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Genetic Improvement of Chickpea (*Cicer arietinum* L.) Cultivar 'Vijay' (Phule G 81-1-1) through Induced Mutagenesis

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ABSTRACT

In the present attempt three well known mutagens, sodium azide (SA), ethyl methane sulphonate (EMS) and gamma radiation (GR) were employed to induce genetic variability for improvement of locally popular chickpea (*Cicer arietinum* L.) cv. 'Vijay' (Phule G 81-1-1). Seeds were treated with three different concentrations/doses of SA (2, 3 and 4 mM), EMS (8, 12 and 16 mM) and gamma radiation (400, 500 and 600 Gy). Different mutants were isolated and characterized. They included leaf, pod, seed, flower colour and morphological mutants. Five true breeding mutant lines were studied for various quantitative and qualitative traits in the M_4 generation. Induced mutant lines showed both positive and negative increases in quantitative traits. True breeding mutant lines in the M_4 generation differed considerably in their quantitative traits from the control. The early mutant lines matured 10 to 11 days earlier than the parent cultivar. *Gigas* mutant plants obtained after 400 Gy gamma radiation were tallest (44.2 cm), with a 2-3 fold increase in pod and seed size over the control. The protein content in the M_4 generation ranged from 259.11 to 282.18 mg/g vs 255.59 mg/g in the control. Lower TI activity was observed in the compact mutant (2465.4 TIU/min/g) as compared to the control (3091.5 TIU/min/g). Analysis of variance showed both positive and negative significant increase in the quantitative traits among the mutant lines. The higher heritability coupled with high genetic advance was observed for quantitative traits like number of pods/plant and number of seeds/plant for the mutants in the chickpea cv. 'Vijay'. Variation was also observed for number of bands electrophoresed in seed protein of mutants.

Keywords: chickpea, EMS, gamma radiation, heritability, SA, variability
Abbreviations: EMS, ethyl methane sulphonate; **GCV**, genotypic coefficients of variation; **PCV**, phenotypic coefficients of variation; **RBD**, randomized block design; **SA**, sodium azide; **TI**, trypsin inhibitor

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is a widely cultivated and important food grain legume in the Indian sub-continent. It is the second largest grown food legume of the world (Gaur *et al*. 2008). It is a major source of protein in human diet. In spite of its importance, its yield did not witness much appreciation during the past decade (Barshile *et al.* 2006). It has been argued that one of the reasons for failure to achieve a breakthrough in productivity in chickpea is the lack of genetic variability (Wani and Anis 2001). The improvement of chickpea using conventional breeding approaches has been hampered due to lack of sufficient genetic variability. Since genetic variability is a pre-requisite for any successful breeding programme, creation and management of induced variability becomes central base for improvement of any crop species (Sharma *et al.* 2008). Kulthe and Kothekar (2011) opined that treatment of chickpea with mutagen like sodium azide offers great possibilities for evolving higher yield variants through proper selection in subsequent generations. Factors like limited genetic variability, genome plasticity and self pollination have yielded little success in augmenting yield through conventional breeding approaches (Singh 2009). A common and efficient tool to create new desirable genetic variability in chickpea is induced mutagenesis (Micke 1988). Although studies on induced mutations have been undertaken in the past in some legumes, limited reports are available on chickpea (Gaur and Gaur 1999).

In the present investigation, an attempt has been made for genetic improvement of the locally adapted cultivar of chickpea, 'Vijay' (Phule G 81-1-1), through induction of mutations employing potent mutagens like gamma radiation, sodium azide and ethyl methane sulphonate for quantitative and qualitative improvement. The antinutritional factors present in chickpea are known to cause adverse physiological effects and decrease the bioavailability of nutrients. Therefore main target has been to induce genetic variability to develop high yielding varieties, increasing protein content and lowering trypsin inhibitor in the chickpea cv. 'Vijay' (Phule G 81-1-1) through induced mutagenesis.

MATERIALS AND METHODS

Seeds of chickpea cv. 'Vijay' (Phule G 81-1-1) were procured from the Mahatma Phule Krishi Vidyapeeth, Rahuri, India. Healthy seeds containing 10-12% water were treated separately with two chemical mutagens, sodium azide (SA) and ethylmethane sulphonate (EMS) and physical (gamma radiation) mutagens. SA and EMS of Sigma, USA were used. For chemical mutagen treatments, seeds were pre soaked in distilled water for 6 h and then subjected to the concentrations of 2, 3 and 4 mM SA and 8, 12 and 16 mM EMS for 12 h at 25 ± 2°C. The treated seeds were thoroughly washed under running tap water for an hour to terminate the reaction of the chemical. For physical mutagen treatment, dry seeds were irradiated with 400, 500 and 600 Gy from a ^{60}Co source available in the Department of Biophysics, Government Institute of Science, Aurangabad (M.S., India). Each treatment was carried out for 250 seeds. All treated seeds along with control were sown in the field at spacing of 15 cm in rows and 45 cm between rows to raise M_1 generation during the 2002 growing season. For raising the M_2 generation, M_1 plants were harvested separately and seeds sown in 2003 growing season in a randomized block design (RBD) with 3 replications at experiment field of Shri Anand Col-

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lege, Pathardi. In M_3 and M_4 generation mutant seeds were planted in RBD with 3 replications. Data collected in M_4 generation were subjected to the analysis of variance (ANOVA). The parameters of coefficient variability (CV), standard error (SE), critical difference (CD) were calculated following by Snedecor and Cochran (1967). The data were analyzed using 'DRY' software program. Data were collected on 5 agronomic traits viz., plant height (cm), plant spread (cm), number of pods/plant, number of seeds/plant, yield/plant (g) and 100-seed weight (g). The genotypic and phenotypic coefficients of variation were estimated following the method of Burton and De Vane (1953). The heritability and genetic advance were calculated following the methods suggested by Hanson *et al*. (1956) and Johnson *et al*. (1955), respectively.

Extraction and estimation of total protein

Healthy, mature seeds of control and viable mutants were used for extraction of proteins. The seeds were ground in mortar and pestle to make a fine powder. This fine seed powder was defatted with acetone and petroleum ether. After defatting the fine seed powder was air dried and the 500 mg of seed powder was dissolved in 2.5 ml of 0.1 molar phosphate buffer (pH 7.0). The extract was centrifuged at 5,000 rpm for 15 min at 4°C. The clear supernatant was collected and used as the source of protein. Total proteins were estimated following the method of Lowry *et al*., (1951). 0.2 ml extracted protein sample was used to prepare the reaction mixture. To this 0.8 ml distilled water, 3 ml of alkaline mixture and 1 ml folin phenol reagent was added. The whole mixture was shaken well and incubated in dark for 30 min at room temperature. The blue colour developed was read at 660 nm on UV-visible spectrophotometer (Shimadzu-1601). The BSA (Bovine serum albumin fraction V) was used at the concentration of 1 mg/ml as standard protein, to prepare standard curve.

Estimation of nitrate reductase activity

The nitrate reductase (NR; E.C. 1.6.6.1) activity from leaf samples of chickpea at flowering stage was performed as described by Sawhney *et al*. (1978). 200 mg of leaves were weighed and cut into small pieces. They were transferred to test tubes containing 5 ml of assay mixture (2.5 ml of 0.1 molar phosphate buffer at pH 7.2, 0.2 ml *n*-propanol and 2.3 ml distilled water). The tubes were vacuum filtrated for 45 min in the dark at 30°C. The reaction was terminated by keeping the tubes in boiling water bath for 10 min. The assay mixture was cooled and 1.0 ml of this was used for developing colour with sulphanilamide and napthyl ethylenediamine reagent for 10 min. The optical density was read at 540 nm against the blank on UV-visible spectrophotometer (Shimadzu-1601). The amount of NO_2 produced was calculated from the standard curve and the nitrate reductase activity was expressed in terms of µ moles of NO_2 produced g^{-1} fresh weight hr^{-1}. Sodium nitrite ($NaNO_2$) was used at the concentration of 200 µg ml^{-1} as a standard nitrite to prepare the standard curve.

Estimation of trypsin inhibitor (TI) activity

Dry seeds of chickpea made into a fine power, defatted with hexane and the meal is air dried. The defatted flour was added to 6 volumes of distilled water and 1% polyvinyl polypyrrolidone (PVP) and allowed to stand overnight. The suspension was centrifuged at 12,000 rpm at 4°C for 20 min to remove the particulate matter and clear supernatant was used for further studies. Trypsin inhibitor activity was measured by using N-benzoyl DL-arginine P-nitroanilide (BAPNA) as a substrate following Chitra and Sadasivan (1986). Twenty µg of trypsin was mixed with an appropriate quantity of inhibitor containing extract (so as to inhibit 50-60% trypsin) and incubated at 25°C for 5 min; the residual trypsin activity was measured by using 1ml of 1 mM BAPNA. After 10 min, reaction was stopped by adding 200 µl of 30% acetic acid. The liberated P-nitroanilide was measured at 410 nm on a spectrophotometer. Trypsin was assumed to be 100% active.

Protein electrophoresis

Analysis and comparison of proteins profile were carried out by the SDS-PAGE following the method of Laemmli (1970). The resolving gel zone was 32 mm in height and contained 30% (w/v) acrylamide, 2% (w/v) BIS in 0.112 M Tri and 0.112 M acetate pH 6.5 solution. The stacking gel zone was 13 mm in height and contained 7.5 % (w/v) acrylamide, 3% (w/v) BIS in the same solution as the resolving gel. After polymerization stacking gel solution was poured quickly on top of the polymerized resolving gel. A comb was placed to form the sample well and the gel was allowed to polymerize. After the stacking gel was polymerized, the comb was removed without distorting the shapes of the wells. The reservoirs of the migration unit were filled with electrode buffer and the glass plates with the gel were installed in the migration unit. 30 µl protein samples and standard protein molecular weight markers (PMW-M of Genei Ltd, Bangalore, India) were mixed with equal volume of sample buffer in 1:1 (v/v) ratio and were heated in boiling water for 2-3 min to ensure complete interaction between proteins and SDS. They were loaded in the sample wells. Electrophoresis was performed at 15°C with a constant current of 15 mA for gel until the bromophenol blue ran out of gel. Proteins were fixed and stained in a solution of 1g of coomassie brilliant blue R 250 in 1 liter of 60% methanol to which was added a 20% solution of acetic acid just before use, the ratio (v/v) was 1:1. After the completion of run, the gel was removed and fixed in 10% TCA for 30 min. They were stained with coomassie brilliant blue R 250 overnight and destained in destaining solution till the background became colourless. The relative mobility values (Rm) and molecular weights of protein bands were calculated.

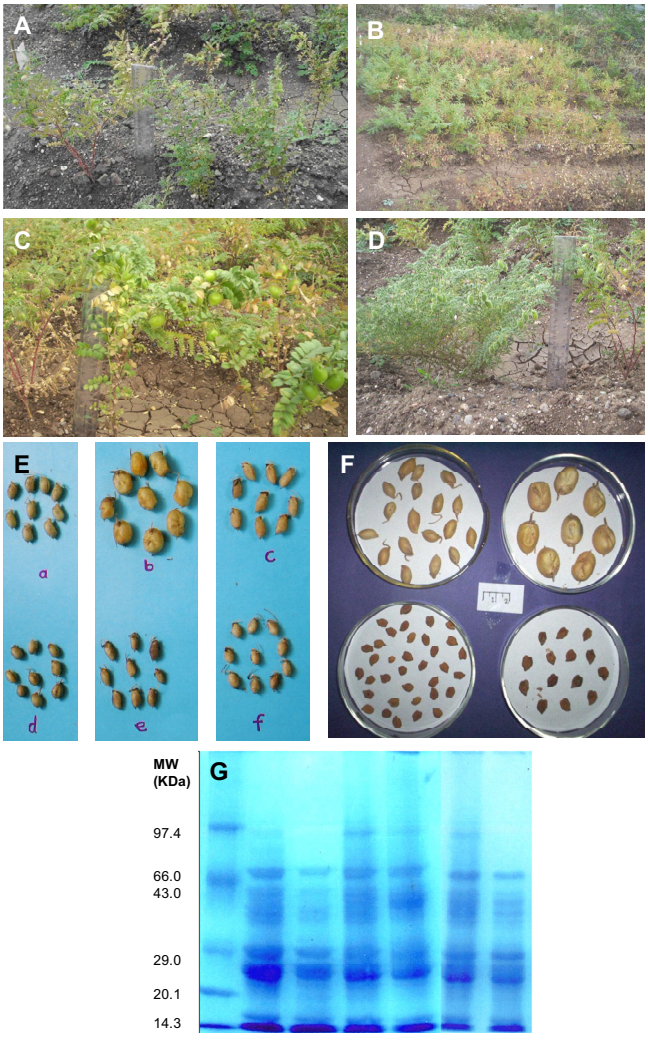

Fig. 1 Parent (control) and *small leaf* mutant in (**A**), early mutant and parent (**B**), parent (left) and *gigas* mutant (right) in (**C**), parent (right) and compact mutant (left) in (**D**), pod of parent a, b to f mutants pod in (**E**), parent (left) and *gigas* mutant (right) pods and seeds in (**F**), SDS-PAGE protein profile of chickpea in induced mutants of Vijay in M_4 generation (**G**).

Table 1 Mean performance for quantitative traits among selected M_4 mutant lines of chickpea.

Mutant / control	Mutagenic treatment	Plant height (cm)	Plant spread (cm)	Number of pods/plant	Number of seeds/plant	Seed yield/plant (g)	100-seed weight (g)
Control	--	29.47	25.43	36.14	37.2	8.40	22.49
small leaf	12 mM EMS	32.47	30.7	70.66	75.96	11.19	15.54
early	500 Gy GR	30.2	21.64	41.33	47.4	9.18	20.33
gigas	400 Gy GR	44.2	24.75	15.66	15.66	5.91	37.5
compact	4 mM SA	31.33	26.00	36.00	36.6	7.19	16.73
bold seeded	500 Gy GR	32.79	26.12	30.66	31.6	6.88	32.09
C V		6.1	6.66	8.61	4.19	10.36	5.44
SE ±		0.71	0.59	1.14	0.59	0.29	0.45
CD (p = 0.05)		2.12	1.77	3.40	1.75	0.86	1.35
CD (p = 0.01)		2.91	2.42	4.66	2.41	1.18	1.85

Data are means (n = 30). CV: coefficient of variation, SE: standard error, CD: critical differences are derived from analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Evaluation of quantitative traits

Five mutants were compared and evaluated for mean values of quantitative traits with parental cultivar 'Vijay' (Phule G 81-1-1) in the M_4 generation. Both positive and negative variation occurred in the quantitative traits as compared to parental cultivar (**Table 1; Fig 1**). Of the five mutants, the mean plant height ranged from 30.2 to 44.2 cm in M_4 generation as compared to 29.47 cm in the parent. Most of the mutants showed both a positive and negative plant spread. A significant reduction in plant spread was observed in the *early* mutant. Conversely, *small leaf* mutant showed a significant increase in plant spread over the control. The maximum number of pods per plant compared to the control was observed for *small leaf* mutant. Among all the mutant lines, highest number of seed per plant was observed in *small leaf* mutant (75.96) as compared to the control (37.2). Among the five mutant *small leaf* mutant showed significantly higher yield per plant (11.19) over parental cultivar (8.40), whereas it was reduced in the *bold seeded* mutant in M_4 generation. Small leaf and compact mutants had lower 100-seed weight. *Gigas* and *bold seeded* mutants showed significantly higher 100-seed weight, which was attributed to the increased cotyledonary cell volume whilst retaining a similar cell number per unit area (Joshua and Bhatia 1983). Early mutant matures earlier than the parent and this will cope better with the late season and moisture stress that is usually encountered in the chickpea growing areas in the state of Maharashtra. Early maturing mutants can be of great importance in the areas with short rainfall also. Early mutants have been reported earlier in chickpea (Toker and Cagirgan 2004) and pigeonpea (Ravikesavan *et al*. 2001). These findings showed that the induced mutations have generated variability for quantitative traits that offers wide scope for genetic improvement of chickpea in forthcoming breeding programme.

Estimation of protein content and nitrate reductase activity

The selected five mutants lines were evaluated for seed protein and NR content in comparison with parent cultivar. Protein content in the mutant ranged between 259.11 and 282.18 mg/g. The *bold seeded* mutant showed highest seed protein (282.18 mg/g) as compared to the parental cultivar (255.59 mg/g). *Compact* and *gigas* mutants also showed higher protein content in M_4 generation (**Table 2**). The highest NR activity was observed in the *bold seeded* mutant (2.87 µmoles/g FW) followed by *compact* mutants (2.70 µmoles/g FW). A lower value was observed in small leaf mutant in the leaves examined. NR activity showed a positive correlation with protein content. Therefore, the results indicate that NR could be used as a tool to correlate with protein content and overall productivity of mutants at an early stage. Our result for NR activity is in agreement with those obtained by Aparna *et al*. (2007).

Table 2 Mean performance of selected M_4 mutant lines for maturity, total crude protein and trypsin inhibitor activity of chickpea.

Mutant/ control	Days to maturity	Protein (mg g^{-1})*	Nitrate reductase (µmoles/g FW)	Trypsin inhibitor (TIU/min/g)*
Control	100	255.59	2.08	3091.5
small leaf	105	259.11	2.17	2530.3
early	89	260.62	2.19	2872.0
gigas	113	273.97	2.59	2927.0
compact	102	274.66	2.70	2465.4
bold seeded	100	282.18	2.87	3530.3

* n = 3, mean values of three replications. Total protein, Mg g^{-1}; µmoles/g FW as nitrate reductase activity. TIU/min/g as trypsin inhibitor content

Trypsin inhibitor activity

The pertinent studies of mutant lines exhibited considerable variation in the TI levels (**Table 2**). Three mutants namely, *small leaf* (2530.3 TIU/min/g meal), *early* (2872.0 TIU/min/g meal) and *compact* mutant (2465.4 TIU/min/g meal) showed lowest amount of TI content as compared to parental cultivar (3091.5 TIU/min/g meal) among the M_4 mutants lines. The *bold seeded* mutant showed higher TI content as compared to the parent. The results indicated that mutagenesis study has been found to be highly effective in reducing the levels of TI content in chickpea. A lower level of TI as a result of treatment with mutagens has been reported by Harsulkar (1994). Low TI-containing mutants identified using induced mutagenesis can be used in the cross-breeding programme for developing low TI lines.

Heritability and variability components for quantitative traits among the mutants

Data in **Table 3** indicates that a consistently greater phenotypic coefficient of variation (PVC) was observed than the genotypic coefficient of variation (GCV) in different quantitative traits among the induced mutants. Comparison among the traits indicated that number of seeds recorded greater PCV (14.2%) followed by number of pods. Plant height and plant spread had lower PCV among the mutant. Because of the enhanced reproductive growth in term of number of seed/plant, diversion of the photosynthates towards vege-

Table 3 Heritability and variability components for quantitative traits among the mutants in M_4 generation of chickpea.

Trait	PCV (%)	GCV (%)	h^2 (%)	Genetic advance as % of mean
Plant height (cm)	11.88	18.85	99.5	48.56
Plant spread (cm)	11.68	11.66	98.58	39.43
Number of pods/plant	14.07	14.06	98.88	65.38
Number of seeds/plant	14.2	14.19	98.84	70.10
Seed yield/plant (g)	12.21	12.21	97.94	12.18
100-seed weight (g)	13.02	13.00	98.80	38.09

Table 4 Presence/absence (+ / -) and molecular weight for various bands electrophoresed in seed protein extracts of different mutants of chickpea cultivar 'Vijay' (Phule G 81-1-1).

Control/ Mutants	Number of bands and molecular weight (kDa)														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	95.5	63.1	59.57	54.95	48.98	45.19	39.36	31.26	28.51	24.83	21.63	20.65	19.05	14.45	13.80
Control	+	+	-	+	+	+	+	+	+	+	-	+	+	+	-
small leaf	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
early	+	-	-	+	+	+	+	+	+	+	+	-	+	+	-
gigas	+	+	-	+	+	+	+	+	+	+	-	+	+	+	-
compact	+	-	-	+	+	+	+	+	+	+	+	-	+	+	-
bold seeded	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+

tative growth probably was minimized resulting into least PCV and GCV for plant height and plant spread. Higher values of PCV and GCV for seed yield/plant indicated further scope of yield improvement through selection of donor for breeding in chickpea.

High heritability coupled with high genetic advance was observed for quantitative traits like number of seeds/plant (**Table 3**), may be due to additive genes. On the contrary, both heritability and genetic advance were less for seed yield/plant. The high heritability with high genetic advance for number of seeds/plant and plant height observed in the present investigation, which may be due to additive genes. Badigannavar and Murty (2007) has reported a high heritability associated with a high genetic advance for plant height, pod yield and seed yield in gamma rays-induced mutants of M_8 generation. We are of the opinion that selection based on heritability and genetic advance for number of seeds/plant and number of pods/plant may be effective for induced mutations for improvement of chickpea.

Total protein profiles

Protein banding pattern revealed variation in number of bands and molecular weights in the viable mutants. Control plants of 'Vijay' (Phule G 81-1-1) exhibited 13 polypeptide bands for total proteins. The *small leaf* and *bold seeded* mutants showed 14 protein bands (**Table 4**). A band with molecular weight 59.57 kDa was found extra in these mutants. The early and compact mutants showed the presence of only 11 polypeptide bands (two bands less than the control). One band having a molecular weight (MW) of 63.1 kDa and another band having MW 13.80 kDa were found missing in these mutants. No difference in protein banding pattern was observed in *gigas* mutants. The present studies on the electrophoretic banding pattern of seed protein could provide quick identification of genetic variability useful for genetic improvement of chickpea.

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