 0.10
14	73.75 ± 0.98	43.50 ± 2.07	9.65 ± 0.11	57.81 ± 1.06	69.91 ± 3.92	22.64 ± 0.81	2.42 ± 0.07
15	81.52 ± 0.80	59.89 ± 1.80	8.24 ± 0.26	46.40 ± 1.07	103.06 ± 4.36	14.77 ± 0.58	1.68 ± 0.06
16	64.86 ± 1.12	40.08 ± 2.22	8.92 ± 0.15	56.39 ± 1.18	64.89 ± 4.24	23.70 ± 0.91	2.37 ± 0.08
17	68.19 ± 1.26	63.79 ± 2.80	10.53 ± 1.15	41.39 ± 1.70	89.62 ± 6.38	14.01 ± 1.07	1.42 ± 0.09
18	78.10 ± 2.38	80.81 ± 3.26	9.15 ± 0.23	27.38 ± 3.09	121.50 ± 15.50	6.51 ± 1.22	0.80 ± 0.12
19	82.31 ± 1.88	20.52 ± 1.66	8.60 ± 0.12	58.25 ± 1.41	104.10 ± 10.48	24.58 ± 0.92	2.73 ± 0.09
20	71.68 ± 1.42	20.03 ± 2.15	8.82 ± 0.56	56.81 ± 1.53	64.44 ± 5.94	27.35 ± 1.25	2.77 ± 0.10
21	87.65 ± 1.59	62.16 ± 3.24	7.54 ± 0.09	42.37 ± 1.98	125.50 ± 7.84	12.85 ± 0.87	1.57 ± 0.10
22	82.08 ± 1.51	30.06 ± 1.48	7.21 ± 0.09	56.35 ± 0.86	117.89 ± 8.40	22.79 ± 0.65	2.50 ± 0.06
23	77.25 ± 1.13	35.70 ± 2.21	7.31 ± 0.08	55.99 ± 1.04	103.09 ± 6.00	21.91 ± 0.68	2.48 ± 0.07
24	70.14 ± 0.83	63.07 ± 2.78	8.49 ± 0.08	48.91 ± 1.72	86.14 ± 5.62	15.93 ± 0.78	1.70 ± 0.09
25	74.47 ± 0.94	45.52 ± 1.82	8.29 ± 0.31	57.28 ± 1.05	104.03 ± 5.05	18.30 ± 0.57	2.35 ± 0.06
26	68.64 ± 1.68	40.20 ± 3.58	9.24 ± 0.29	58.85 ± 1.72	82.58 ± 10.60	28.32 ± 2.13	2.61 ± 0.11
27	73.00 ± 1.46	45.26 ± 2.61	9.87 ± 0.15	60.37 ± 1.06	96.60 ± 7.87	19.31 ± 0.88	2.53 ± 0.09
28	67.73 ± 1.26	39.24 ± 3.08	9.14 ± 0.54	53.83 ± 1.73	72.08 ± 5.59	21.98 ± 1.08	2.50 ± 0.10
29	85.13 ± 1.31	57.98 ± 2.55	7.81 ± 0.08	45.66 ± 1.28	128.78 ± 7.21	12.74 ± 0.62	1.73 ± 0.07
30	98.09 ± 1.97	64.86 ± 3.52	9.84 ± 0.12	30.82 ± 1.44	166.83 ± 13.72	7.22 ± 0.50	1.14 ± 0.07
31	94.00 ± 2.10	42.14 ± 3.97	7.52 ± 0.08	50.38 ± 1.53	171.50 ± 12.99	17.79 ± 0.75	2.24 ± 0.11
32	74.61 ± 1.40	30.45 ± 2.00	7.19 ± 0.08	55.50 ± 1.02	90.55 ± 7.03	26.67 ± 0.94	2.58 ± 0.06
33	74.84 ± 1.40	57.10 ± 2.01	10.66 ± 0.13	48.41 ± 1.18	129.44 ± 9.19	16.62 ± 0.61	1.86 ± 0.09
34	80.00 ± 5.77	80.91 ± 7.62	11.29 ± 0.31	37.02 ± 3.47	185.85 ± 40.28	8.75 ± 0.89	0.96 ± 0.14
35	81.51 ± 1.51	49.52 ± 2.53	8.28 ± 0.09	51.53 ± 1.23	126.22 ± 9.38	20.72 ± 1.37	2.23 ± 0.09
36	80.17 ± 1.40	54.80 ± 2.50	8.06 ± 0.08	46.11 ± 1.29	113.43 ± 6.93	16.25 ± 0.97	1.90 ± 0.08
37	88.81 ± 3.33	71.42 ± 3.79	10.91 ± 0.30	30.78 ± 2.56	209.57 ± 27.73	8.09 ± 1.24	1.06 ± 0.13
38	83.17 ± 1.69	40.95 ± 3.13	9.74 ± 0.15	52.29 ± 1.54	130.98 ± 12.23	18.03 ± 1.38	2.32 ± 0.09
39	93.79 ± 1.60	64.47 ± 2.54	10.76 ± 0.14	35.86 ± 1.67	211.94 ± 13.29	10.62 ± 1.02	1.43 ± 0.10
40	74.29 ± 1.99	87.89 ± 2.88	12.49 ± 1.39	25.19 ± 2.52	171.61 ± 19.89	4.71 ± 1.33	0.93 ± 0.15
41	71.36 ± 1.71	80.38 ± 3.43	11.41 ± 0.40	36.68 ± 2.54	100.83 ± 12.58	10.11 ± 1.39	1.21 ± 0.12
42	69.88 ± 1.57	53.14 ± 3.54	8.71 ± 0.11	55.56 ± 1.77	75.53 ± 7.59	22.31 ± 1.24	2.30 ± 0.11
43	65.83 ± 1.63	74.22 ± 3.29	10.10 ± 0.19	42.21 ± 2.90	87.07 ± 9.43	13.52 ± 2.61	1.42 ± 0.13
44	71.54 ± 1.94	42.49 ± 2.92	10.75 ± 1.14	54.36 ± 1.61	99.38 ± 11.16	24.35 ± 1.83	2.51 ± 0.11
45	77.21 ± 1.50	68.73 ± 2.63	10.28 ± 0.16	45.17 ± 2.08	145.44 ± 13.02	14.08 ± 1.49	1.70 ± 0.12
46	77.95 ± 2.15	71.81 ± 3.66	8.83 ± 0.20	43.26 ± 2.63	109.56 ± 11.32	14.07 ± 1.66	1.67 ± 0.16
47	64.30 ± 1.38	48.32 ± 3.10	8.75 ± 0.18	57.05 ± 1.73	76.06 ± 5.83	23.56 ± 1.98	2.20 ± 0.11
48	96.16 ± 1.78	47.79 ± 2.16	10.47 ± 0.11	46.41 ± 1.26	134.47 ± 8.92	16.00 ± 0.77	2.11 ± 0.08
49	81.15 ± 2.33	44.00 ± 4.63	9.65 ± 0.14	57.55 ± 1.93	152.36 ± 14.64	21.35 ± 1.30	2.55 ± 0.13
50	75.41 ± 2.19	39.50 ± 3.27	9.64 ± 0.26	52.36 ± 2.34	89.56 ± 11.00	19.99 ± 1.61	2.53 ± 0.22
51	85.39 ± 1.42	41.38 ± 2.42	7.75 ± 0.13	55.99 ± 1.33	107.44 ± 8.73	22.72 ± 1.31	2.60 ± 0.09
52	76.11 ± 2.00	59.05 ± 5.84	8.15 ± 0.13	49.75 ± 3.22	77.95 ± 10.57	17.34 ± 1.21	2.01 ± 0.18
53	69.78 ± 2.76	54.57 ± 6.92	6.75 ± 0.16	48.34 ± 3.33	64.33 ± 10.24	19.81 ± 2.49	2.09 ± 0.23
54	76.00 ± 2.08	13.42 ± 4.08	8.34 ± 0.40	58.99 ± 6.46	98.18 ± 24.91	31.83 ± 3.57	2.90 ± 0.42
55	84.55 ± 4.55	31.41 ± 4.70	7.07 ± 0.31	57.92 ± 2.94	130.13 ± 25.01	19.86 ± 2.19	2.88 ± 0.15
56	80.00 ± 4.08	50.45 ± 6.63	8.02 ± 0.22	52.70 ± 0.76	116.87 ± 33.17	17.14 ± 1.23	2.10 ± 0.08
57	66.11 ± 3.09	55.35 ± 8.27	6.75 ± 0.21	51.81 ± 4.54	60.42 ± 17.99	26.51 ± 3.79	2.24 ± 0.28
58	95.00 ± 4.08	55.42 ± 4.32	10.95 ± 0.13	50.30 ± 4.76	229.56 ± 45.88	21.29 ± 7.23	1.89 ± 0.36
59	85.71 ± 3.52	75.73 ± 5.44	11.40 ± 0.20	40.24 ± 4.84	125.07 ± 29.35	13.53 ± 3.37	1.36 ± 0.20
60	81.32 ± 2.00	84.51 ± 1.95	11.16 ± 0.13	32.17 ± 1.61	141.09 ± 13.05	9.62 ± 1.14	1.01 ± 0.08
61	80.00 ± 10.00	85.03 ± 4.14	10.98 ± 0.49	21.10 ± 3.41	209.27 ± 87.43	3.59 ± 0.81	0.61 ± 0.14

No. = somaclone identity; All values are means ± SE

The frequency of plants variant for height was observed to be 49.2% relative to the expected plant height of 89 cm for cv ICPL 87 and was seen in 58% of explants regenerated. The frequency of R1 plants with a seed mass greater than the control (9 g) was observed to be 29.5% and was seen in progeny of 45.2% of explants regenerated.

The standard deviation for various traits in the different somaclones appears to change with the calculated means (TABLE 12). Therefore the coefficient of variation (C.V) was used to detect the variance in these populations. Very high C.Vs are observed for the traits biomass and *Helicoverpa* damage. Also very large differences in C.V was observed for seed mass.

Genotypic variation for quantitative traits: From the high F-values (TABLE 13) in the analysis of variance, the variations are estimated with a single probability among the two sources of variables which are somaclones (R1 plants) and the donor explants (source of R1 plants). For the quantitative traits under consideration, highly significant F-values are observed for the different somaclones and also for the R1 families each originating from separate explants.

The square of the correlation co-efficient (TABLE 14) was used to obtain the proportion of variation that was caused by the two independent variables which are cotyledonary explants and somaclones, as effected by tissue culture. The R square value of 0.68 for plant height over both the independent variables at 90 degrees of freedom indicates that 68% of the variation in plant height is due to the two independent variables or 32% of variation is due to factors other than the two independent variables. For the traits like *Helicoverpa* damage (53%), shelling % (59%), and seed number per pod (58%), the independent variables as well as the other sources contributed almost equally, while for seed mass (36%), biomass (34%), and harvest index (37%), a higher proportion of variation is due to sources other than the two variables.

Table 12

The extent of somaclonal variation as seen from the following standard summary statistics of the various yield parameters in the R2 somaclonal populations

Soma-clone	Plant height			*Helicoverpa* damage			100 seed weight			Shelling percent			Biomass			Harvest index			Seed number per pod		
No.	Mean	S.D	C.V	Mean	S.D	C.V	Mean	S.D	C.V	Mean	S.D	C.V	Mean	S.D	C.V	Mean	S.D	C.V	Mean	S.D	C.V
1	92.43	11.04	11.94	25.61	19.54	76.30	8.55	0.94	11.03	67.95	11.18	16.45	160.16	81.19	50.69	19.97	6.40	32.04	3.11	0.74	23.76
2	94.85	12.78	13.47	40.55	22.96	56.63	8.30	0.82	9.89	63.55	12.47	19.62	171.12	84.42	49.33	18.99	6.34	33.40	2.59	0.72	27.73
3	97.53	12.56	12.88	31.98	22.10	69.11	7.90	2.71	34.33	62.43	19.78	31.68	141.01	77.75	55.14	21.08	7.66	36.35	2.95	0.80	26.99
4	86.37	13.05	15.11	42.05	21.53	51.19	10.12	1.38	13.63	58.11	12.31	21.19	123.64	75.22	60.84	17.29	7.77	44.93	2.42	0.73	30.31
5	90.60	12.75	14.08	49.23	26.36	53.54	9.49	0.98	10.35	55.33	16.67	30.12	137.50	82.06	59.68	15.67	7.83	50.00	2.19	0.89	40.68
6	78.84	13.66	17.33	33.42	18.25	54.59	9.77	1.22	12.50	67.04	6.25	9.32	115.99	80.70	69.57	22.53	9.46	41.98	3.01	0.61	20.40
7	77.51	16.24	20.95	34.99	19.22	54.93	7.46	0.93	12.49	60.83	11.12	18.28	103.24	66.16	64.08	22.89	10.40	45.44	2.63	0.69	26.27
8	74.96	11.75	15.67	36.98	20.69	55.96	9.77	1.07	10.91	59.62	11.73	19.67	78.24	51.17	65.40	19.92	6.32	31.74	2.48	0.69	27.79
9	74.78	9.86	13.19	74.20	10.89	14.67	8.45	0.99	11.69	45.68	9.79	21.43	91.17	45.51	49.92	14.46	7.95	55.02	1.48	0.50	33.69
10	93.19	17.22	18.48	40.64	21.81	53.66	8.79	0.69	7.86	54.43	11.35	20.85	142.94	98.63	69.00	17.13	6.40	37.34	2.21	0.67	30.04
11	94.24	8.58	9.11	70.70	23.14	32.73	11.85	7.61	64.19	41.67	18.32	43.97	178.77	91.64	51.26	10.50	8.23	78.35	1.41	0.82	57.91
12	93.40	12.18	13.04	59.60	19.67	33.00	11.03	0.93	8.45	46.70	10.70	22.90	189.92	122.95	64.74	13.15	7.12	54.14	1.69	0.55	32.39
13	85.61	10.42	12.17	45.97	19.25	41.87	7.86	1.60	20.37	59.04	20.14	34.12	87.76	57.86	65.93	23.50	7.04	29.98	2.60	0.88	34.01
14	73.75	11.61	15.75	43.19	24.47	56.66	9.65	1.29	13.39	57.81	12.55	21.72	69.91	46.22	66.12	22.64	9.49	41.93	2.42	0.78	32.10
15	81.52	8.85	10.86	59.42	20.83	35.05	8.24	2.96	35.94	46.40	12.07	26.02	103.06	48.91	47.46	14.77	6.51	44.05	1.68	0.71	42.24
16	64.86	10.78	16.61	39.21	21.71	55.36	8.92	1.45	16.26	56.39	11.41	20.24	64.89	40.91	63.04	23.70	8.76	36.96	2.37	0.78	32.99
17	68.19	11.22	16.46	62.98	25.63	40.69	10.53	10.24	97.26	41.39	15.03	36.33	89.62	56.02	62.51	14.01	9.42	67.25	1.42	0.83	58.22
18	78.10	10.89	13.95	80.81	14.92	18.46	9.15	1.07	11.64	27.38	14.14	51.66	121.50	71.04	58.47	6.51	5.60	86.02	0.80	0.54	67.50
19	82.31	14.30	17.37	20.52	12.75	62.13	8.60	0.96	11.16	58.25	10.86	18.65	104.10	80.50	77.33	24.58	7.04	28.64	2.73	0.65	23.98
20	71.68	12.30	17.16	15.55	16.77	107.86	8.82	4.90	55.59	56.81	13.36	23.51	64.44	51.47	79.87	27.35	10.83	39.59	2.77	0.83	30.15
21	87.65	11.39	12.99	62.16	23.17	37.27	7.54	0.67	8.93	42.37	14.15	33.40	125.50	55.98	44.60	12.85	6.20	48.26	1.57	0.69	43.89
22	82.08	16.20	19.74	29.54	16.19	54.81	7.21	0.94	12.99	56.35	9.31	16.52	117.89	90.48	76.75	22.79	7.01	30.76	2.50	0.61	24.59
23	77.25	11.97	15.49	33.20	23.75	71.54	7.31	0.82	11.26	55.99	11.15	19.92	103.09	64.04	62.13	21.91	7.29	33.27	2.48	0.70	28.06
24	70.14	6.97	9.93	62.17	24.15	38.85	8.49	0.71	8.34	48.91	14.35	29.35	86.14	46.68	54.19	15.93	6.48	40.69	1.70	0.74	43.77
25	74.47	11.12	14.93	44.55	22.11	49.63	8.29	3.62	43.74	57.28	12.48	21.78	104.03	59.51	57.20	18.30	6.69	36.58	2.35	0.76	32.48
26	68.64	11.12	16.20	39.29	23.97	61.01	9.24	1.95	21.07	58.85	11.38	19.34	82.58	70.30	85.12	28.32	14.16	50.00	2.61	0.72	27.75
27	73.00	11.75	16.10	45.26	21.03	46.46	9.87	1.23	12.45	60.37	8.56	14.19	96.60	63.43	65.66	19.31	7.09	36.73	2.53	0.69	27.30
28	67.73	10.94	16.16	34.72	27.16	78.23	9.14	4.77	52.26	53.83	15.26	28.35	72.08	49.04	68.04	21.98	9.48	43.15	2.50	0.86	34.36
29	85.13	11.69	13.73	57.98	22.78	39.30	7.81	0.74	9.49	45.66	11.47	25.12	128.78	64.46	50.05	12.74	5.50	43.21	1.73	0.64	36.77
30	98.09	13.50	13.76	64.86	24.16	37.26	9.84	0.84	8.55	30.82	9.85	31.95	166.83	94.08	56.39	7.22	3.40	47.13	1.14	0.51	44.59

continued...

Table 12 continued from previous page:

Soma-clone	Plant height			*Helicoverpa* damage			100 seed weight			Shelling percent			Biomass			Harvest index			Seed number per pod		
No.	Mean	S.D	C.V	Mean	S.D	C.V	Mean	S.D	C.V	Mean	S.D	C.V	Mean	S.D	C.V	Mean	S.D	C.V	Mean	S.D	C.V
31	94.00	10.51	11.18	42.14	19.86	47.13	7.52	0.42	5.61	50.38	7.63	15.14	171.50	64.97	37.88	17.79	3.74	21.03	2.24	0.55	24.71
32	74.61	13.24	17.75	30.12	19.28	64.00	7.19	0.75	10.46	55.50	9.79	17.64	90.55	67.39	74.42	26.67	8.99	33.70	2.58	0.59	22.77
33	74.84	13.36	17.86	57.10	19.14	33.53	10.66	1.22	11.48	48.41	11.24	23.21	129.44	87.63	67.70	16.62	5.81	34.97	1.86	0.91	48.77
34	80.00	10.00	12.50	80.91	13.20	16.32	11.29	0.53	4.71	37.02	6.01	16.25	185.85	69.77	37.54	8.75	1.54	17.66	0.96	0.24	24.77
35	81.51	10.99	13.48	49.52	18.45	37.26	8.28	0.68	8.18	51.53	8.94	17.35	126.22	68.28	54.10	20.72	9.99	48.21	2.23	0.67	30.01
36	80.17	10.79	13.45	54.80	19.20	35.03	8.06	0.58	7.19	46.11	9.92	21.52	113.43	53.27	46.96	16.25	7.46	45.92	1.90	0.65	34.01
37	88.81	15.24	17.16	71.42	17.35	24.29	10.91	1.38	12.64	30.78	11.75	38.18	209.57	127.09	60.64	8.09	5.66	69.93	1.06	0.60	56.32
38	83.17	13.12	15.77	40.95	24.62	60.12	9.74	1.21	12.47	52.29	12.13	23.20	130.98	96.27	73.50	18.03	10.86	60.24	2.32	0.73	31.57
39	93.79	12.57	13.40	64.47	20.15	31.26	10.76	1.08	10.03	35.86	13.25	36.93	211.94	105.49	49.77	10.62	8.12	76.45	1.43	0.76	53.60
40	74.29	10.52	14.16	81.61	27.04	33.14	12.49	7.20	57.67	25.19	13.12	52.09	171.61	103.35	60.22	4.71	6.93	147.17	0.93	0.77	83.12
41	71.36	9.03	12.66	80.38	18.16	22.59	11.41	2.10	18.42	36.68	13.43	36.61	100.83	66.59	66.05	10.11	7.38	73.01	1.21	0.61	50.77
42	69.88	9.90	14.17	53.14	22.37	42.10	8.71	0.71	8.14	55.56	11.22	20.20	75.53	47.98	63.52	22.31	7.84	35.12	2.30	0.68	29.62
43	65.83	6.91	10.50	74.22	13.96	18.80	10.10	0.80	7.92	42.21	12.29	29.12	87.07	40.01	45.96	13.52	11.06	81.79	1.42	0.55	38.91
44	71.54	12.09	16.91	40.36	19.88	49.25	10.75	7.23	67.26	54.36	10.21	18.78	99.38	69.68	70.11	24.35	11.46	47.06	2.51	0.70	27.74
45	77.21	8.72	11.30	68.73	15.80	22.99	10.28	0.99	9.62	45.17	12.49	27.66	145.44	78.14	53.73	14.08	8.94	63.47	1.70	0.72	42.20
46	77.95	10.08	12.93	71.81	17.53	24.42	8.83	0.96	10.88	43.26	12.62	29.17	109.56	54.27	49.54	14.07	7.97	56.64	1.67	0.76	45.59
47	64.30	9.74	15.15	47.38	22.73	47.98	8.75	1.26	14.43	57.05	12.32	21.60	76.06	41.65	54.76	23.56	14.11	59.89	2.20	0.79	36.15
48	96.16	15.63	16.26	47.79	18.96	39.66	10.47	0.96	9.13	46.41	11.09	23.90	134.47	78.26	58.20	16.00	6.73	42.04	2.11	0.69	32.89
49	81.15	11.86	14.61	44.00	23.63	53.70	9.65	0.74	7.64	57.55	9.84	17.09	152.36	74.65	49.00	21.35	6.62	31.03	2.55	0.67	26.31
50	75.41	12.41	16.46	39.50	19.09	48.33	9.64	1.54	15.95	52.36	13.62	26.01	89.56	64.15	71.63	19.99	9.38	46.92	2.53	1.26	49.75
51	85.39	11.55	13.52	40.75	20.06	49.21	7.75	1.02	13.14	55.99	10.78	19.26	107.44	70.96	66.05	22.72	10.62	46.74	2.60	0.74	28.55
52	76.11	6.01	7.90	59.05	17.51	29.65	8.15	0.39	4.83	49.75	9.66	19.42	77.95	31.72	40.69	17.34	3.62	20.88	2.01	0.54	27.07
53	69.78	11.72	16.80	51.70	31.17	60.28	6.75	0.72	10.61	48.34	14.50	30.00	64.33	44.65	69.40	19.81	10.87	54.87	2.09	1.01	48.20
54	76.00	3.61	4.74	13.42	7.06	52.63	8.34	0.69	8.23	58.99	11.18	18.96	98.18	43.15	43.95	31.83	6.19	19.43	2.90	0.72	24.86
55	84.55	15.10	17.86	28.55	16.95	59.49	7.07	1.02	14.41	57.92	9.77	16.86	130.13	82.95	63.74	19.86	7.25	36.50	2.88	0.51	17.58
56	80.00	8.16	10.21	50.45	13.26	26.28	8.02	0.45	5.59	52.70	1.52	2.88	116.87	66.34	56.77	17.14	2.46	14.37	2.10	0.16	7.69
57	66.11	9.28	14.04	49.20	28.62	58.17	6.75	0.64	9.55	51.81	13.61	26.26	60.42	53.97	89.33	26.51	11.38	42.94	2.24	0.83	37.07
58	95.00	8.16	8.59	55.42	8.64	15.60	10.95	0.26	2.35	50.30	9.52	18.93	229.56	91.76	39.97	21.29	14.47	67.95	1.89	0.72	38.01
59	85.71	9.32	10.88	75.73	14.40	19.02	11.40	0.52	4.60	40.24	12.81	31.82	125.07	77.66	62.10	13.53	8.92	65.98	1.36	0.54	39.40
60	81.32	11.64	14.31	84.51	11.34	13.42	11.16	0.77	6.88	32.17	9.41	29.24	141.09	76.10	53.93	9.62	6.64	69.04	1.01	0.45	43.98
61	80.00	14.14	17.68	85.03	5.86	6.89	10.98	0.69	6.31	21.10	4.82	22.83	209.27	123.64	59.08	3.59	1.14	31.76	0.61	0.21	33.89

S.D = standard deviation
C.V = co-efficient of variation

Table 13

Analysis of variance for various yield parameters in the seed progeny of tissue cultured *Cajanus cajan* (L.) Millsp. cv ICPL 87 plants.

Source of variation	df	Mean Squares						
		plant height	*Helicoverpa* damage	100 seed mass	Shelling percent	Biomass	Harvest index	Seed number per pod
explant	30	11655.88	22707.98	183.35	9645.05	161236.81	1775.65	31.15
F-value		150.69**	73.44**	41.36**	98.94**	36.58**	34.55**	91.80**
Somaclone	60	7100.91	15456.68	98.91	6144.00	97285.88	1452.59	20.96
F-value		91.80**	49.99**	22.31**	63.03**	22.07**	28.27**	61.79**
Error		77.35 (4633)	309.19 (4672)	4.43 (4672)	97.48 (4671)	4407.19 (4664)	51.39 (4664)	0.34 (4664)

df=degrees of freedom
**=significance at 1% level
figures in parenthesis are error degrees of freedom

Table 14

Source of variation for the yield parameters in the somaclonal populations derived from different cotyledonary explants of *Cajanus cajan* (L.) Millsp. cv ICPL 87

Trait	R^2	Variation due to the independent variable[a] (%)	Variation due to other sources[b] (%)
Plant height	0.684	68.4	31.6
Helicoverpa damage	0.527	52.7	47.3
100 seed mass	0.356	35.6	64.4
Shelling %	0.591	59.1	40.9
Biomass	0.342	34.2	65.8
Harvest index	0.369	36.9	63.1
Seed number per pod	0.581	58.1	41.9

a independent variables: explants and somaclones
b various environmental factors
df = degrees of freedom = 90
R^2 = square of correlation co-efficient

4.2.3 GAMMA-IRRADIATED VS UN-IRRADIATED SOMACLONAL POPULATIONS

In a separate experiment a small sample each of progeny lines of somaclones derived from gamma irradiated cultures (9 R1 plants) and unirradiated cultures (3 R1 plants) were compared for phenotypic and genotypic variation in quantitative traits. The γ-irradiated plants were however not variant for any qualitative traits.

Phenotypic variation: Cluster analysis was performed separately for the two treatments using the computer programme INDOSTAT (INDOSTAT Services Hyderabad). The traits studied are -- plant height, *Helicoverpa* damage, seed mass, harvest index, shelling percentage and seed number per pod. Considering all these traits Ward's minimum variance grouped the putative somaclones from the γ-irradiated and the unirradiated treatments into 6 and 3 groups each respectively. From the dendrogram produced (FIG 6) it can be seen that IR 5 (IR refers to irradiated), the control (ICPL 87) and IR 6, each of which form separate clusters, are variant form each other as well as from the other 3 clusters. Similarly in FIG 7, SC 64 and the control (ICPL 87) fall into separate clusters while the other two somaclones are grouped.

From the cluster means of the irradiated sample (TABLE 15) cluster III has the shortest plants with a mean of 57.5 cm. As expected this group also has the lowest value for average biomass (87.8 g) and highest mean values for harvest index (22.8), shelling percent (57.9) and seed number per pod (3.33). In addition this also has the lowest incidence of *Helicoverpa* (only 27.8% as compared to 43.7% in the control). Incidentally, all the somaclonal populations show lower average pest incidence as compared to the control ICPL 87 from which they were derived. Clusters I, V and VI have average seed mass of 11.1 to 11.5 g, while cluster IV with ICPL 87 has an average seed mass of 10.6 g. None of the somaclonal populations exceeded the control for the trait biomass. Although cluster II has an average harvest index (17.4%)

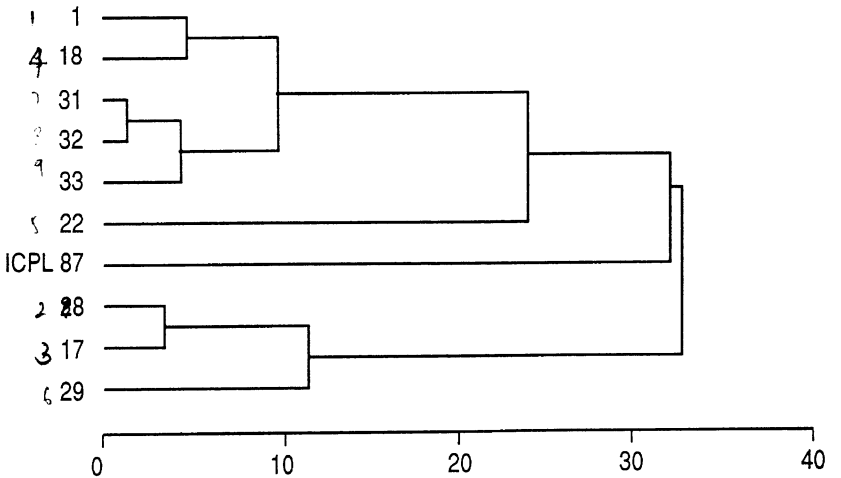


Figure 7: Ward's minimum variance dendrogram showing the clustering of seed progeny of tissue culture plants derived from gamma irradiated seedling cotyledons.

Table 15

Cluster means for the yield parameters on seed progeny of tissue cultured R1 plants derived from in vitro gamma irradiated seedling cotyledons of *Cajanus cajan* (L.) Millsp. cv ICPL 87.

Trait	Cluster[a] means					
	I	II	III	IV	V	VI
Plant height (cm)	61.95	64.86	57.47	63.21	60.90	65.55
Helicoverpa damage (%)	30.53	26.36	27.85	43.70	43.42	27.38
100 seed mass (g)	11.24	10.78	10.58	10.57	11.13	11.48
Biomass (g)	124.98	131.00	87.85	183.61	105.13	123.83
Harvest index	16.10	17.44	22.80	17.30	15.41	16.62
Shelling percent	57.78	57.89	57.93	56.20	56.17	57.43
Seed number/pod	3.29	3.22	3.33	3.31	3.02	2.93

a. Ward's minimum variance dendrogram was used for cluster analysis. Cluster I=IR 1, IR 4; Cluster II=IR 7, IR 8, IR 9; Cluster III=IR 5; Cluster IV=control ICPL 87; Cluster V=IR 2, IR 3; Cluster VI=IR 6. Data represented in percentage are values after arcsin transformation.

closer to control (17.3%), its average biomass (131.0 g) is lower than the control (183.6 g). Phenotypic variation for the same traits as mentioned above is seen for tissue culture derived plants (TABLE 16). Cluster I has plants with smaller average value for plant height (61.9 cm) than control (63.2 cm), but considerably larger average value than seen from gamma irradiated cultures (57.5 cm). Cluster II has SC 64 with only 27.1% *Helicoverpa* damage which is almost equal to that seen for gamma irradiated sample (27.8%). Clusters I and II have an average seed mass of 12.2 to 12.5 g which is higher than for control (10.6 g) and for gamma irradiated (11.1 to 11.4 g). The average harvest index was modestly higher with values of 18.2 to 17.4% than the control (17.3%). Cluster II has a higher shelling percentage (57.1%) than cluster III (56.2%). Unlike the gamma irradiated sample the unirradiated sample did not show increased average seed number per pod for the somaclonal populations.

The extent of somaclonal variation in the different yield parameters which reflects the quantitative phenotypic traits in the gamma irradiated population is shown in TABLE 17. The values of standard deviation tends to change with change in means for all traits except seed mass and seed number per pod, which are also stable in conventional breeding. The variation as measured by co-efficient of variation is also very high for the irradiated somaclone IR 5 for the traits plant height, *Helicoverpa* damage, seed mass and biomass. Low mean values are observed for the traits harvest index and seed number per pod in somaclone IR 6 making it obviously different from the other somaclones and the control, while for shelling percentage, IR 2 and IR 3 are significantly different from the others with very low values. From the respective means it is clear that somaclone IR 5 is varying from the other somaclones as well as the control for the traits plant height, biomass, harvest index, shelling percentage and seed number per pod. It is also showing a significantly low percent of *Helicoverpa* damaged pod when compared to control. This is also evident from Duncan's grouping where it is grouped all by itself for all traits except damaged pods percent which however has values closer to the

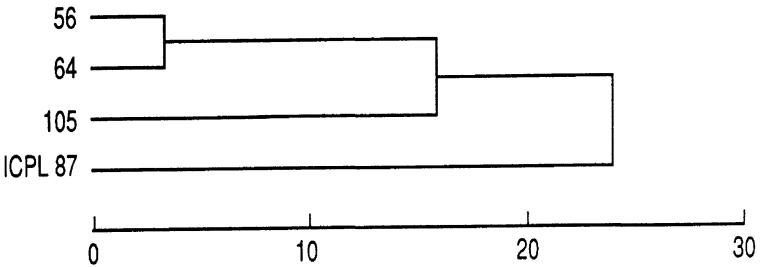


Figure 3: Ward's minimum variance dendrogram showing the clustering of seed progeny of selfed tissue culture R1 plants of *Cajanus Cajan* (L.) Millsp. cv ICPL 87 for various yield parameters

Table 16

Cluster means for the yield parameters on seed progeny of tissue cultured R1 plants derived from seedling cotyledons of *Cajanus cajan* (L.) Millsp. cv ICPL 87.

Trait	Cluster[a] means		
	I	II	III
Plant height (cm)	61.89	66.84	63.21
Helicoverpa damage (%)	33.72	27.11	43.70
100 seed mass (g)	12.24	12.47	10.57
Biomass (g)	100.02	107.40	183.61
Harvest index	18.20	17.38	17.30
Shelling percent	53.37	57.09	56.20
Seed number/pod	2.87	3.07	3.31

a. Ward's minimum variance dendrogram was used for cluster analysis. Cluster I=SC 62, SC 63; Cluster II=SC 64; Cluster III=control ICPL 87. Data represented in percentage are values after arcsin transformation.

Table 17

The extent of somaclonal variation in the R2 progeny of regenerants obtained from gamma irradiated seedling cotyledons of *Cajanus cajan* (L.) Millsp. cv ICPL 87

		Irradiated somaclone number									
Trait		1	2	3	4	5	6	7	8	9	C
PH	MN	62.02	61.35	60.46	61.88	57.47	65.55	63.87	64.55	66.16	63.21
	SD	8.85	8.68	9.03	8.72	9.97	9.71	8.98	6.84	6.35	6.48
	CV	14.28	14.15	14.93	14.08	17.35	14.81	14.06	10.60	9.60	10.25
ADP	MN	31.04	31.85	34.98	30.01	27.85	27.38	25.58	26.96	26.53	43.70
	SD	11.51	15.04	15.08	13.55	12.74	14.05	12.18	11.46	10.49	21.63
	CV	37.09	47.22	43.12	45.17	45.74	51.30	47.63	42.51	39.53	49.50
SDM	MN	11.39	10.87	11.39	11.08	10.58	11.48	10.99	10.71	10.65	10.57
	SD	0.83	0.83	0.80	0.86	0.93	1.04	0.73	0.74	0.83	0.75
	CV	7.27	7.65	7.04	7.74	8.75	9.04	6.65	6.94	7.79	7.07
BIO	MN	108.63	107.19	103.08	141.32	87.85	123.83	125.62	134.28	133.11	183.61
	SD	28.69	40.55	49.86	65.54	52.93	63.13	64.26	62.60	45.63	58.74
	CV	26.41	37.83	48.37	46.37	60.25	50.98	51.15	46.62	34.28	31.99
HI	MN	17.05	15.10	15.71	15.14	22.80	16.62	18.36	17.93	16.03	17.30
	SD	8.62	9.70	8.88	9.51	13.63	12.09	12.87	12.41	8.40	4.46
	CV	50.55	64.24	56.51	62.85	59.78	72.77	70.11	69.24	52.42	25.82
SH	MN	57.68	56.45	55.89	57.88	57.93	57.43	57.93	57.84	57.89	56.20
	SD	2.35	3.65	3.60	2.32	2.45	2.88	3.37	2.63	1.80	2.29
	CV	4.07	6.47	6.44	4.01	4.23	5.02	5.81	4.54	3.10	4.07
SNP	MN	3.28	3.04	2.99	3.29	3.33	2.93	3.17	3.22	3.27	3.31
	SD	0.53	0.60	0.55	0.54	0.61	0.65	0.50	0.60	0.55	0.43
	CV	16.31	19.76	18.46	16.55	18.34	22.24	15.79	18.63	16.78	12.88

C=control ICPL 87; PH=plant height (cm); HD=*Helicoverpa* damage (%); SDM=seed mass (g); BIO=biomass (g); HI=harvest index; SH=shelling percentage; SNP=seed number per pod; MN=mean; SD=standard deviation; CV=coefficient of variation.

lowest pest incidence groups. Although IR 7 has lower incidence of *Helicoverpa* damage than IR 5, the latter has other agronomically improved traits like high harvest index, shelling percentage and seed number per pod. All somaclones show higher or lower mean values than the control except for the traits biomass and *Helicoverpa* damage. While all the somaclones have lower pest incidence than the control, none equalled the control in biomass.

The extent of somaclonal variation in the unirradiated population is shown in TABLE 18. High variance (as seen from C.V) is noted for SC 62 for *Helicoverpa* damage, seed mass, biomass, harvest index, shelling percentage and seed number per pod. The somaclones show mean values greater than or less than the control mean for the traits plant height and shelling percent. All somaclones showed less incidence of *Helicoverpa*, higher seed mass and harvest index, lower biomass and seed number per pod than the control. With respect to *Helicoverpa* damage SC 64 shows only 27% (same as the lowest in irradiated sample). Higher seed mass is evident in irradiated sample. Gamma irradiated somaclones (IR 5) show higher seed yield as seen from seed number per pod. The Duncan's grouping show SC 64 as significantly different from the other somaclones as well as from the control and also all the somaclones differ from the control.

Genotypic variation: General linear model (GLM) was used for analysis of variance in the computer programme SAS. The computed F-values in the gamma irradiated treatment for the traits plant height, *Helicoverpa* damage, seed mass, biomass, harvest index, shelling percent and seed number per pod are all significant at the 1% level (TABLE 19). The variance due to error is small as compared to variance due to other source (i.e., somaclones) in all the traits under study. Therefore high F-values are observed. In the unirradiated sample (TABLE 20), the GLM for analysis of variance also shows significant F-values for all the traits except harvest index which shows a variance due to error to be very high (109.88). In both the treatments

Table 18

The extent of somaclonal variation in the R2 progeny of regenerants obtained from seedling cotyledons of *Cajanus cajan* (L.) Millsp. cv ICPL 87

Trait		Somaclone Number			
		62	63	64	C
PH	MN	61.24	62.53	66.84	63.21
	SD	8.60	8.98	9.08	6.48
	CV	14.05	14.36	13.58	10.25
HD	MN	33.83	33.61	27.11	43.70
	SD	21.58	20.63	13.05	21.63
	CV	63.80	61.39	48.12	49.50
SDM	MN	12.28	12.19	12.47	10.57
	SD	1.56	1.07	0.94	0.75
	CV	12.69	8.75	7.52	7.07
BIO	MN	89.71	110.33	107.40	183.61
	SD	48.70	54.22	37.98	58.74
	CV	54.29	49.14	35.36	31.99
HI	MN	17.86	18.53	17.38	17.30
	SD	13.55	15.08	8.13	4.46
	CV	75.87	81.38	46.77	25.82
SP	MN	53.01	53.72	57.09	56.20
	SD	8.35	9.72	1.92	2.29
	CV	15.74	18.10	3.37	4.07
SNP	MN	2.87	2.86	3.07	3.31
	SD	1.11	1.08	0.60	0.43
	CV	38.62	37.78	19.53	12.88

C=control ICPL 87; PH=plant height (cm); HD=*Helicoverpa* damage (%); SDM=seed mass (g); BIO=biomass (g); HI=harvest index; SH=shelling percentage; SNP=seed number per pod; MN=mean; SD=standard deviation; CV=coefficient of variation.

Table 19

Analysis of variance (GLM) for various yield parameters in the seed progeny of tissue cultured plants derived from in vitro gamma irradiated seedling cotyledons of *Cajanus cajan* (L.) Millsp. cv ICPL 87 plants.

Source of variation	df	Mean Squares						
		plant height	*Helicoverpa* damage	seed mass	Biomass	Harvest Index	Shelling Percent	Seed number per pod
Somaclone	9	1132.19	2809.56	18.11	72386.27	823.26	89.15	3.23
(F-value)		15.62**	15.66**	25.47**	24.36**	7.17**	11.21**	10.01**
Error		72.49 (1542)	179.44 (1543)	0.71 (1543)	2971.20 (1543)	114.89 (1543)	7.95 (1542)	0.32 (1543)

df=degrees of freedom
**=significance at 1% level
figures in parenthesis are error degrees of freedom

Table 20

Analysis of variance (GLM) for various yield parameters in the seed progeny of tissue cultured plants derived from seedling cotyledons of *Cajanus cajan* (L.) Millsp. cv ICPL 87 plants.

Source of variation	df	Mean Squares						
		plant height	*Helicoverpa* damage	seed mass	Biomass	Harvest Index	Shelling Percent	Seed number per pod
Somaclone	3	647.01	4179.35	56.25	117535.94	22.53	358.39	2.95
(F-value)		8.86**	12.68**	46.88**	52.23**	0.21	10.78**	4.53**
Error		73.00 (344)	329.67 (346)	1.20 (346)	2250.48 (346)	109.88 (346)	33.23 (346)	0.65 (346)

df=degrees of freedom
**=significance at 1% level
figures in parenthesis are error degrees of freedom

with harvest index having the least F-values (7.17 and 0.21) thus suggesting a high variance due to error than due to the explant. However in the γ-irradiated sample, inspite of a higher proportion of variance due to sources other than the somaclones, the variation due to the somaclones is still significant. In the irradiated treatment high F-values are seen for seed mass (24.47) and biomass (24.36) indicating a significantly high variation due to the somaclones themselves. Similarly the unirradiated sample also shows a high variation in the somaclones for the two traits seed mass (F-value=46.88) and biomass (F-value=52.23).

From the square of the correlation coefficient, the proportion of variation due to the independent variable i.e., the somaclones in the γ-irradiated sample ranged from 4% to 24.5% for the various traits (TABLE 21) and for the unirradiated sample the range was between 0.2% and 28.9% (TABLE 22) for the same traits. However, when compared to a larger sample of unirradiated plants (TABLE 14), the proportion of variation due to the independent variables (explant and the individual somaclones) a higher range of 34% to 68% is observed.

4.2.4 SOMACLONAL VARIATION IN R3 GENERATION

Phenotypic and genotypic variation for qualitative traits: The Chi-square test for the goodness of fit to expected Mendelian ratios was studied for strophiolate and white seed coat variants. In the R2 somaclonal population strophiolation segregated as both dominant and recessive traits. The inheritance of white seed coat was studied in the R3 progeny of individual R2 plants selected for the trait. While the R2 plants showed epistatic gene interactions, the trait segregated in a 3:1 ratio (TABLE 23). Its segregation in the R3 generation indicates its heterozygous state. For both the traits (strophiole and white seed) the probability of a 3:1 ratio was between 5 to 100%. PLATE 8B shows the obtusely lanceolate leaf shape variant to be stably inherited without any segregation.

Table 21

Source of variation for the yield parameters in the somaclonal populations derived from different gamma irradiated cotyledonary explants of *Cajanus cajan* (L.) Millsp. cv ICPL 87

Trait	R^2	Variation due to the independent variable[a] (%)	Variation due to other sources[b] (%)
Average flowers per raceme	0.2446	24.46	75.54
Raceme per plant	0.0931	9.31	90.69
Average PBL	0.2421	24.21	75.79
Plant height	0.0835	8.35	91.65
Helicoverpa damage	0.0847	8.47	91.53
100 seed mass	0.1293	12.93	87.07
Shelling %	0.0614	6.14	93.86
Biomass	0.1244	12.44	87.56
Harvest index	0.0401	4.01	95.99
Seed number per pod	0.0552	5.52	94.48

a independent variables: explants and somaclones
b various environmental factors
df = degrees of freedom = 90
R^2 = square of correlation co-efficient
PBL = pod bearing length per branch

Table 22

Source of variation for the yield parameters in the somaclonal populations derived from different cotyledonary explants of *Cajanus cajan* (L.) Millsp. cv ICPL 87

Trait	R^2	Variation due to the independent variable[a] (%)	Variation due to other sources[b] (%)
Average flowers per raceme	0.1830	18.30	81.70
Raceme per plant	0.0247	2.47	97.53
Average PBL	0.2093	20.93	79.07
Plant height	0.0717	7.17	92.83
Helicoverpa damage	0.0990	9.90	90.10
100 seed mass	0.2890	28.90	71.10
Shelling %	0.0855	8.55	81.45
Biomass	0.3117	31.17	68.83
Harvest index	0.0017	0.17	99.83
Seed number per pod	0.0378	3.78	96.22

a independent variables: explants and somaclones
b various environmental factors
df = degrees of freedom = 90
R^2 = square of correlation co-efficient
PBL = pod bearing length per branch

Table 23

Chi-square analysis of qualitative characters for inheritance in the R3 generation of tissue culture derived plants of *Cajanus cajan* (L.) Millsp. cv ICPL 87

Trait	Presence	Absence	X^2 for 3:1	Probability
Strophiole	10	8	3.630	0.050 - 0.075
	14	3	0.400	0.400 - 0.500
	14	4	0.074	0.800 - 0.900
	7	3	0.133	0.700 - 0.080
	16	1	3.314	0.075 - 0.100
	15	8	1.174	0.250 - 0.300
White seed coat	17	3	1.007	0.300 - 0.400
	11	8	2.965	0.070 - 0.100
	15	2	1.588	0.200 - 0.150
	6	4	1.200	0.150 - 0.300
	8	3	0.030	0.500 - 0.600
	12	2	0.857	0.300 - 0.500
	16	2	1.852	0.100 - 0.150
	15	5	0.000	1.000
	15	4	0.158	0.600 - 0.700

Phenotypic and genotypic variation for quantitative traits: The inheritance of quantitative traits like plant height, seed mass, high and low biomass was studied in the R3 generation. Although the traits plant height, seed mass and high biomass were maintained in the selected progeny lines in the subsequent R3 generation, plants selected for low biomass appeared to be segregating (TABLE 24). The progeny lines selected for seed mass and plant height maintained the improvements over the control population. While biomass was maintained in the somaclonal populations, it did not perform as well as the control, but gave lower values than control.

The other experiment in inheritance studies involved progeny lines selected for low and high *Helicoverpa* incidence as measured by percentage of damaged pods. The lines selected for high *Helicoverpa* incidence showed the same level of incidence as the control. The somaclones SC 19 and SC 20 showed 56.7% and 54.5% damage respectively under unsprayed conditions while with spraying they had only 22.1% and 28.8% damage (TABLE 25). PLATE 8A shows SC 19 with mature and dry pods from the first flush, while the control ICPL 87 plants are seen to have only the second flush (green) pods. Thus both somaclones had less *Helicoverpa* damage than the control in both sprayed and unsprayed conditions.

PLATE 8

8A Progeny of SC 19 selected for low *Helicoverpa* incidence (on far right), showing many first flush pods which are absent in the control ICPL 87 (the rows on the left). The central row was pruned to check outcrossing.

8B Progeny of SC 40 (selected for large seed) showing the true breeding trait of obtusely lanceolate leaves.

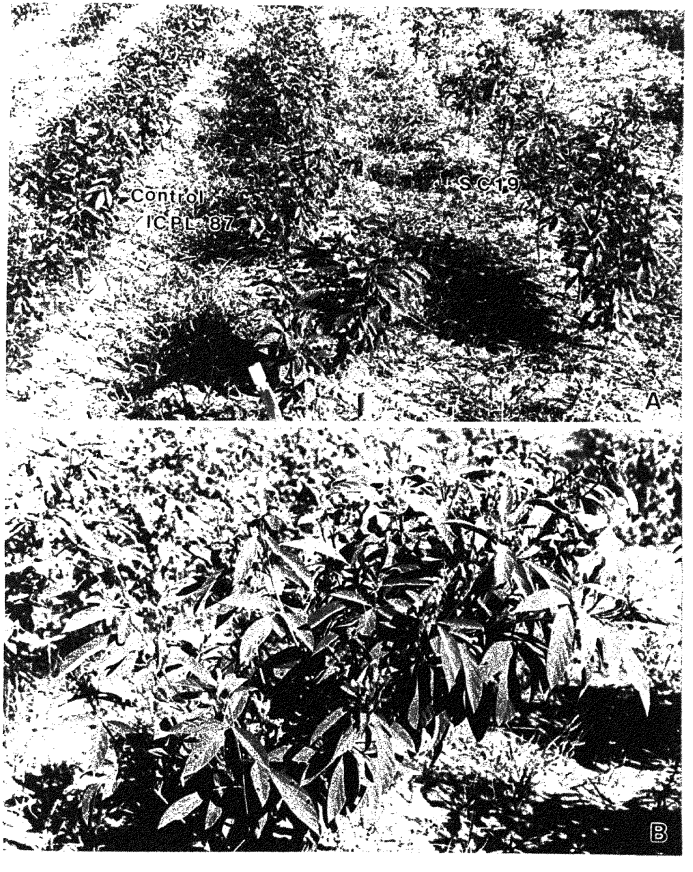


PLATE 8

Table 24

Inheritance of quantitative traits in the progeny of tissue culture regenerated plants of *Cajanus cajan* (L.) Millsp. cv ICPL 87

Trait	Somaclone	Generation R2	Generation R3
Seed mass (g)	SC12	11.03	14.72
	C	9.00*	10.53
Plant height	SC47	64.30	55.72
	C	82.00*	61.85
High biomass	SC3	141.01	142.17
Low biomass	SC42	75.53	168.76
	C	NA	178.39

C = seed derived control ICPL 87; R = Tissue culture derived population; SC = somaclone; * = Data from ICPL 87 Plant material description No. 42, ICRISAT (1993); NA= Data not available; All values in R3 are means of 6 rows.

Table 25

Comparison of *Helicoverpa* damage in R3 generation of tissue culture derived plants of *Cajanus cajan* (L.) Millsp. cv ICPL 87 under insecticidal spray and unspray conditions as compared with seed derived control material

	Percentage damage in the treatment	
Somaclone	Unspray	Spray
SC 19	56.67	22.14
SC 20	54.52	28.87
SC 40	55.67	33.28
SC 60	60.45	33.21
C	60.73	30.02

All values are after arcsin transformation; C = control material; SC = somaclone.

4.3 DISCUSSION

4.3.1 SOMACLONAL VARIATION FOR QUALITATIVE TRAITS IN R1 GENERATION

Although an array of phenotypic variation involving floral morphology, architecture, twin and triple pods was observed in the R1 generation, its perpetuation in the subsequent selfed sexual generation was not evident. This reversion in the R2 generation indicates that the apparent variations are manifestations of tissue culture effect and are therefore epigenetic and not under complete genetic control. This conformity of the in vitro propagated plants in the second generation for morphological characters was also observed in sugarcane by Lourens and Martin (1987). Barbier and Dulieue (1983) also found instability for marker genes in tobacco while Groose and Bingham (1986) found a high frequency of reversion for anthocyanin mutation. However, the occurrence of homozygous mutations in the primary regenerants were also reported. In alfalfa, Reisch and Bingham (1981), Bingham and McCoy (1986) and Groose and Bingham (1986) reported altered variations for flower colour, leaf morphology, branching patterns, fertility, regeneration ability and ploidy level in the primary regenerants. The abnormal and rudimentary flowers and simple leaves were found to be monogenically recessive variants. In soybean, Barwale and Widholm (1987) observed lethal sectorial albinos in the initial regenerants.

4.3.2 SOMACLONAL VARIATION FOR QUALITATIVE IN R2 AND R3 GENERATIONS

Phenotypic and genotypic variation for both qualitative and quantitative traits were observed in the selfed sexual generation of R1 plants.

Phenotypic variation for qualitative traits: Many of the variant phenotypes which were not seen in the R1 were assumed to be heterozygous recessive for these variant phenotypes. Various workers have shown that the flower and seed colour in pigeonpea is a very complex trait (Saxena and Sharma, 1990). However, the expression of purple colour flowers and white seed coat colour in the progeny (R2 plants) of self fertilized R1 plants which was not observed in the donor material suggests that tissue culture influenced the expression of these traits. This change in pigmentation as a consequence of tissue culture could be due to the possible activation of transposable elements (Groose and Bingham, 1986). The observation of a partially male sterile (which is also female sterile) plant in the progeny of the regenerated plant (SC 25) suggests of cytoplasmically encoded changes.

Generaliy 10 to 20% of progeny of determinate pigeonpea (cv ICPL 87) could segregate for indeterminate as a result of genetic heterogeneity in the initial material possibly due to cross-pollination. However, the observation of 4.3% of the progeny of one out of seven somaclones derived from a single explant to segregate for indeterminate plants can be explained to be either due to the occurrence of a dominant mutational event during the tissue culture cycle or that the residual heterozygosity was altered by the tissue culture cycle in the other 6 somaclones but not in the somaclone segregating for indeterminate type. Segregation of a semi-determinate type in an otherwise determinate population has not been reported. The occurrence of semi-determinate plants in 1.9% of progeny of another somaclone therefore evidences a different dominant mutational event. Leaf shape variants in the R2 generation is yet another mutation from a recessive obtuse shaped to obtusely-lanceolate type (lanceolate is dominant over obtuse).

The variant phenotypes for qualitative characters (presence of strophiole, seed shape and colour, seed coat pattern, colour around hilum, flower colour and flowering habit) ranged from

0.25 to 4.00 (TABLE 6) in a set of R1 families arising from a single explant, when the variants of a similar phenotype were considered to have arisen from a single mutational event. This method of frequency calculations was also followed for sugarcane by Lee and Phillips (1987) and for maize by Edallo et al. (1981). However, the frequency of a somaclonal mutational event could be even higher because the variants of a similar phenotype may not have arisen from a single mutational event as these could have occurred independently in different regenerated plants due to the presence of mutation sensitive regions in the chromosomes. With increase in culture time, Lee et al. (1988) observed an increase in the frequency of variant phenotypes in regenerated maize plants from 0.5 to 1.3 variants per regenerated plant while in wheat, Ryan et al. (1987) recovered potentially useful variation for quality characters like increases in kernel weight, hardness and protein content and reduced yellow pigmentation. In soybean, Barwale and Widholm (1990) reported segregation for sterility, wrinkled leaf morphology, multiple branching, twin seeds and leaflet number in at least three generations of selfing. They also found the frequency of qualitative variation per initial regenerant to range from 0.05 to 1.00. Also in maize, Zehr et al. (1986) reported average mutation frequency for qualitative variation per regenerated plant to range from 0.18 to 0.71.

Among the somaclonal population (R1 plants), the highest frequency of variants was observed for the trait seed coat pattern (41% of somaclones) followed by strophiolation (31% of somaclones). About 2% each of somaclones were variant for the traits indeterminate and semi-determinate. White seed coat colour was observed in 16.4% of the somaclones while colour around hilum was observed in 18% of somaclones. Thus a considerable amount and type of variation has occurred in the tissue culture generated putative somaclones.

All the qualitative mutations that apparently occurred were visible in the first self-pollinated generation. The traits such as flowering habit, presence of strophiole and leaf shape are

mutations from recessive to dominance. However, the first two appear to be heterozygous as they are seen to segregate in the next (R3) selfed generation. The progeny of obtusely-lanceolate shaped leaf variant did not segregate. Homozygous mutations otherwise were however not observed.

Genotypic variation for qualitative traits: Chi-square analysis determined that the variant traits under consideration (colour around hilum and presence of strophiole) followed Mendelian ratios. Clonally related plants segregated either as dominant or recessive. The other prominent feature is the production of different mutational events in R1 siblings derived from different explants resulting in both dominant as well as recessive traits under the same cultural conditions.

The R3 progeny lines of individual R2 plants with white seeds or strophiolation segregated in a 3:1 ratio, with white seed coat or presence of strophiole inherited as monogenic dominant. The R2 plants show co-dominant inheritance wherein the mutant genes are expressed in the heterozygotes which segregated for presence or absence of trait in the R3. Further the observation of digenic segregation ratios suggests the probable involvement of two unlinked epistatic loci, the segregation of which could explain the different ratios observed.

In mung bean, Bhatia and Mathews (1988), reported the recovery of monogenically inherited recessive as well as dominant mutations from tissue culture raised plants whereas in wheat, Cheng et al. (1992), observed both recessive and dominant gene mutations at one, two or three loci in the selfed progeny variants as indicated by segregation data. Mutations as a result of changes from dominance to recessive (awns and grain colour) and from recessive to dominance and co-dominance (glume colour and gliadins) were also observed in wheat by Larkin et al. (1984). From this it could be deduced that somaclonal variation is entirely

random and the same cultural conditions can produce different mutational events resulting in both dominant and recessive mutant alleles. This substantiates the above observations on the possibility of higher mutational frequencies. Since plant differentiation occurred early in the tissue culture cycle, the appearance of these variations in siblings of different explants suggests that they occurred at a very early stage, probably prior to plant differentiation. This is supported by earlier observations of Evans and Sharp (1983), who presume that tissue culture mutations observed by them apparently occurred before shoot formation, especially as there were no chimeras. Cheng et al. (1992), also suggest the occurrence of variation prior to plant differentiation as the different R2 families from the same explant gave rise to similar type of variants.

In contrast to the traits presence of strophiole and colour around hilum, the seed coat colour was a mutation from a dominant brown seed to recessive white. Progeny analysis in R3 generation for presence of strophiole, white colour seed coat using chi-square test for a 3:1 model revealed the two traits to be segregating as single gene dominants. Its heterozygous status is evidenced by its further segregation in R3. Therefore mutational events involving the loci governing the traits have apparently occurred and were perpetuated in succeeding generations. In maize, Zehr et al. (1986) and Lee and Phillips (1987) observed culture induced mutant phenotypes to be inherited mostly as single gene recessives. In mung bean, Bhatia and Mathews (1988) recovered monogenically inherited recessive (green cotyledon) as well as dominant (dull seed surface) mutations from tissue cultured plants. In wheat, Ryan et al. (1987), reported significant improvements in quality traits like protein content, seed moisture and reduction in yellow pigmentation. In the present investigation, further inheritance studies need to be taken up for confirmation of inheritance patterns and possible fixation of characters in R4 and test crosses for possible transposable element activity.

The plants regenerated from gamma irradiated explants did not exhibit any qualitative trait variation suggesting that the dosage of 1 KR was not sufficiently large to cause mutations. However, since tissue culture alone did induce mutations in seed coat colour (the one trait among others of great value to breeders), it is difficult to explain the apparently stabilizing effect of gamma irradiation on induction of mutations. Studies at the molecular level may throw light on this aspect. Although the lines selected for strophiolation and white seed coat have been segregating in R3, individual lines were identified for presence of strophiole wherein all the plants showed the character and are presumably fixed for the trait. Thus there is a possibility for the observed traits to breed true in R4 generation.

4.3.3 SOMACLONAL VARIATION FOR QUANTITATIVE TRAITS IN R2 AND R3 GENERATIONS

Phenotypic variation for quantitative traits: Cluster analysis identified somaclones with variations in quantitative traits. It grouped the putative somaclones arising from the same or different original cotyledonary explants, which infers that considerable variability was generated for these quantitative traits. Somaclones with more than one improved agronomic trait, like high biomass, harvest index, seed mass, shelling percentage or reduced plant height or low *Helicoverpa* damage, were grouped together. Cluster I and V were influenced by the yield parameters like plant height, biomass, pod bearing length per plant and per branch and seed mass; cluster II was based mainly on low *Helicoverpa* damage and high shelling percentage and harvest index; cluster IV identified plants with low biomass but high harvest index and shelling percentage, where high harvest index and shelling percentage can be explained by the long raceme length. Although clusters IV and VI had R2 plants with low biomass and high harvest index, they differed in traits like plant height, raceme number, raceme length and pod bearing length per plant. The short raceme length in cluster VI appeared to have been compensated by higher numbers of flowers per raceme; cluster IV

had longer raceme length but fewer flowers per raceme. These observations thus enabled single R2 plant selections from the R1 plants of each cluster for one or more agronomically useful traits. Thus the best performing somaclones were selected for high biomass, seed mass and raceme number from cluster I, low *Helicoverpa* damage, high shelling percentage, harvest index and raceme number from cluster II and for low biomass and high harvest index from cluster VI. The lines selected in R2 represent extremes in variation observed.

From the changing standard deviation with the change in mean values it is clear that the somaclonal population was very heterogeneous for the traits under consideration (plant height, seed mass, shelling percentage, biomass, harvest index, seed number per pod and low *Helicoverpa* damage). The high values of C.V for the traits *Helicoverpa* damage and biomass indicate high variance for the two traits in the somaclonal population. In a normal breeding experiment the seed mass is the least effected. However the extensive differences in the C.V for this trait indicate the tissue culture induced variation.

In a subsequent experiment the following year, regenerated plants derived from tissue culture alone and those derived from explants subjected to gamma-irradiation followed by a tissue culture cycle were compared. For the different parameters studied, shifts to both higher and lower mean values from the control were noticed. Significant improvements over the control as seen in both treatments for *Helicoverpa* damage (SC 19, SC 20, SC 64, IR 5 and IR 7), seed mass (SC 40, SC 64, IR 6) and seed number per pod (IR 5). Thus in the two treatments significant improvements over the control for more than one trait are noted in the regenerated plants (SC 64 for low *Helicoverpa* damage and high seed mass and IR 5 for low *Helicoverpa* damage and high harvest index, shelling percentage and seed number per pod). Tissue culture system of pigeonpea has therefore produced transgressive lines i.e., those superior to parent for one or more traits in addition to performing as well as the parent in other traits. The

recovery of such agronomically useful variants in a population as small as that examined here as comparable to a conventional breeding experiments, should therefore consider somaclonal variation as a potentially useful breeding tool.

Genotypic variation for quantitative traits: The R2 progeny lines selected for reduced plant height, high biomass with high harvest index, low biomass with high harvest index, and high seed mass were analyzed for stable inheritance in the R3 generation. Lines selected for plant height and seed mass maintained the improvements while those selected for biomass and harvest index did not. The short statured plants also differed significantly from parent values for other yield related traits like biomass (lower than control), and harvest index (higher than control). The genotypic variances in all the quantitative traits studied in R2 was evident from the analysis of variance. The high F-values observed in the two sources of variation -- somaclones (R1 plants) and the cotyledonary explants-- for many characters indicate that tissue culture did induce highly variant and random alterations. The square of correlation coefficient was employed in assessing the sources and proportion of variation caused by the independent variables. Tissue culture induced significant variation in plant height. It is also clear that for the traits shelling percentage and *Helicoverpa* damage only about 50% of variation due to the two independent variables (explants and somaclones) is due to tissue culture effects. Most of the variation observed in seed mass, biomass and harvest index cannot be attributed to tissue culture effect, but it could be due to other sources like the various environmental factors and inherent variation. The polygenically controlled quantitative trait variation could therefore occur due to tissue culture or irradiation or it could be due to heterozygosity in the initial plant material. In the course of a tissue culture cycle (with or without in vitro irradiation), mutations could occur in the nucleotide sequences of one or more genes that together affect a particular trait. These could be due to point mutations or due to chromosome rearrangements involving translocations or inversions. From the plant breeders

perspective, the heritable variation occurring in gene(s) during tissue culture and, with large effects in a desirable quantitative trait, would be agriculturally useful.

The sources of variation for plant height, seed mass and biomass in even aged stands of plants was studied extensively by Benjamin and Hardwick (1986), who reported that the weight of plant accumulates during two physiologically distinct periods of growth -- pre- and post-seedling emergence -- and can be expressed as a function of the variances and covariances of the three physiological variables-- the growth rate, duration of growth, and size of plant at start of growth period. It is well known that the variation in availability of environmental resources is accommodated by variation in size while variation in size of one plant affects the environmental resources available to its neighbouring plants thus affecting size. Therefore for the traits related to plant height like plant weight (biomass) and for the traits seed mass a higher proportion of variation due to epigenetic/environmental effects is as expected. This could also be the reason for the non-maintenance of significant improvements for biomass and the other related yield components i.e., harvest index in the subsequent selfed generation.

The traits of agronomic value that were significantly improved over the control besides reduced plant height are seed mass and low *Helicoverpa* damage. Larger seeds are desirable as they have lower polyphenol levels, reduced thickness of pericarp (testa) and are also easier to dehull with less dhal breakage. The data (R3 generation) of the somaclones selected for both low and high incidence of *Helicoverpa* revealed the low incidence lines to perform better than the high incidence lines and the controls in both the spray and unspray treatments. Earlier the control (ICPL 87) has been suggested to be used as infestor rows as it generally has a high incidence of this pest. Improvement in *Helicoverpa* incidence should therefore be viewed as a potential source of resistance.

The differential expression of somaclonal variation is evident in the two treatments -- irradiated and unirradiated-- from the type and amount of variation induced. While irradiation did not produce any qualitative trait variation, significant variation for quantitative traits was observed. Apparently the lower irradiation levels of 1 KR was sufficient to cause changes in at least some of the genes (which make up a polygenic trait) involved in that particular phenotype. The extent of variation is evident in higher coefficient of variation in unirradiated populations as compared to the irradiated populations. Also significantly larger differences in the coefficient of variation are visible in the unirradiated populations. This suggests that the irradiated populations were more homogeneous in the extent of variation. A greater percentage of variation is due to the independent variables -- explant and somaclones -- in the unirradiated explant derived populations, while in the irradiated explant derived populations, the variation is more due to environmental effects. This same trend is also evident from the higher F-values in the populations derived from cultures subjected to tissue culture alone. Therefore the variance due to error (environmental effect) is greater in irradiated than in the unirradiated populations. Higher levels of irradiation are expected to result in chromosome breakage thus causing irreversible changes, while lower doses cause point mutations. In the present study lower doses were used which could have resulted in point mutations which however may not have the desired effect as the affected traits are polygenically controlled. It thus appears that the genome of ICPL 87 was sufficiently affected by irradiation to cause epigenetic variations which could possibly affect its adaptation to local conditions. Higher F-values indicate lower variance due to error or environmental factors and higher variance (as seen from high mean squares) due to changes or mutations that have occurred in the tissue cultured component i.e., the explant, or the R1 plants derived from these explants. The maintenance of the significant improvements for the traits seed mass, low *Helicoverpa* damage and white seed coat colour, all of which are useful agronomically, in the next selfed sexual generation demonstrates a genetic basis for the observed somaclonal variation.

The observations made in this study are in conformity with those in other crops by various other workers. Examples of successful utilization of quantitative traits i.e., those controlled by many genes each with a small effect, are few. In potato Shepard et al. (1980) reported high frequency of variations for several horticultural and disease resistance characters in clonal populations. They also observed stable changes in tuber shape, yield, maturity date, photoperiod requirement for flowering and in plant morphology. Evans and Sharp (1983) recovered several monogenic qualitative traits thus evidencing that plant tissue culture can be mutagenic. In sorghum, Bhaskaran et al. (1987) observed significant reduction in plant height, increases in seed yield and seed number. They also observed that while some of the somaclones did not maintain the original differences, other somaclones showed stable inheritance. In *Triticale*, Jordan and Larter (1985) reported greatest variability for spike length, fertility and plant height. They also observed the second generation plants to have a significant increase in percent kernel protein relative to control. In wheat, Galiba et al. (1985) reported that among the various traits observed, early or late variants and dwarfs were promising for practical breeding purposes. Also in wheat, Chen et al. (1987) observed significant variations for heading date, plant height, 100 kernel weight, tiller number, seed yield and fertility. While most variation was negative some lines exceeded the control for most of the variables examined. Lee et al. (1988) reported lower grain yield and moisture in the tissue cultured maize lines although the highest yielding line per se in 3 of 6 trials and top ranking grain moisture in 5 of 6 trials were tissue culture derived. Male sterility was reported in rice by Ling et al. (1987) and Nowick et al. (1988). An example of pest resistance as somaclonal variation is from sorghum (Isenhour et al., 1991) where significantly higher levels of resistance to fall armyworm as comparable to the non-regenerated and susceptible cultivar was reported.

Early maturity was observed in maize by Lee et al. (1988) and in sorghum by Bhaskaran et al., (1987). Significant increases in dry matter in potato were reported by Evans et al. (1986). Increase in yield per se for oats was reported by Dahleen et al. (1991). In wheat Ryan et al. (1987) identified significant variations for agronomic characters like height, grain number per spike, kernel weight, yield, total dry weight and harvest index which were not maintained in the next generation of the lines selected for high yield or harvest index. However qualitative characters like kernel weight, hardness, protein content and reduced yellow pigmentation were significant and viewed as potentially useful. In barley (*Hordeum vulgare*), Dunvell et al. (1986) found little or no useful agronomic variation. In maize, Lee et al. (1988) and Zehr et al. (1987) have shown hat most of the variation generated is undesirable. Earlier Lee et al. (1988) found early maturing lines with no loss in yield. Other workers like Galiba et al. (1985) and Carver and Johnson (1989) found useful agronomic variation which include heading date, plant height, seed weight, biomass, grain protein and number of spikes per plant. In potato, Shepard et al. (1980) found increases in tuber weight with no significant increase in tuber yield. Significant heritable quantitative trait variation was also observed in rice (Sun et al., 1983), wheat (Maddock and Semple, 1986, Chen et al., 1987, Lazar et al., 1988) and *Lotus corniculatus* (Damiani et al., 1985). In these studies although the variation is deleterious in most cases, a good number of traits performing better than the control are also found. In soybean, (Barwale and Widholm, 1990) reported quantitative trait variation for both early and late maturity, oil content (increase by ca. 25%), protein (both higher and lower values than control). Nagarajan and Walton (1989) reported the somaclonal population of alfalfa to give dry matter yields about 29% lower than the control and also lower values for plant height, stem length, number of nodes per stem and internode length.

Studies on quantitative and qualitative genetics in pigeonpea have been rather limited (Saxena and Sharma, 1990). It is difficult to explain genetically the observed quantitative and

qualitative trait variation based on the available data, and further work would be needed to study the genetics of the variant somaclones. Niizeki et al. (1990), suggest that the quantitative trait variation observed in *Lotus corniculatus* could be caused by polygenic changes and also by minor structural alterations in somatic chromosomes. Alteration of gene expression resulting from changes in DNA methylation during tissue culture (Meins, 1983), also lead to phenotypic variation for both qualitative and quantitative traits (Peschke and Phillips, 1992).

Although the natural outcrossing of 2-70% (Saxena et al., 1993) in pigeonpea allows for a high genetic variability with many diverse genotypes in the primary gene pool, very limited success in identifying *Helicoverpa* tolerance and resistances to major diseases and insect pests have been identified. The observation of low *Helicoverpa* damage in the somaclonal progeny could provide additional genes for pest tolerance for use in crop improvement. The somaclones with white colour seeds which has a market preference or with improved quantitative traits like large seeds, high harvest index and reduced plant height may also be of value for the improvement of this crop, as they may be different alleles to those already known.

5 MOLECULAR ANALYSIS OF SOMACLONAL PROGENY (RAPD ANALYSIS)

5.1 MATERIALS AND METHODS

5.1.1 PLANT MATERIAL

Seed of the best sexual derivatives from the best performing somaclonal variants of *Cajanus cajan* (L.) Millsp. cv ICPL 87 evaluated for improvements in qualitative and quantitative traits was sown in a randomized complete block design. For the experiment on pest resistance a split plot design was employed. To initiate randomly amplified polymorphic DNA (RAPD) analysis, total genomic DNA was extracted from freeze dried leaf material of thirteen individual R3 plants identified for white seed coat colour, high seed mass, reduced plant height, indeterminate flowering habit, strophiolate seeds, high or low biomass with high harvest index,

high or low raceme number, small or large pod bearing length per plant and high or low incidence of *Helicoverpa* damage.

A total of 5 positive controls with the genomic DNA of the parent ICPL 87 from which the putative somaclones were derived was used in this analysis. Three of these were from individual ICPL 87 plants, the fourth was a bulk of the DNA from these three plants and the fifth was a different bulk of DNA from eight other ICPL 87 plants.

5.1.2 GENOMIC DNA ISOLATION

Total genomic DNA was isolated following the CTAB method of Saghai Maroof et al. (1984). Two to three grams of lyophilized leaf powder was dispersed in 15 ml of extraction buffer (1 M Tris-HCl, pH 8.0; 2% hexadecyltrimethyl ammonium bromide (CTAB); 5 M EDTA and 2% fresh β-mercaptoethanol) preheated to 65°C in 30 mL capped bottles. The suspension was incubated for 3 h with occasional mixing. After cooling, 20 ml of chloroform-isoamyl alcohol (24:1) was added and centrifuged at 6 K for 20 min. To the aqueous phase chloroform-octonol was added and centrifuged at 6 K in a refrigerated centrifuge. DNA was then precipitated from the aqueous phase with isopropanol. The fibrous DNA precipitate was spooled out and rinsed in 76% ethanol, 0.2 M sodium acetate and air dried at 37°C. The DNA was dissolved in 400 µL of Tris-EDTA (TE) buffer with RNAse (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Polysaccharides were further removed by addition of NaCl, phenol/chloroform (1:1), and chloroform/octonol (24:1) sequentially with centrifugation between each addition. DNA was finally precipitated in cold absolute alcohol. Precipitated DNA was washed in absolute alcohol, air dried and dissolved in 400 µL TE buffer. DNA was quantified in a RNA/DNA Calculator (GeneQuant, Pharmacia) at 280/260 nm.

5.1.3 DNA AMPLIFICATION AND PCR CONDITIONS

The template for PCR consisted of total genomic DNA. The high molecular weight DNA was used as template for amplification with nineteen decamer oligonucleotide primers of arbitrary sequences using polymerase chain reaction (PCR). The primers used in this study are OPA-01 to 07 and OPA-09 to OPA-20 from KIT A of Operon Technologies Inc. The PCR amplification mixture comprised of 200 ng genomic DNA, 0.2 µM each primer, 50 µM each dNTP (dATP, dCTP, dGTP, dTTP from Perkin-Elmer Cetus), 2 µL of reaction (PCR) buffer (0.05 M KCl, 1.5 mM $MgCl_2$, 0.01 M Tris-HCl pH 8.2 and 0.1 mgL^{-1} gelatin) and 1.5 units of Taq polymerase in a 20 µL reaction volume taken in a 1.5 mL PCR tube. The reaction mixture was overlaid with approximately 0.05 mL of paraffin oil. Forty one amplification cycles of PCR were performed in a sixty- well thermal cycler (MJ Research Inc.) with the following temperature conditions: 94°C for 4 min, 38°C for 1 min, 72°C for 2 min, followed by forty cycles of 93°C for 1 min, 40 °C for 1 min, 72 °C for 1 min, and ending with 10 min at 72°C. The fastest available transition between each temperature was used.

5.1.4 FRACTIONATION OF PCR PRODUCTS

The separation of amplification products was by electrophoresis through 1.4 % agarose (Sigma) gel made up in 1x TBE (89 mM Tris - Borate, 89 mM Boric acid, 2 mM EDTA). The wells were loaded with 20 µL of PCR mixture and electrophoresed initially at 60 Volts/cm for a half hour and the rest of the run at 40 Volts/cm. They were then stained with ethidium bromide and visualized using an ultraviolet transilluminator. Gels were photographed and the bands were scored from 4x5" polaroid film.

5.2 RESULTS

Eighteen of the nineteen 10-mer oligonucleotide primers tested amplified 3 to 7 fragments each. Six of these eighteen primers revealed 1 to 5 polymorphic bands each. These 6 primers showed distinct polymorphism between 2 to 12 somaclones as well as with the control.

Primer OPA-07 (5'-GAAACGGGTG-3') distinguished the indeterminate, white seeded, high seed mass and the small and large pod bearing length variants from the five positive controls (PLATE 9A). The indeterminate and the white seed coat variants amplified the same fragments while the other two variants were polymorphic between each other and with the controls.

Primer OPA-10 (5'-GTGATCGCAG-3') was suitable for distinction between indeterminate, strophiolate, small pod bearing length, high biomass, low biomass, high *Helicoverpa* damage and high and low raceme number variants (PLATE 9B). All the variants are also polymorphic with the five positive controls.

Polymorphism between the controls and the white seed coat, high seed mass, and high and low raceme number variants was noticed with the primer OPA-13 (5'-CAGCACCCAC-3') (PLATE 9C). However while the white seed coat and the high seed mass variants amplified the same fragments, they were polymorphic to high and low biomass variants which were polymorphic to each other.

The somaclonal variants for high seed mass, large pod bearing length, high biomass and high *Helicoverpa* damage were distinguished from each other as well as from the controls when the primer OPA-15 (5'-TTCCGAACCC-3') was used (PLATE 9D).

PLATE 9

Fractionation of PCR products of total genomic DNA of selected somaclones through a 1.4% agarose gel. Lane1: negative control; Lanes2 to 6: positive controls: 5 and 6 contain bulks; Lane7: indeterminate; Lane8: strophiolate; Lane9: white seed coat; Lane10: short statured; Lane11: high seed mass; Lane12: small pod bearing length; Lane13: large pod bearing length; Lane14: high biomass with high harvest index; Lane15: low biomass with high harvest index; Lane16: low *Helicoverpa* damage; Lane17: high *Helicoverpa* damage; Lane18: high raceme number; Lane19: low raceme number; Lane20: lambda marker. The following primers show polymorphism for the agronomically important somaclones.

9A Primer OPA-07 showing polymorphism for white seed coat and high seed mass.

9B Primer OPA-10 showing polymorphism for high biomass with high harvest index.

9C Primer OPA-13 reveals polymorphic bands for white seed and high seed mass variants.

9D Primer OPA-15 reveals polymorphism for the variant high biomass with high harvest index.

9E Primer OPA-19 showing polymorhism for the traits white seed, short statured and high seed mass variants.

9F Primer OPA-20 shows polymorphic bands for white seed, short statured, high seed mass, high biomass and low *Helicovarpa* damaged variants.

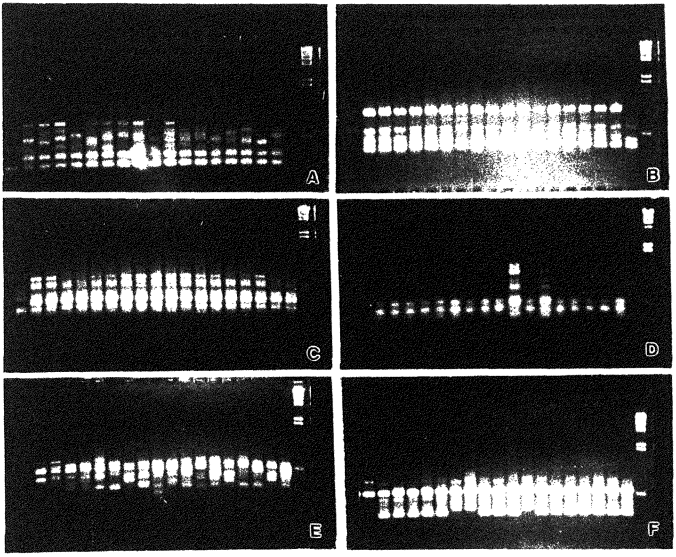


PLATE 9

Indeterminate, strophiolate, white seeded, reduced plant height, high seed mass, small and large pod bearing length, low and high *Helicoverpa* damage and low raceme number variants were polymorphic to each other and to the controls with the primer OPA-19 (5'-CAAACGTCGG-3') (PLATE 9E). However, there was no polymorphism between low and high *Helicoverpa* incidence variants.

All the variants were polymorphic to each other as well as from the positive controls when RAPD analysis was conducted with the primer OPA-20 (5'-GTTGCGATCC-3') (PLATE 9F). The results reported here were reproducible when the experiment was repeated.

5.3 DISCUSSION

Among the variants studied in this RAPD analysis, the white seed coat, reduced plant height, high seed mass, high biomass and low *Helicoverpa* incidence variants are of agronomic use. These along with other quantitative and qualitative trait variants were studied with the objective of identifying RAPD markers (using PCR) which may be linked to the gene of interest. Primers OPA-07, 13, 19 and 20 appear to be suitable for the observation of polymorphism between the white seeded variant and the parent ICPL 87. Primers OPA-19 and 20 distinguished polymorphic fragments between reduced plant height and ICPL 87. For the traits high seed mass, polymorphism was evident on using primers OPA-07, 13, 15 19 and 20. The high biomass variants were distinguished from the ICPL 87 with the primers OPA-10, 15 and 20.

One primer i.e., OPA-20 identified the somaclonal variant with low *Helicoverpa* incidence to be polymorphic to high *Helicoverpa* incidence variant and also to the control ICPL 87. The results of the screening for pest incidence in R3 generation agree with this as the somaclones with low *Helicoverpa* damage (SC19 and SC20) maintained lower mean values than SC40 and SC60 which show higher pest incidence. Besides, the two variants are also polymorphic to ICPL 87. This too is in conformity with the field trials of the R3 generation where both the variants selected for extremes of pest incidence also showed lower mean values for *Helicoverpa* incidence than the control ICPL 87.

High level of polymorphism is evident with the primer OPA-20 followed by primers OPA-19, 15 and 10. A low level of polymorphism is seen with the primer OPA-07. This high level of polymorphism with certain primers could be explained by the capability of individual RAPD primers to amplify less conserved and highly repeated regions of the DNA. Devos and Gale

(1992) and Paran and Michelmore (1993) have reported earlier that there is a high probability for the amplified fragments to contain repeated sequences.

Detection of markers for quantitative traits would be a great asset to the plant breeder for improvement of pigeonpea crop. Breeding for *Helicoverpa* resistance which is a complex trait has thus far yielded little success. Resistance could occur either due to a thick pod wall, hairy pod or due to antibiosis. Development of markers for components of resistance, especially those which are difficult to score for e.g., antibiosis would be of immense value to the pigeonpea breeder.

In this study somaclonal variants could be distinguished at the molecular level by using specific arbitrary sequences of 10-mer primers, which helped in verification of their variant nature from earlier experiments. Since it has been demonstrated that RAPDs segregate in a Mendelian fashion, each fragment scored could indicate a unique locus (Reiter et al., 1992 and Tinker et al., 1993). Thus the somaclonal variants such as the white seeded (which has a market preference due to lower polyphenol content), high seed mass (which reduces the thickness of the pericarp or testa enabling easier dehulling) and the reduced plant height, high biomass and low *Helicoverpa* incidence variants (which affect yield) all appear to have additional alleles which may be of great value for the improvement of this crop. Thus tissue culture has produced somaclones with better agronomic performance than the donor plant which could be agriculturally useful when integrated into the existing pigeonpea breeding programmes.

6 GENERAL DISCUSSION

The present investigation resulted in optimization of a high frequency regeneration protocol from seedling cotyledons of pigeonpea which enabled identification of putative somaclonal variants of agronomic value. Variation at the molecular level was also detected using RAPD analysis.

6.1 TISSUE CULTURE AND REGENERATION

Initial efforts in morphogenesis of pigeonpea using MS medium supplemented with various auxins and BA alone or in combination succeeded in induction of somatic embryogenesis from embryo explants such as immature cotyledonary stage zygotic embryo, mature zygotic embryo and immature and mature cotyledon. However whole plant regeneration was not

achieved. While the first two explants produced embryogenic cultures in the presence of auxin alone, the immature and mature cotyledons produced the globular stage somatic embryos only in the presence of BA. With very few exceptions, initiation of regenerable embryogenic cultures from immature embryo explants in large seeded legumes has to date relied almost exclusively on high auxin and no cytokinin growth regulator regimes. Most workers on somatic embryogenesis in soybean evidence the exclusive use of auxin alone for induction (Phillips and Collins, 1981, Christianson et al., 1983, Li et al., 1985, Lazzeri et al., 1985, Ranch et al., 1985, Barwale et al., 1986, Ghazi et al., 1986, Hammatt and Davey, 1987 and Parrott et al., 1988). Similar observations were made in other crops like peanut (Ozias-Akins, 1989; Sellars et al., 1989; Hazra et al., 1989) Chickpea (Rao and Chopra, 1989 and Kumar et al., 1994), *Vicia faba* (Griga et al., 1987) and pigeonpea (George and Eapen, 1994). High concentrations of auxins like picloram (Kysely and Jacobson, 1990, Sellars et al., 1990 and George and Eapen, 1994), NAA (Lazzeri et al., 1987, Ozias-Akin, 1989) and 2,4-D (Hazra et al., 1989, Sellars et al., 1990, George and Eapen, 1994) have been reported to promote somatic embryo formation in grain legumes. Addition of cytokinins like zeatin, kinetin and BA to media containing 2,4-D inhibited embryogenic tissues and embryoid induction in cotyledon explants of soybean (Lippmann and Lippmann, 1984). Lazzeri et al. (1987) also showed that inclusion of BA in an auxin containing medium reduced somatic embryo induction, or, had no effect in cotyledonary explants of soybean. However, Gill and Saxena (1992) and Murthy et al. (1995) reported somatic embryogenesis in peanut using thidiazuron, a substituted phenylurea with cytokinin-like activity.

Seedling cotyledons of pigeonpea cv ICPL 87 regenerated efficiently in the presence of BA alone at 8.9 µM. This indicates that the in vitro application of BA activates the morphogenic potential as was also observed in peanut by Diamon and Mii (1991). Zaerr and Mapes (1982) suggested that this promotive effect of BA could be attributed either to the abilities of the plant

tissues to metabolize BA quite efficiently or to the ability of BA to induce endogenous production of zeatin. The cultures initiated on L2 medium with 8.9 µM BA were regularly subcultured on maintenance medium with 0.44 µM BA where shoot buds proliferated extensively. On transfer to L2 medium with 0.44 µM BA, 1% activated charcoal and 2% sucrose these shoot buds elongated. However, additional shoot buds elongated only on removal of these earlier shoots which could be due to apical dominance, as was also observed in pea by Nielsen et al. (1991). Other reports on pigeonpea regeneration using BA alone are from Mehta and Mohan Ram (1981), Kumar et al. (1983, 1984) and Prakash et al. (1994). All other reports included auxins in the regeneration media. All these workers used either MS or B5 medium.

Although longitudinally cut pigeonpea stem segments cultured on L2 medium produced whole plant regeneration, the frequency of responding cultures was only 40% in comparison to 100% from seedling cotyledon. Kumar et al. (1984a) reported 38% of cultures to produce shoot formation from the cut region of epicotyl segments when cultured on Blaydes medium supplemented with BA alone. It is observed that in pigeonpea, regeneration from explants other than cotyledon occurs at low frequencies (Shama Rao and Narayanaswamy, 1975, Kumar et al., 1984b, Reena et al., 1992 and Eapen and George, 1993, 1994). This implies that morphogenic potential differs significantly with the source of explant (Murashige, 1974).

Although MS medium is basically known to produce organogenic mode of regeneration, it has been widely employed for studies on somatic embryogenesis in most crop species for e.g., groundnut (Gill and Saxena, 1992), pigeonpea (George and Eapen, 1994) and chickpea (Kumar et al., 1994). L2 medium was effectively used by several workers to obtain somatic embryogenesis in *Vicia faba* (Griga et al., 1987) peanut (Sellars et al., 1990) and *Trifolium* (Collins and Phillips, 1982). In the present study L2 medium was superior to either MS or B5 in

producing an organogenic system of regeneration. Although L2 medium was developed by Phillips and Collins (1979) as an embryogenic system for red clover, it was highly effective for plant regeneration via organogenesis in all six genotypes of alyceclover (*Alyscicarpus vaginalis*) that were studied by Wofford et al. (1992). They were unable to recover plants from MS and B5 media from all these genotypes.

The major salts composition of L2 is similar to MS except for increased levels of K, P, Ca and Mg and decreased levels of NH_4NO_3. Among the major elements ammonium per se is critical for the growth of cells. Gamborg and Shyluk (1970) reported a reduction in cell growth of some plant systems when cultured in the presence of high ammonium concentration. Further ammonium is utilized rapidly and before nitrate when the two are in combination (Gamborg, 1984). This would mean that in high ammonium nitrate media (MS medium), there would be an increase in nitrate ions which leads to acidic state which effectively hinders further growth. In suspension cultures of white clover (White, 1984) lowering the NH_4NO_3 concentration in the MS medium from 20.6 mM to 2.6 mM (the concentration in SH medium) or 10 mM (the concentration present in L2 medium), produced increased meristemoid formation. Addition of potassium phosphate to the MS medium (MS has lower levels of potassium and phosphate than L2 medium) increased the shoot forming ability of chickpea callus culture (Barna and Wakhlu, 1994). The minor salts composition is similar to Schenke and Hildebrandt (SH) medium with generally high levels excepting B and Mn. High levels of B and Mn can produce toxicity symptoms (Collins and Phillips, 1982).

L2 medium has higher levels of myoinositol and lower levels of sucrose concentrations. Since higher concentrations of sucrose increase the levels of polyphenols which result in browning, L2 with its lower levels of sucrose could have been beneficial for promoting high morphogenesis in the apparent absence of polyphenols. Also the use of activated charcoal,

which adsorbs hydroxymethyl-furfural, an inhibitor formed by sucrose degradation due to autoclaving could have enhanced shoot proliferation and also shoot elongation by eliminating the residual effects of BA (Maene and Debergh, 1985).

It is now well known that regeneration in tissue culture is genetically controlled (Bhojwani et al., 1984). In *Vigna* (Gulati and Jaiwal, 1992) the frequency of shoot regeneration and the number of shoots per explant varied among cultivars. Such genotypic differences were also reported in *Pisum* (Malmberg, 1979 and Rubiuo et al., 1984), *Phaseolus* (Rubiuo and Kartha, 1985) and peanut (McKently et al., 1991).

Studies on embryogenic capacity documented the influence of genotype in producing a differential embryogenic response. Both high and low frequency of regenerating genotypes were observed in clovers (Arcioni and Mariotti, 1982, Arcioni et al., 1990), alfalfa (Williams et al., 1990, Atanassova and Brown, 1984). This response was exploited to breed genotypes with high regeneration capacity (67% regeneration frequency from an initial 12% in 'Regen S') in alfalfa (Bingham et al., 1975). In soybean, Komatsuda and Ohyama (1988) found a strong influence of genotype on embryogenic capacity of immature explants of soybeans. The germination of induced somatic embryos was also influenced by the genotype. In alfalfa, Saunders and Bingham (1972) reported genotype dependency of regeneration. In *Arachis*, Ozias-Akins et al. (1992) reported significant differences among genotypes for somatic embryo formation, subculture capacity and plant regeneration using a single media sequence for immature cotyledon and embryo axis explants. George and Eapen (1993, 1994) also observed the frequency of response and the average number of somatic embryos per explant to vary with genotype in peanut as well as in chickpea. Wofford et al. (1992) observed genotypic variation in alyceclover for callus induction and growth, shoot initiation and development, root development and plant regeneration. However, Barwale et al. (1986) report that different

genotypes with differing maturity groups, seed coat colour etc. did not influence plant regeneration to any substantial degree.

Wan et al. (1988) studied tetraploid alfalfa and concluded that somatic embryogenesis was under the control of 2 dominant genes, *Rn*3 and *Rn*4 with complementary effects. Similarly, Hernandez-Fernandez and Christie (1989) observed somatic embryogenesis to be under the control of two complementary loci *Rn*a and *Rn*b acting in a dominant fashion. Kielly and Bowley (1992) in their studies on alfalfa, confirmed these findings that somatic embryogenesis was dominant and governed by two loci. The regeneration capacity is also believed to be determined by the interaction of many genes. Using diploid germplasm, Reisch and Bingham (1980) concluded that 2 genes *Rn*1 and *Rn*2 controlled bud differentiation from callus of alfalfa. Galiba et al. (1986), Mathias and Fukui (1986), Felsenberg et al. (1987), and Kaleikau (1989 a,b), observe that nearly all the chromosomes of the genome may be involved in regulation of plant morphogenesis. Further, it is also believed that one or two genes may have a major influence on plant regeneration (Reisch and Bingham, 1980, Hodges et al., 1985 and Ma et al., 1987). Thus it can be deduced that depending on the environmental components, developmental stage of explants, and the genotype, the regulation of regeneration is determined by the interaction of one to many genes.

From the reports thus far it is clear that regeneration of different pigeonpea genotypes from diverse explants was obtained on using either MS, B5 or L2 media. It is evident that just as in other crop plants, regeneration of pigeonpea is also governed by the environmental factors such as inorganic or organic salts and hormonal inclusions, age of explant and the genotype. In the present study an efficient and high frequency regeneration was developed from seedling cotyledon of ICPL 87. This regeneration system enabled the recovery of useful somaclonal variants viz. white seed coat, large seed and low *Helicoverpa* damage. Prior to

this study there has been no report on somaclonal variation in pigeonpea. Therefore, screening of different pigeonpea genotypes could provide regenerating cultures with higher frequencies of useful somaclonal variants.

6.2 SOMACLONAL VARIATION

Tissue culture techniques were developed to exploit somaclonal variation which can generate plants with improved agronomic traits. Regenerated plants were grown in the glasshouse where seed was collected on maturity. Field testing of regenerated plants began in 1993. The best sexual derivatives from the best performing somaclonal variants were evaluated for improvements in the selected qualitative and quantitative traits of agronomic importance.

Somaclonal variation ensuing from plant cell, tissue and organ culture is well documented in literature. It is one major product of plant tissue culture which can make significant impact on agriculture. However, for the somaclonal variants to be useful to the agricultural sector, their identification and evaluation under field conditions is mandatory. A sound field testing design in conjunction with a vigorous plant breeding program is essential for the final release of the agronomically useful regenerated plant material.

Although considerable genetic variability exists in the primary gene pool of pigeonpea, the current cultivated crop is based to a large extent on only a small portion of the available germplasm. Sufficient resistance to important diseases (e.g., Fusarium wilt) and pests (e.g., *Helicoverpa armigera*) which are the major threats to the resource poor farmer is still found lacking. Lines released as improved varieties for insect or disease resistance or high yielding have more often than not lost their improvements over a few generations. This could be due to low pest or disease pressure at selection time or non-adaptability to changing

environmental conditions. Wide crossing has featured significantly as one of the options to introduce variability. *Cajanus platycarpus*, a wild species of pigeonpea with annuality, extra early flowering and maturity, photoperiod insensitivity and also resistance to phytophthora blight, was used as the female parent to wide hybridize with *C. cajan*; the hybridity of the F1 was confirmed (Mallikarjuna and Moss, 1995). Although mutation breeding can provide the desired additional genes, the formation of chimeras and low mutation frequencies are its main drawbacks. On the other hand, plant transformation by transfer of DNA using *Agrobacterium* or by direct gene delivery can efficiently overcome the many obstacles in obtaining elite germplasm. However, efforts in this direction are still in the initial stages. It is in this context that somaclonal variation with its potential to improve an already improved genotype by altering a gene or loci governing the desired trait can provide the much needed variability via the additional gene.

Resistances to some of the diseases and insect pests have been found within the pigeonpea gene pool. However, additional genes for pest resistance resulting from in vitro selection would be an important addition to the breeders arsenal. The pigeonpea plant breeding programmes require greater variability than is available to handle problems such as pest or salt and drought tolerance. Tissue culture can therefore come to the rescue by providing additional genes or alleles as a result of higher incidence of mutations than in conventional mutation breeding.

In the present study considerable variation was observed for qualitative and quantitative traits of agronomic importance. The source of quantitative variation in tissue cultured plants was primarily due to the original explants and the individual somaclones which gave rise to the R2 families. However in the γ-irradiated populations the environmental factors effected the apparent variation to a greater extent which would mean that crosses would be necessary to

further develop somaclonal lines usable in breeding programmes. It is suggested that the major mutational events leading to the observed variation among the regenerated plants and their progeny could have occurred during the course of culture and plant regeneration. Most of this variation was heterozygous and therefore a continuous segregation within the families was a common occurrence for all qualitative traits except leaf shape. Stable changes for the quantitative characters like strophiolation, white seed coat colour, plant height, seed mass, biomass, and *Helicoverpa* damage were observed in the R3 generation. The genetic basis of these variant traits needs to be further studied for ensuring effective integration of desirable characters in subsequent generations of plant breeding programmes.

In a conventional breeding programme, transgressive lines i.e., those superior to the parent lines in at least one important characteristic are selected. In this investigation such transgressive lines superior for more than one trait (low *Helicoverpa* infestation, high seed mass, white seed coat colour, high seed number) were identified and are a potentially useful source of additional genes for possible implementation into pigeonpea crop improvement programmes.

Bajaj et al. (1990) described a mixed reaction from plant breeders regarding the usefulness of somaclonal variation which, they also opine requires rigorous selection and full trials before being accepted. Quereshi et al. (1992) reported that somaclonal variation is not a reliable tool for spring wheat improvement as the variants do not perform any better than the controls. Ryan et al. (1987) recovered only limited agronomically useful variation in wheat. The negative aspect of somaclonal variation is highlighted in the efforts of McCoy (1987), to obtain NaCl tolerant lucerne, wherein the regenerated tolerant plants exhibited high levels of variation with one plant failing to flower while the other was completely sterile. However, Larkin et al. (1989) reported agronomically useful variation by recovering superior outliers for a number of

characters. Maddock and Semple (1986) reported stable changes in height and morphological traits. They propose that the application of tissue culture techniques will be influenced by the amount of variability or uniformity that can be expected in plants regenerated from tissue culture. Although most plant breeding programmes require somaclonal variation which can produce variability that is different from that obtained by existing procedures, where tissue culture is used as part of a process of genetic manipulation of plants in vitro (e.g., transformation), it would however be desirable to eliminate the background somaclonal variation.

Despite the controversy regarding the usefulness of somaclonal variation for crop improvement, the current results indicate a definite gene for white seed coat colour and the possibility of additional genes for pest tolerance and high seed mass in an already adapted background of *Cajanus cajan* (L.) Millsp. cv ICPL 87. However, just as in a breeding programme, somaclonal variants -- SC19, SC20, SC64 and IR5 -- with an acknowledged improvement in one or more traits need to be exposed to adequate environmental pressure to eliminate possible epigenetic effects. The preliminary results can help the breeders in terms of varietal improvement, since the variants observed resemble the parent genotype with the exception of the specific trait for which they are superior. Ryan et al. (1987) suggested that single gene changes produced in specific genetic background may be one of the most useful application of somaclonal variation to plant breeding. Thus, even the advanced lines with one useful trait but with single character faults may not have to be discarded if the somaclonal variant for the character fault can be conveniently isolated. This would require crosses to further develop somaclonal lines for use in breeding programmes. Further they also suggest that if the different variants recovered represent changes at more than one locus, then it could be possible to recombine the variants to produce even further variation than was originally present in the regenerated plants or their progeny.

Due to the lack of sufficient genetical studies in the pigeonpea crop in general and since such a study is presently beyond the scope of this investigation, the derivation of the genetical basis of the observed random array and extent of variation in tissue culture generated plants, especially in the absence of intentional mutagenesis proves to be a little difficult. Also with the present lack of knowledge in the cellular basis of many agronomically important traits, it is as yet not possible to control and direct the variability that occurs in culture. For most crops the difficulty in identifying and isolating desired recombinants still remains as a limiting factor. Therefore it would be desirable to regulate the degree of variation in addition to establishing the source and nature of variation before identification of the varieties as a useful adjunct to conventional breeding. With this achievement somaclonal variation can indeed prove to be very valuable for crop improvement.

In conventional breeding stable lines can be obtained only after 6 to 8 generations due to gene segregation and recombination in the hybrid progenies. However, with the occurrence of homozygous mutations during the course of a tissue culture cycle, it is possible to fix the characters in the first regenerated plant or otherwise it may take about 2 to 4 generations. The homozygosity of the mutant genes in later generations could be as a result of the phenomenon of gene conversion (Holliday, 1964) where non-reciprocal transfer of genetic information can occur between repeated DNA sequences.

Two possible sources exist for tissue culture induced variability. The expression and perpetuation of inherent somatic variation already existing in explant and the mutational event induced as a result of tissue culture. Although much attention was directed towards generation of somaclonal variation, knowledge on the genetic mechanisms that give rise to these variations is still limited. Keyes et al. (1980) believe the variation in red clover is due to the additive effect of mutant genes. Shepard et al. (1980) considered the changed traits to

be a result of expression of the genes rather than the gene mutation. Larkin *et al.* (1989) reviewed the genetic consequences of somaclonal variation to be due to single base changes, altered gene copy number, altered expression of multigene families, chromosome fragment interchanges and mobilization of transposable elements. The action of transposable elements as a source of genetic variation among the regenerated plants was suggested by Larkin and Scowcroft (1981), Karp and Bright (1985), Groose and Bingham (1986) and Peschke and Phillips (1992).

In the present investigation somaclonal variants of the released pigeonpea variety ICPL 87 were recovered. The white seed coat mutant has provided a specific additional gene. White seed coat being a recessive trait, its segregation in the later (R3) generation (and at the time of writing this thesis the R4 generation), indicate its reverse mutation to dominance. For the qualitative traits *Helicoverpa* resistance and high seed mass, the mutations could have occurred for 1 or more genes that make up the trait(s). Thus the tissue culture system of pigeonpea cultivar ICPL 87 has provided additional genes for further use in plant breeding programmes.

6.3 SOMACLONAL VARIATION AT MOLECULAR LEVEL

The molecular changes associated with tissue culture appear mostly to involve alterations in heterochromatin regions of chromosomes or regions composed of repetitive DNA sequences. Deamplification of repetitive sequences was detected among somaclones of potato (Landsmann and Uhrig, 1985), wheat (Breiman *et al.*, 1987), and *Triticale* (Brettel *et al.*, 1986), while amplification of DNA as a result of tissue culture was reported in tobacco (Reed and Wernsmann, 1989) and in *Triticale* (Lapitan *et al.*, 1988). Both amplification and deamplification

of DNA sequences have been observed in maize callus (Brown et al., 1991). Although the function of most of this repetitive DNA is unknown, the majority of these sequences do not code for protein. Thus they can be transposed, amplified or even deleted without any adverse effect on the organism (Flavell, 1982). However, rearrangements involving these repeated sequences may effect the expression of genes in adjacent regions. The genetic stability in regenerated plants is also believed to be due to the changes in the repeated DNA sequences (Kidwell and Osborne, 1993). In legumes Siva Raman (1984) observed repetitive DNA with both long as well as short sequences comprising of 25 to 90% of the genome. RAPD markers are capable of detecting polymorphisms in both single copy and repetitive DNA resulting from insertions, deletions, rearrangements or single base changes (William et al., 1990).

Alterations in plant DNA methylation are reported to be another molecular event in somaclonal variation. Most studies involve the use of the two isochizomers Hpa II and Msp I, the first cleaves the CCGG sequence when the internal cytosine is methylated and the latter cuts only when the external C is methylated. Brown and Lörz (1986) reported overmethylated maize somaclones along with those which were preferentially digested by Hpa II. Ball (1990) suggests that undermethylation could as well lead to activation of transposable elements which in turn generate a vast array of changes seen in somaclonal variation.

This study detected molecular variation for presence or absence of fragment(s) when the putative somaclones were primed with six specific primers from Operon Kit A. Significant among these is the polymorphism detected in the somaclone SC20 for low *Helicoverpa* damage and SC4 for white seed coat colour. This may eventually lead to the development of molecular markers for these two traits of utmost importance to the pigeonpea breeder. If desirable the gene of interest may be cloned for use in the development of a linkage map of pigeonpea.

Other components of the genome subject to somaclonal variation are the two cytoplasmic genomes i.e., chondriosome and plastosome. The chloroplast genome which is well conserved during evolution shows lower rate of variation as compared to the mitochondrial genome. This could be due to the presence of two inverted repeat sequences (Fluhr and Edelman, 1981 and Palmer and Thompson, 1982). While Kemble and Shepard (1984) found no diffusion for chloroplast DNA (cpDNA) in tissue cultured potato, Day and Ellis (1984, 1985) observed large deletions (upto 80% loss of sequences) in a culture of wheat. Mitochondrial DNA variation was reported in maize by Chourey and Kemble (1982) who found a loss of the S1 and S2 linear mitochondrial plasmids. However, this was not observed in another genotype of maize studied by McNay et al. (1984). Other reports on mitochondrial DNA variability were from Gengenbach et al. (1981) and Umbech and Gengenbach (1983). In this study the progeny of a single tissue culture regenerated plant (SC25) of pigeonpea cultivar ICPL 87) contained a partially male sterile and possibly completely female sterile plant which could be the result of cytoplasmically encoded changes.

As a result of the present study somaclonal lines with additional gene for white seed coat colour, and possibly additional genes for the traits encoded by multiple loci e.g., high seed mass and low *Helicoverpa* incidence were selected. The observed variation for quantitative traits indicates a great potential for additional variability in ICPL 87 since more than one gene could have been affected simultaneously. The R4 generation was grown in the breeders field where it was compared with material generated by hybridization. The somaclones appear to have integrated the traits for which they were selected. From this material it would therefore be convenient to develop molecular markers for the important trait *Helicoverpa* resistance especially so if the resistance is due to antibiosis which is a difficult trait to score. Thus tissue cultured pigeonpea was altered at the nuclear or extranuclear DNA (gene) level which provide additional variability for integration into pigeonpea breeding programmes.

7 SUMMARY AND CONCLUSIONS

The present study addressed the problem that the extent of somaclonal variation in tissue culture regenerated pigeonpea had not been investigated. The previously low regeneration frequencies due to the difficulties in overcoming recalcitrance problems precluded such a study. The present investigation optimized a high frequency regeneration for maximal identification of somaclonal variation.

Whole plant regeneration was achieved from 6-day old seedling cotyledons when cultured on L2 medium supplemented with 8.9 µM BA and from longitudinally cut stem segments cultured on the same medium incorporating 10 µM BA and 0.1 µM NAA. Shoot buds from seedling cotyledons were elongated on transfer to 0.44 µM BA, 1% activated charcoal and 2% sucrose. The elongated shoots were rooted on L2 hormone free medium. For additional variability precultured seedling cotyledons were exposed to a range of γ-irradiation. Explants subjected

to 2.5 KR and above lost their morphogenic ability. Exposure to 1 KR irradiation provided a regeneration ability equivalent to LD30, thus a dose of 1 KR was employed in all irradiation studies.

Phenotypic variations from parental morphology for qualitative traits were observed for leaf shape (obtusely lanceolate), flower colour (purple), flowering types (indeterminate and semi-determinate), white seed coat colour, colour around hilum and strophiolation. The average frequency of these variant phenotypes varied from 0.25 to 4.00 per regenerated plant per explant, while the frequency of variant R1 plants in the somaclonal population for seed coat colour was 0.16, colour around hilum was 0.18, seed coat pattern was 0.41, strophiolation was 0.31, indeterminate and semi-determinate were 0.016 each. A single R2 plant from a putative somaclone flowered profusely but completely failed to set pod. 4.3% of progeny of SC7 segregated for indeterminate type of flowering habit while 1.9% of the progeny of SC25 segregated for semi-determinate type. Genotypic variation for qualitative traits colour around hilum and presence of strophiole was evident from Chi-square tests. Clonally related R1 plants segregated as both dominant and recessive traits. Also for a particular trait, one set of clonally related plants segregated as dominant while the other set segregated as recessive.

Phenotypic variations for quantitative traits such as reduced plant height, seed size, low *Helicoverpa* incidence, variation in raceme number and length, pod bearing length per branch and per plant were also observed. Data was scored separately for each individual R2 plant. Cluster analysis of the data using Ward's minimum variance grouped the putative somaclones into six clusters. From cluster information and cluster means, somaclones were identified for high biomass, seed mass, *Helicoverpa* damage and raceme number from cluster I, for low *Helicoverpa* damage, high shelling percentage from cluster II and for reduced plant height, low biomass and high harvest index from cluster VI. The best performing sexual derivatives from

the putative somaclones with agronomically useful variation for plant height, seed mass, biomass, harvest index and low *Helicoverpa* incidence were selected. In addition progeny plants identified for the other extreme values of the traits in question were also selected for inheritance studies in the R3 generation.

The genotypic variation in quantitative traits was studied by conducting analysis of variance. Highly significant F-values were observed among the somaclones as well as between the R1 families originating from different explants. The square of the correlation coefficient provided the source and the proportion of variation caused by the independent variables-- somaclones and explants. Sixty eight percentage of variation for the trait reduced plant height appeared to be due to the two independent variables. For the traits *Helicoverpa* damage (53%), shelling percentage (59%) and seed number per pod (58%), the independent variables as well as the other sources appear to contribute almost equally while for seed mass (36%), biomass (34%) and harvest index (37%) a higher proportion of variation could be due to sources other than the two variables.

The R1 plants regenerated from explants exposed to γ-irradiation did not exhibit any variation for qualitative traits. However, phenotypic and genotypic variation for quantitative traits was observed. In cluster analysis of both irradiated and un-irradiated treatments, the somaclonal populations were grouped in clusters distinct from the control ICPL 87 which formed a cluster by itself. Similar results were observed with *Helicoverpa* resistance where both populations have lower *Helicoverpa* incidence (27.8% for the irradiated IR5 and 27.1% for the unirradiated SC64) than the control ICPL 87 (43.7%). Besides low *Helicoverpa* incidence, IR5 was also identified for reduced plant height, low biomass, high harvest index, shelling percentage and seed number per pod compared to the control values. The unirradiated sample displayed a shift in mean values in both positive and negative directions for plant height and shelling

percentage. A shift towards higher values than the control was observed for seed mass and harvest index while a shift to lower mean values than control was observed for *Helicoverpa* incidence, biomass and seed number per pod. SC64 was identified for low *Helicoverpa* incidence and high shelling percentage. Variance as measured by the coefficient of variation is higher for unirradiated populations than the irradiated populations. Significantly higher genotypic variation is seen for unirradiated tissue culture derived population than the irradiated and tissue cultured population as evidenced from the higher F-values in the analysis of variance.

R3 seed selected from the best sexual derivatives of the putative somaclonal R2 progeny for the traits white seed coat, seed strophiolation, reduced plant height, high seed mass and high and low biomass was field sown the following year in randomized complete block design while seed from the plants selected for the traits high and low *Helicoverpa* incidence was sown in a split plot design. Observations were again scored for individual plants in the progeny lines. Chi-square analysis for the qualitative traits white seed coat colour and seed strophiolation indicated a good fit to 3:1 ratio with the presence of the trait segregating as dominant. R3 plants selected for reduced plant height, seed mass and high biomass maintained the traits while the progeny from the plants selected for low biomass did not. In both sprayed and unsprayed treatments in the experiment to study *Helicoverpa* incidence, both the progeny lines selected for high and low *Helicoverpa* incidence maintained their improvements over the control in the R3 generation.

Somaclonal variation at the molecular level was studied by randomly amplified polymorphic DNA (RAPD) analysis using polymerase chain reaction (PCR). Total genomic DNA from somaclones variant for 13 traits --reduced plant height, high seed mass, white seed coat colour, strophiolation, high or low biomass with high harvest index, high or low raceme number, small

or large pod bearing length per branch, indeterminate flowering type and high or low *Helicoverpa* incidence-- and 5 control DNA, the first 3 of which are from individual ICPL 87 plants, the fourth, a bulk of the DNA from these three controls and the fifth a different bulk of the DNA from eight other ICPL 87 plants were used in the study. Nineteen 10-mer oligonucleotide primers of arbitrary sequences were used to amplify the template DNAs. While 18 primers displayed amplification with 3 to 7 bands each, 6 of these revealed 1 to 5 polymorphic bands between 2 to 12 putative somaclones. Five different primers detected polymorphism with the control for traits high seed mass and pod bearing length per branch. The traits white seed coat and indeterminate type were each distinguished from the controls by four different primers each. Similarly the traits biomass and raceme number were polymorphic to the control with 3 primers each. Only one primer distinguished polymorphic fragments for the traits reduced plant height, strophiolation and low *Helicoverpa* incidence.

The present investigations on application of somaclonal variation for pigeonpea crop improvement identified seedling cotyledon as the best explant for in vitro organogenesis. A high frequency regeneration system was then optimized for maximal identification of somaclonal variants. L2 medium was superior to MS or B5 medium. The use of activated charcoal enhanced the frequency of whole plant regeneration. Tissue cultured pigeonpea was sufficiently altered to effect the generation of a random array of variant plants. The floral alterations observed in the R1 generation are believed to be due to transient effect of tissue culture. Variants of a similar phenotype were counted as a single mutation to arrive at average mutation frequencies. However in the present study higher frequencies of phenotypic variations for qualitative traits are conceivable as the variants of a similar phenotype may not have arisen from a single mutational event as these could have occurred independently in different regenerated plants. This is amply substantiated form the analysis of segregation pattern wherein the same cultural conditions produced different mutational events resulting in both dominant

and recessive alleles. Segregation of the traits in the R2 indicate their heterozygous status in the R1. The R2 plants show codominant inheritance wherein the mutant genes are expressed in the heterozygotes which segregated for presence or absence of trait in the R3. Further the digenic ratios suggests the involvement of two unlinked epistatic loci. The spontaneous mutations in somaclonal variation appears to occur at higher frequencies than the conventional mutagenesis which is an attractive possibility for crop improvement programmes. Generation of considerable variability for quantitative traits are observed for plant height, low *Helicoverpa* incidence, seed mass, shelling percentage, biomass, harvest index and seed number per pod. This is noticeable from cluster analysis which placed plants originating from the same cotyledonary explant in different clusters; and the analysis of variance which showed highly significant F-values. The proportion of variation induced through tissue culture in the two independent variables was high for plant height, about 50% for the traits low *Helicoverpa* damage, shelling percentage and seed number per pod and to a much lesser extent for seed mass and biomass. The traits selected as reduced plant height, high seed mass and biomass in the R2 generation were maintained in the R3 while the traits white seed coat and strophiolation were stably integrated. Progeny lines selected for extremes in *Helicoverpa* incidence also maintained their improvements over the control in both the sprayed and unsprayed treatments in the R3 generation. The use of γ-irradiation for additional variability produced best results for a single quantitative trait i.e., seed number per pod. A differential expression of variability was observed in the irradiated and unirradiated samples as seen from the type (only quantitative or both qualitative and quantitative) and the extent of variation (as seen from the respective coefficient of variation and F-values). Both the treatments resulted in mean value shifts towards both higher and lower values than the controls. Putative somaclones from both treatments performed better than the control with respect to the agronomically useful traits such as reduced plant height, high seed mass and low *Helicoverpa* incidence.

Preliminary analysis of the putative somaclones at the molecular level by RAPD using PCR indicates the possibility of identification of molecular markers for the agronomically valuable traits like white seed coat colour, high seed mass and low *Helicoverpa* incidence. This possibility allows for further molecular analysis where, if deserving, the mutated gene can be cloned and sequenced to aid in saturating the genetic linkage map of pigeonpea with molecular markers. The tissue culture system of pigeonpea therefore has produced somaclonal variants of agronomic importance which could provide additional genes for improvement of this crop. The variants recovered could help enhance the breeders arsenal for varietal improvement as they may be possessing different alleles to those already known.

Heritable variation was demonstrated for the traits white seed coat colour, strophiolation, obtusely lanceolate leaf shape, flower colour, flowering type, seed size, reduced plant height, and low *Helicoverpa* incidence in at least two sexual generations studied. The variation observed in the present investigation therefore appears to have a genetic basis which could open up possibilities of introduction of genetic variability rapidly in the sexually propagated crop plants. This new source of genetic variability would be a valuable asset in pigeonpea plant breeding programmes. These variants could be used for varietal development or improvement in a breeding programme as they resemble the parental phenotype with the exception of one or more specific trait (white seed coat, high seed mass or low *Helicoverpa* incidence) for which they are superior.

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List of abbreviations

ABA	Abscisic acid
BA	5-Benzylaminopurine
NAA	α-Naphthaleneacetic acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
GA_3	Gibberellic acid
KT	Kinetin
pCPA	p-Chlorophenoxyacetic acid
mCPA	m-Chlorophenoxyacetic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
2,4,5-T	2,4,5-Trichloroacetic acid
2-iP	2-Isopentyladenine
NOA	β-Naphthoxyacetic acid
TDZ	Thidiazuron
MS	Murashige and Skoog, 1962
LS	Linsmaer and Skoog, 1965
B5	Gamborg, 1968
L2	Phillips and Collins, 1979
FAA	Formalin acetic acid
KR	Kilo Rads
Gy	Gray units
LD	Lethal dose
DNA	Deoxyribonucleic acid
CTAB	Cetyl triethylammonium bromide
TE	Tris EDTA
EDTA	Ethelenediamine tetra-acetic acid
dNTPs	Deoxyribonucleotide triphosphates
PCR	Polymerase chain reaction
RAPD	Randomly amplified polymorphic DNA

Nomenclature

The letter 'R' in this study indicates the in vitro plants regenerated from seedling cotyledon explants of *Cajanus cajan* (L.) Millsp. cv ICPL 87.

The initial regenerants are termed R1 plants since these are equivalent to F1 plants resulting from sexual crossing.

R2 progeny was grown from R2 seed produced by the selfing of R1 plants.

R3 progeny was grown from R3 seed produced by the selfing of R2 plants.

The letters SC prefixed to numerics denotes 'somaclone' and, somaclones arising from different R1 plants are identified as SC1, SC2, SC3, etc.

The letters IR prefixed to numerics is designated to identify regenerants obtained from precultured and gamma irradiated seedling cotyledons and, somaclones arising from different R1 plants obtained after γ-irradiation are designated as IR1, IR2, IR3, etc.

CURRICULUM VITAE

Name	PRASANNA LATHA CHINTAPALLI
Father's Name	Peter Chintapalli
Date of Birth	20 May 1965
Marital Status	Single
Address for Correspondence	Cellular and Molecular Biology Division ICRISAT, Patancheru 502 324, A.P., INDIA
E-mail	IN%"RSCSEV@ICRISAT.CGNET.COM"
Tel. No.	+91 40 596161 Ext 2698 (work) +91 40 283359 (home)
FAX No.	+91 40 241239/238451

Education

Ph D	Osmania University, Hyderabad 500 007, A.P., INDIA and ICRISAT, Patancheru 502 324, A.P., INDIA April 1990 to December 1995 Ph D studies on 'Application of Somaclonal Variation for Crop Improvement in Pigeonpea (*Cajanus cajan* (L.) Millsp.)'.
Pre-Ph D	Osmania University, Hyderabad 500 007, A.P., INDIA (1991) Passed in I Division with distinction Secured 1st rank in University
M Sc	Osmania University, Hyderabad 500 007, A.P., INDIA (1987 to 1989) Botany--specialized in Plant Physiology Passed in I Division with Distinction Secured 3rd rank in University
B Sc	Osmania University, Hyderabad 500 007, A.P., INDIA (1982 to 1985) Botany, Zoology and Chemistry Passed in I Division with Distinction

Awards:

Awarded the Research Scholarship of ICRISAT for Ph D Studies from December 1990 to October 1995 providing all Laboratory, Glasshouse and Field facilities.

Awarded the Senior Research Fellowship of the Council of Scientific and Industrial Research (CSIR)- for pursuing Ph D studies from November 1992 to October 1995.

Presentations:

PRASANNA LATHA, CH., JP Moss, KK Sharma and JK Bhalla. (1995). Induced Variability in Pigeonpea (*Cajanus cajan* (L.) Millsp) Somaclones Through In Vitro Culture. Paper communicated to "In Vitro Cell and Developmental Biology-Plant".

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