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IN VITRO CULTURE PROVIDES ADDITIONAL VARIATION FOR PIGEONPEA [*CAJANUS CAJAN* (L.) MILLSP.] CROP IMPROVEMENT[1]

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SUMMARY

The present study is an attempt to exploit somaclonal variation for the varietal improvement of pigeonpea [*Cajanus cajan* (L) Millsp.]. The pigeonpea plants were regenerated from cotyledon explants, and their progeny was screened for variability. The regenerated R1 plants exhibited a spectrum of alterations in floral morphology and architecture that were absent in the control population. The field-sown R2 plants segregated for traits such as flower color, leaf shape, seed size, color and strophiolation, flowering habit, and fertility. Tissue culture produced different mutational events resulting in both dominant and recessive alleles. Significant variation was observed for plant height, seed mass, and damage due to the insect pest *Helicoverpa armigera*. The R3 plants, obtained from seed of R2 generation selected for traits such as white seed coat, strophiolation, reduced plant height, seed mass and low damage due to *Helicoverpa*, maintained the traits when compared with the seed-derived control populations. The results indicate a definite gene for white seed coat and the possibility of additional genes for pest tolerance and high seed mass in an adapted background.

Key words: tissue culture; regeneration; somaclonal variation; insect resistance; *Cajanus cajan*.

INTRODUCTION

Crop improvement depends on the availability of adequate genetic variability. In pigeonpea [*Cajanus cajan* (L.) Millsp.], not all the variation required by the plant breeder is readily available in the primary gene pool. Additional genes for disease and insect resistance would enhance the breeders' arsenal. This could be achieved by mutation breeding, wide hybridization, tissue culture, or by the production of transgenic plants. The major constraints to productivity of this crop are the various biotic stresses such as phytophthora blight, fusarium wilt, sterility mosaic, and insect pests such as *Helicoverpa armigera* (Hübner), *Maruca testulalis* (Geyer), *Melanogromyza obtusa* (Malloch), and *Myrabilis pustulata* (Thunberg). Although ample resistance to wilt and sterility mosaic is available, resistance or tolerance to insect damage is not readily available in the cultivated pigeonpea germplasm. Some degree of resistance to *Helicoverpa armigera* through wide hybridization has been reported (Reddy, 1979); however, some of the resistance in the wild relatives may be associated with undesirable characters like hard pod wall. Somaclonal variation, a consequence of plant regeneration *in vitro*, is a potential source of genetic variability that can be used to widen the genetic base and therefore to develop new breeding lines particularly for resistance to insect pests in further crop improvement programs (Larkin et al., 1989).

Somaclonal variation has been successfully exploited in tomato for early blight resistance (Shepard and Sohndhal, 1986), in maize for *Helminthosporium* resistance (Gengenbach et al., 1977), and in sugarcane for resistance to Fiji virus (Krishnamurthi and Tlaskal, 1974). Fusarium wilt resistant tomato plants (Shahin and Spivey, 1986), *Fusarium oxysporium* resistant celery somaclones (Heath-Pagliuso et al., 1988), and fall armyworm resistant sorghum (Isenhour et al., 1991) were recovered from plants regenerated without any deliberate selection pressure. Somaclonal variants have also been reported in some important legume species. These include resistance to *Fusarium oxysporium* sp. *medicaginis* in *Medicago sativa* (Hartman et al., 1984) and chlorophyll and morphological variants in *Vigna radiata* (Mathews et al., 1986). Significant levels of somaclonal variation, either useful or deleterious, were recorded in soybean for male sterile phenotype (Ranch and Palmer, 1987), yield parameters (Greybosch et al., 1987), and morphological variations for wrinkled leaves, chlorophyll deficiency, and dwarfism (Barwale and Widholm, 1987). The present study, therefore, addressed the possibility of identification of useful somaclonal variation for pigeonpea crop improvement.

MATERIALS AND METHODS

Seed of Pigeonpea [*Cajanus cajan* (L.) Millsp. cv. ICPL 87] was obtained from the seed stock maintained by the Pigeonpea Breeding Unit of ICRISAT. Seed was surface sterilized by treatment with 0.1% mercuric chloride for 6 min, followed by six serial washes with sterile distilled water to remove traces of mercuric chloride. Sterilized seed was soaked in sterile distilled water for 4 h and germinated on filter paper bridges in culture tubes sealed with kaputs and containing 10 ml hormone-free liquid L2 medium (Phillips and Collins, 1979), with 1% sucrose. Efficient *in vitro* organogenesis was obtained from 6-d-old seedling cotyledons. Adventitious shoot buds were induced in 100

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× 15 mm sterile disposable polystyrene petri dishes containing 25 ml L2 medium with 8.9 μ*M* of 6-benzylaminopurine (BA). These shoot buds were then subcultured at 3-wk intervals on maintenance medium containing 9 ml of L2 medium with 0.44 μ*M* BA taken in 12 mm × 150 mm culture tubes sealed with cotton plugs. The cotyledonary explants bearing shoots >1 cm were transferred to tubes containing shoot elongation medium, which was comprised of L2 salts, 0.44 μ*M* BA, 2% sucrose, and 1% activated charcoal. The elongated shoots were rooted in hormone-free L2 medium. The rooted shoots were transferred to sterile sand contained in 7 × 7 × 7.5 cm plastic pots. Broughton's nutrient solution was applied to these plants at regular intervals. The plantlets were hardened under 90% humidity in an incubator maintained at 16/8 h light/dark regime for 3–4 wk, before transplanting into larger pots containing three parts sterile soil:one part farmyard manure:one part sand in the greenhouse where they flowered in isolation and R2 seed was collected on maturity.

The initial regenerants were termed R1 plants and R2 plants were grown from seed produced by the selfing of R1 plants under isolated conditions. The first field study was conducted on these R2 plants that were sown in the rainy season 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 13 selected putative somaclones. These were sown in the rainy season of 1994. Non-tissue-cultured parent lines obtained from cv. ICPL 87 seed stock used for regeneration studies were designated as control (C) plants.

Design of Experiment

R2 generation. All R2 seed collected was planted in 61 different plots in an alfisol field. Each plot was 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.

R3 generation. Eleven putative somaclones selected for various agronomic traits and the control cv. ICPL 87 lines were sown in the field in a randomized complete block design (RCBD) with three replications. 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 experiment studying the incidence of *Helicoverpa* damage had two somaclonal lines each of low and high *Helicoverpa* incidence and the control ICPL 87 in a split plot design with three replications. Each block had two treatments—one sprayed with endosulfan and the other without any insecticidal spray. The field specifications were the same as described above for RCBD.

Measurements of Qualitative Characters

R1 generation. The R1 plants grown to maturity in the glasshouse were only scored for morphological variations (flower color, morphology and architecture, and pod-bearing length per branch and per plant) that could be rapidly assessed on single plants in pots. Quantitative characters (e.g., yield per se) were not assessed.

R2 generation. Observations were recorded for qualitative traits like seed coat color and pattern, seed shape, strophiolation, color 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.

R3 generation. Data on inheritance and segregation of the selected putative variants were collected for the altered traits like seed coat color and strophiolation.

Measurement of Quantitative Characters

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 3–4 mo., and the plant height differed drastically with the seasonal variation. Variation for pod-bearing length per branch and per plant was noted but measurements were not taken.

R2 generation. Data on the R2 generation were collected for individual plants (border plants were excluded for statistical analysis) to enable single plant selections to be made. The data included plant height, number of racemes per plant, number of flowers per raceme, pod-bearing length per branch and per plant, stalk yield, number and weight of *Helicoverpa* damaged and undamaged pods, and number and weight of seed obtained from *Helicoverpa* damaged and undamaged pods. From this data, 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; 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.

Statistical Analysis

Chi-square test for goodness of fit to Mendelian ratios was performed using GENSTAT version 5 (GENSTAT, 1987). The summary statistics like mean, standard deviation, and coefficient of variation were calculated in SAS (SAS Institute, 1985) for the traits including 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. In addition to cluster analysis, Duncan's multiple range test was used to identify somaclones differing significantly. This further 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 (SAS Institute, 1985) to determine the genetical variances between and within the somaclones/somaclonal progeny, and that due to the explants giving rise to somaclones.

Results

Somaclonal Variation in R1 Generation

Phenotypic variation for qualitative traits. The R1 plants exhibited a spectrum of floral alterations and architecture that included supernumerary wing and standard petals associated with gross variations in shape, anthers protruding from buds, flowers arising from within a flower, petaloid sepals, and petaloid stamen. Some plants continuously produced a few flowers with two or three gynoecia that formed twin or triple pods. These floral variations were not observed in the R2 generation.

Somaclonal Variation in R2 Generation

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% and the anthers generally contained fewer pollen grains. Somaclonal variant SC7 segregated for indeterminate flowering habit (4.3% of the progeny) while SC24 segregated for semideterminate (1.9% of the progeny). Leaf shape variants (obtusely lanceolate) were observed in certain R2 plants. The R2 plants also segregated for reduced plant height, white seeds, seed coat pattern, color around hilum, seed shape, and strophiolation. These traits are not present in the parental population. The possible frequencies of these variant phenotypes arising in different families of R1 plants originating from different cotyledonary explants are seen to range from 0.25 to 4.00 per regenerated plant per explant (Table 1). 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. Further, the frequencies of variant R1 plants for the various types in the somaclonal population ranged from 0.02 to 0.41 (Table 2). Among the

TABLE 1

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
—	86[d]	0	0.00

[a]The culture time prior to regeneration from the different cotyledonary explants ranged from 9–50 wk.

[b]The variant phenotypes recorded for calculating the frequency are the qualitative characters: presence of hilum, seed shape, seed coat color and patter, color around hilum, flower color 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 or R1 plants from that particular explant.

[d]The number of non-tissue-cultured plants derived from the same seed lot as used for tissue culture studies.

somaclonal population (R1 plants), the highest frequency of variants was observed for seed coat pattern (41% of somaclones) followed by strophiolation (31% of somaclones). About 2% each of somaclones were variant for indeterminate and semideterminate traits. White seed coat color was observed in 16.4% of the somaclones while color around hilum was observed in 18% of somaclones. For the traits color around hilum and strophiolation, chi-square test (Table 3) revealed all clonally related R1 plants to segregate 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.

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. Based on Ward's minimum variance dendrogram, 52 somaclones originating from 31 different cotyledonary explants were grouped into six clusters (Fig. 1). Cluster I included 29 putative somaclones, while clusters III, VI, II, V, and IV, had 8, 6, 4, and 2 somaclones, respectively (Table 4). 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 with contrasting characters, or those originated from cotyledons with two or more regenerants distributed in different clusters. The rest of the somaclones that were single regenerants from different cotyledons largely distributed in cluster I, followed by clusters VI and III. From the cluster means it was 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). Further, this group also had R2 plants with low *Helicoverpa* damage (20%). Cluster I included R2 plants that 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 standard deviation for various traits in different somaclones appeared to change with the calculated means. Therefore, the coefficient of variation (CV) was used to detect the variance in these populations. Very high CVs were observed for the biomass (89.33), seed mass (97.26), and *Helicoverpa* damage (107.86). Also, very large differences in CV were observed for seed mass (4.60 to 97.26) and *Helicoverpa* damage (6.89 to 107.86).

For the quantitative traits under consideration, ANOVA indicated highly significant F-values for the different somaclones and also for the R1 families each originating from separate explants.

In a subsequent experiment the following year, three R2 plants (SC62, SC63, and SC64) were compared with non-tissue-cultured control ICPL 87 plants derived from the same seed lot as used for tissue culture studies. The control plants (ICPL 87) and SC64 fall into separate clusters while the other two are grouped (Fig. 2). All these somaclones show mean values greater than or less than the control mean for plant height and shelling percent (Table 5). With respect to damage due to *Helicoverpa*, SC64 shows only 27% while with respect to seed mass all somaclones had higher values than control. The Duncan's grouping showed SC62 and SC63 to differ from control for all traits except harvest index. SC64 differed from the control for plant height, frequency of damaged pods, seed mass, and biomass. It also differed from the other somaclones for plant height, frequency of damaged pods, and shelling percent. It was also observed that all somaclones had less incidence of *Helicoverpa*, higher seed mass, and lower biomass than the control.

Somaclonal Variation in R3 Generation

While epistatic gene interactions were observed in the R2 plants for color around hilum and strophiolation (Table 3) and white seed coat, the progeny of R2 plants with white seed coat and strophiolation segregated in R3 to give a 3:1 ratio (Table 6). Although the 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 7). The progeny lines selected for seed mass and plant height maintained the differences over the control population. Lower values than the control were observed for biomass, which, however, was maintained in the selected somaclonal lines.

The somaclones SC19 and SC20 showed 56.7% and 54.5% *Helicoverpa* damage, respectively, under unsprayed conditions, while with spraying they had only 22.1% and 28.8% damage. Thus, both somaclones had less *Helicoverpa* damage than the control in both sprayed and unsprayed conditions.

TABLE 2

COMPARISON OF FREQUENCY OF VARIANT PHENOTYPES IN THE PROGENY 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]
Color around hilum	2	6	3	0.5	0.18
	3	7	7	1.0	
	26	1	1	1.0	
Seed coat color	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
All abvove	—	86[c]	—	0	—

[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.
[c]The number of non-tissue-cultured plants derived from the same seed lot as used for fissue culture studies.

DISCUSSION

Although an array of phenotypic variations involving floral morphology, architecture, and twin and triple pods were observed in the R1 generation, their perpetuation in the subsequent sexual generation was not evident. This would mean that the apparent variations were epigenetic manifestations of tissue culture and were, therefore, not under complete genetic control, or that they were genetic changes in cell layers that did not contribute to the germ cells. This conformity of the *in vitro* propagated plants in the second generation for morphological characters was also observed in sugarcane (Lourens and Martin, 1987). Instability for marker genes in tobacco (Barbier and Dulieu, 1983) and a high frequency of reversion for anthocyanin mutation (Groose and Bingham, 1986) have also been reported.

Phenotypic and genotypic variation for both qualitative and quantitative traits was observed in the selfed sexual generation of R1 plants. Many of the variant phenotypes that were not seen in the R1 were assumed to be heterozygous recessive. The expression of the recessive white seed coat in the progeny (R2 plants) of self-fertilized R1 plants, which was not observed in the donor material, suggests the possible activation of transposable elements during tissue culture. The observation of a partially male sterile (which is also female sterile) plant in the progeny of the regenerated plant (SC25) suggests cytoplasmically encoded changes.

TABLE 3

GENOTYPIC VARIATION IN TWO QUALITATIVE TRAITS IN PROGENY OF TISSUE-CULTURED *CAJANUS CAJAN* (L.) MILLSP. CV. ICPL 87

Variant phenotype	Explant number[b]	R1 plant identity	R2 plants segretating[a] for the variant phenotype		
			Absent	Present	Ratio
Color around hilum	3	SC8	10	199	1 : 15
		SC14	19	120	3 : 13
	3	SC7	79	54	9 : 7
		SC22	61	55	9 : 7
		SC23	72	39	9 : 7
	86[c]	—	86	0	
Presence of strophiole	2	SC6	33	112	1 : 3
		SC8	43	166	1 : 3
		SC14	25	114	3 : 13
	3	SC7	101	32	3 : 1
		SC22	88	28	3 : 1
		SC23	85	29	3 : 1
	86	—	86	0	

[a]Segregating ratios of only those R1 plants with large progeny number are shown.

[b]Explant number from Table 1.

[c]The number of non-tissue-cultured plants derived from the same seed lot as used for tissue culture studies.

Generally, 10 to 20% of progeny of determinate pigeonpea (cv. ICPL 87) could segregate for indeterminate flowering habit as a result of genetic heterogeneity in the initial material. However, the observation of 4.3% of the progeny of only one out of a total of seven somaclones derived from a single explant to segregate for indeterminate plants can be due to the occurrence of a dominant mutational event during the tissue culture cycle. Alternatively, the residual heterozygosity could be altered by the tissue culture cycle in the other six somaclones but not in the somaclone segregating for indeterminate type. Segregation of a semideterminate type in an otherwise determinate population has not been reported. The occurrence of semideterminate plants in 1.9% of progeny of the 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).

Although the frequencies of variant phenotypes ranged from 0.25 to 4.00 per regenerated plant per explant, 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. In soybean, Barwale and Widholm (1990) reported the frequency of qualitative variation per initial regenerant to range from 0.05 to 1.00, while in maize, Zehr et al. (1987) reported average mutation frequency for qualitative variation per regenerated plant to range from 0.18 to 0.71. The high frequencies of phenotypic variation in the somaclonal populations as compared to those observed in a conventional mutagenesis study could be due to the screening of small somaclonal populations, which is sufficient for isolation of variants.

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 dominant. 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. However, homozygous mutations were not observed. Although a considerable amount and type of qualitative variation has occurred in the tissue-culture-generated somaclones, it should be noted that the variations, except white seed, are not of agronomic importance and are only of academic interest.

In mung bean, Bhatia and Mathews (1988) reported the recovery of monogenically inherited recessive (green cotyledon) as well as dominant (dull seed surface) 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 dominant to recessive (awns and grain color) and from recessive to dominant and co-dominant (glume color and gliadins) were also observed in wheat by Larkin et al. (1984). Thus, somaclonal variation is entirely random and the same cultural con-

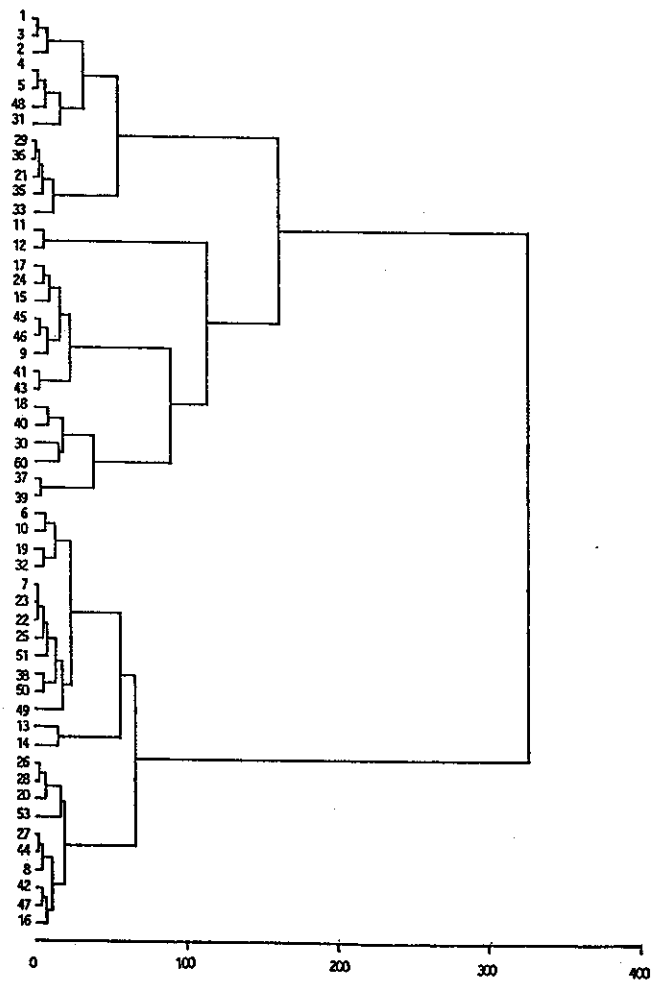

FIG. 1. Ward's minimum variance dendrogram showing the clustering of R1 plants of *Cajanus cajan* (L.) Millsp. cv. ICPL 87 for various yield parameters measured on R2 progeny plants from individual R1 plants.

TABLE 4

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]
I	29	SC4	1	III	8	SC38	2
		SC17	1			SC7	3
		SC18	1			SC22	3
		SC45	4			SC23	3
		SC11	5			SC49	4
		SC12	5			SC51	6
		SC34	5			SC25	17
		SC41	5			SC50	26
		SC15	6				
		SC21	6	IV	2	SC14	2
		SC29	6			SC13	6
		SC35	6				
		SC36	6				
		SC46	6	V	4	SC20	1
		SC30	7			SC26	1
		SC48	7			SC28	1
		SC37	8			SC53	3
		SC1	10				
		SC2	11				
		SC3	12	VI	6	SC8	2
		SC5	13			SC27	2
		SC9	14			SC16	15
		SC24	16			SC42	22
		SC31	18			SC44	24
		SC33	19			SC47	25
		SC39	20				
		SC40	21				
		SC43	23				
		SC60	30				
II	4	SC19	1				
		SC6	2				
		SC32	3				
		SC10	4				

[a]The cotyledonary explant from which the somaclone originated. The group of clusters that identified somaclones similar for the traits of interest in the present study was chosen.

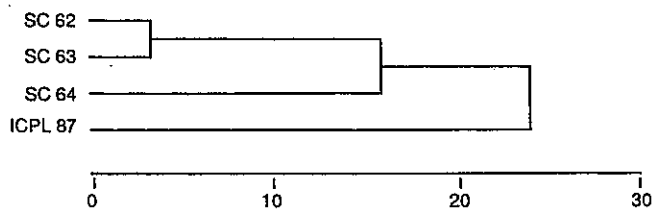

FIG. 2. Ward's minimum variance dendrogram showing the clustering of R1 plants of *Cajanus cajan* (L.) Millsp. cv. ICPL 87 for various yield parameters measured on R2 progeny plants from individual R1 plants.

ditions can produce different mutational events resulting in both dominant and recessive mutant alleles.

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 explains the different ratios observed. 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.

In contrast to presence of strophiole and color around hilum, the seed coat color was a mutation from a dominant brown to recessive white. Progeny analysis in R3 generation for presence of strophiole or white seed coat using chi-square test for a 3:1 model revealed the two traits to be segregating as single gene dominants. Therefore, mutational events involving the loci governing the traits had apparently occurred and were perpetuated in succeeding generations. In maize, Zehr et al. (1987) and Lee and Phillips (1987) observed culture-induced mutant phenotypes to be inherited mostly as single gene recessives. In the present investigation, further inheritance studies must be taken up for confirmation of inheritance patterns and possible fixation of characters in R4 and for test crosses to determine possible transposable element activity.

The grouping of plants originating from the same explant in different clusters and also shifts to both higher and lower mean values from the control for various quantitative traits infers that considerable variability was generated for these quantitative traits. It is apparent that this variation includes both deleterious as well as useful varia-

TABLE 5

THE EXTENT OF SOMACLONAL VARIATION IN THE R2 PROGENY OF REGENERANTS OBTAINED FROM SEEDLING COTYLEDONS OF *CAJANUS CAJAN* (L.) MILLSP. CV. ICPL 87

Trait		Samaclone Number			
		SC62	SC63	SC64	C
PH	MN	61.2[b]	62.5[b]	66.8[a]	63.2[b]
	SD	8.60	8.98	9.08	6.48
HD	MN	33.8[b]	33.6[b]	27.1[c]	43.7[a]
	SD	21.58	20.63	13.05	21.63
SDM	MN	12.3[a]	12.2[a]	12.5[a]	10.6[b]
	SD	1.56	1.07	0.94	0.75
BIO	MN	89.7[c]	110.3[b]	107.4[b]	183.6[a]
	SD	48.70	54.22	37.98	58.74
HI	MN	17.9[a]	18.5[a]	17.4[a]	17.3[a]
	SD	13.55	15.08	8.13	4.46
SH	MN	53.1[b]	53.7[b]	57.1[a]	56.2[a]
	SD	8.35	9.72	1.92	2.29
SNP	MN	2.9[b]	2.9[b]	3.1[a,b]	3.3[a]
	SD	1.11	1.08	0.60	0.43

C = control ICPL 87; PH = plant height (cm); HD = *Helicoverpa* damage (%); SDM = seed mass (g); BIO = biomass (g); HI = harvest indes; SH = shelling percentage; SNP = seed number per pod; MN = mean; SD = standard deviation; Duncan's grouping shows means followed by the same letter are not significantly different.

TABLE 6

CHI-SQUARE ANALYSIS OF QUALITATIVE CHARACTERS FOR INHERITANCE IN THE R3 GENERATION OF TISSUE-CULTURE-DERIVED PLANTS OF *CAJANUS CAJAN* (L.) MILLSP. EV. ICPL 87

Trait	Presence	Absence	X^2 for 3:1	Probability
Strophiole	10	8	3.630	0.050–0.075
(SC7)[a]	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
(SC4)[a]	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

[a]Somaclone variant for the particular trait is given in parenthesis.

tions. The high F-values observed for the polygenically controlled quantitative characters indicate that tissue culture did induce highly variant and random alterations that could be due to polygenic changes or minor structural alterations in somatic chromosomes (Nizeki et al., 1990). Early maturity was reported in maize (Lee and Phillips, 1987) and in sorghum (Bhaskaran et al., 1987). Significant increases in dry matter in potato (Evans et al., 1986) and increase in yield per se for oats (Dahleen et al., 1991) were also reported. In wheat, Ryan et al. (1987) identified significant and potentially useful variations for qualitative characteristics like kernel weight, hardness, protein content, and reduced yellow pigmentation and viewed these characteristics as potentially useful.

TABLE 7

INHERITANCE OF QUANTITATIVE TRAITS IN THE PROGENY OF TISSUE-CULUTRE-REGENERATED PLANTS OF *CAJANUS CAJAN* (L.) MILLSP. CV. ICPL 87

Trait	Somaclone	Generation	
		R2	R3
Seed mass (g)	SC12	11.0	14.7
	C	9.0*	10.5
Plant height	SC47	64.3	55.7
	C	82.0*	61.9
High biomass	SC3	141.0	142.2
Low biomass	SC42	75.5	168.8
	C	NA	178.4

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 six rows.

Significant improvements over the control for more than one trait was noted in SC64 for low *Helicoverpa* damage and high seed mass. It is, therefore, possible for the tissue culture system of pigeonpea to produce 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. These improvements in seed mass and *Helicoverpa* damage should, therefore, be viewed as a potential source of additional variation because they may be different alleles to those already known. The maintenance of the significant improvements in the next sexual generation demonstrates a genetic basis for somaclonal variation. The recovery of such agronomically useful variants justifies the consideration of somaclonal variation as a potentially useful breeding tool.

Conclusions

Although considerable genetic variability exists in the pigeonpea gene pool, the current cultivated crop is based to a large extent on only a small portion of the available germplasm. While wide crossing featured significantly as one of the options to introduce variability (Mallikarjuna and Moss, 1995), efforts in the transformation of pigeonpea are still in their infancy. It is in this context that somaclonal variation with its potential to improve an already adapted genotype by altering a gene or loci governing the desired trait could provide the much needed variability. The spontaneous mutations occurring in somaclonal variation appear at higher frequencies than by conventional mutagenesis, which is advantageous for crop improvement programs provided they are agriculturally useful. Tissue-cultured pigeonpea was sufficiently altered to effect the generation of a random array of variant plants. Most of this variation was heterozygous and, therefore, a continuous segregation was a common occurrence for all qualitative traits except leaf shape. Changes for the characteristics

such as strophiolation, white seed coat color, plant height, seed mass, biomass, and *Helicoverpa* damage were maintained in the R3 generation. The current results indicate a definite gene for white seed coat color 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. The genetic basis of these variant traits must be further studied to ensure effective integration of desirable characteristics in subsequent generations of plant breeding programs. Single gene changes produced in specific genetic background could be one of the most useful applications of somaclonal variation in plant breeding (Ryan et al., 1987). The material generated through the present study would help breeders in terms of varietal improvement, since the variants observed resemble the parent genotype with the exception of the specific trait(s) for which they are superior.

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References

Barbier, M.; Dulieu, H. Early occurrence of genetic variants in protoplast cultures. Plant Sci. Lett. 29:201–206; 1983.

Barwale, U. B.; Widholm, J. M. Somaclonal variation in plants regenerated from cultures of soybean. Plant Cell Rep. 6:365–368; 1987.

Barwale, U. B.; Widholm, J. M. Soybean: plant regeneration and somaclonal variation. In: Bajaj, Y. P. S., ed. Biotechnology in agriculture and forestry. Vol. 10. Legumes and oilseed crops I. Berlin, Germany: Springer-Verlag; 1990.

Bhaskaran, S.; Smith, R. H.; Paliwal, S., et al. Somaclonal variation from *Sorghum bicolor* (L.) Moench cell culture. Plant Cell Tissue Organ Cult. 9:189–196; 1987.

Bhatia C. R.; Mathews, H. Inheritance of the somaclonal variation in Mung bean (*Vigna radiata* (L.) Wikzek. J. Hered. 79(2):122–124; 1988.

Cheng, M.; Hsi, D. C. H.; Phillips, G. C. In vitro regeneration of valencia-type peanut (*Arachis hypogeae* L.) from cultured petiolules, epicotyl sections and other seedling explants. Peanut Sci. 19:82–87; 1992.

Dahleen, L. S.; Stuthman, D. D.; Rines, H. W. Agronomic trait variation in oat lines derived from tissue culture. Crop Sci. 31:90–94; 1991.

Evans, N. E.; Foulger, D.; Farrer, L., et al. Somaclonal variation in explant derived potato clones over three tuber generations. Euphytica 35:353–361; 1986.

Gengenbach, B. G.; Green, C. E.; Donovan, C. M. Inheritance of selected pathotoxin resistance in maize plants regenerated from cell cultures. Proc. Natl. Acad. Sci. USA 74:5113–5117; 1977.

GENSTAT. A general statistical programme reference manual; Statistical department, Rothamsted Experimental Station; Published by Numerical Algorithm Group Ltd., UK; Oxford, U.K.: Clarendon Press; 1987.

Greybosch, R. A.; Edge, M. E.; Delanny, X. Somaclonal variation in soybean plants regenerated from cotyledonary node system. Crop Sci. 27:803–806; 1987.

Groose, R. W.; Bingham, E. T. An unstable anthocyanin mutation recovered from tissue culture of alfalfa (*Medicago sativa*) 1. High frequency of reversion upon reculture. Plant Cell Rep. 5:104–107; 1986.

Hartman, C. L.; McCoy, T. J.; Knous, T. R. Selection of alfalfa (*Medicago sativa*) cell lines and regeneration of plants resistant to the toxin(s) produced by *Fusarium oxysporium* f. sp. *medicaginis*. Plant Sci. Lett. 43:183–194; 1984.

Heath-Pagliuso, S.; Pullman, J.; Rappaport, L. Somaclonal variation in celery: screening for resistance to *Fusarium oxysporium* f. sp. *apii*. Theor. Appl. Genet. 75:446–451; 1988.

Isenhour, D. J.; Duncan, R. R.; Miller, D. R., et al. Resistance to leaf-feeding by the fall armyworm (Lepidoptera: Noctuidae) in tissue culture derived sorghums. J. Econ. Entomol. 84(2):680–684; 1991.

Krishnamurthi, M.; Tlaskal, J. Fiji disease resistant *Saccharum officinarum* var. Pindar subclones from tissue cultures. Proc. Int. Soc. Sugar Cane Technol. 15:130–137; 1974.

Larkin, P. J.; Banks, P. M.; Bhati, R., et al. From somatic variation to variant plants: mechanisms and applications. Genome 31:705–711; 1989.

Larkin, P. J.; Ryan, S. A.; Brettel, R. K., et al. Heritable somaclonal variation in wheat. Theor. Appl. Genet. 67:443–455; 1984.

Lee, M.; Phillips, R. L. Genetic variants in progeny of regenerated maize plants. Genome 29:834–838; 1987.

Lourens, A. G.; Martin, F. A. Evaluation of in vitro propagated sugarcane hybrids for somaclonal variation. Crop Sci. 27:793–796; 1987.

Mallikarjuna, N.; Moss, J. P. Production of hybrids between *Cajanus platycarpus* and *Cajanus cajan*. Euphytica 83(1):43–46; 1995

Mathews, V. H. M.; Rao, P. S.; Bhatia, C. R. Somaclonal variation in cotyledonary plants of mung bean. Z. Pflanzenzuech. 96:169–173; 1986.

Niizeki, M.; Ishikawa, R.; Saito, K. Variation in a single protoplast- and seed-derived population of *Lotus corniculatus* L. Theor. Appl. Genet. 80:732–736; 1990.

Phillips, G. C.; Collins, G. B. *In vitro* tissue culture of selected legumes and plant regeneration from callus cultures of red clover. Crop Sci. 19:59–64; 1979.

Ranch, J. P.; Palmer, R. G. A ploidy variant regenerated from embryogenic tissue cultures of soybean. Soyb. Genet. Newslett. 14:161–163; 1987.

Reddy, L. J. In: Pigeonpea breeding. ICRISAT, Pulse breeding progress report 3, Patancheru, A.P., India; 1979.

Ryan, S. A.; Larkin, P. J.; Ellison, F. W. Somaclonal variation in some agronomic and quality characters in wheat. Theor. Appl. Genet. 74:77–82; 1987.

SAS Institute. SAS user's guide: Statistics. Cary, NC: SAS Institute; 1985.

Shahin, E. A.; Spivey, R. A single dominant gene for *Fusarium* wilt resistance in protoplast-derived tomato plants. Theor. Appl. Genet. 73:164–169; 1986.

Shepard, S. L. K.; Sohndhal, M. K. Selection for early blight disease resistance in tomato: use of tissue culture with *Alternaria solani* culture filtrate. Sixth International Congress Plant Tissue and Cell Culture. Abstract, University of Minnesota, Minneapolis. p. 211; 1986.

Zehr, B. E.; Williams, M. E.; Duncan, D. R., et al. Somaclonal variation in the progeny of plants regenerated from callus cultures of seven inbred lines of maize. Can. J. Bot. 65:491–499; 1987.