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Development of inter-specific hybrid between *Cicer arietinum* and *C. judaicum* and characterization of interspecific derivatives for economic traits

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Abstract

Interspecific hybrids between *Cicer arietinum* and *C. judaicum* were obtained using embryo rescue techniques. *In vitro* techniques of rescuing embryo/ovule were standardized by applying gibberellic acid (50 ppm/l) at the base of flower pedicil for the next three days after pollination to circumvent post-zygotic barriers. The F_{1s} were partially fertile and showed normal meiotic behavior and intermediate morphological characters. These partially fertile F_{1s} were advanced to F_2 and F_3 generations and subsequently advanced to F_{10} generation using single pod descent method. Wide range of genetic variability was recorded for different qualitative (plant growth habit, pod and seed characters) and quantitative (days to flowering, plant height, number of branches/plant, number of pods/pod, number of seeds/pod, 100 seed weight and yield per plant) characters in F_2 generation. Genotypic and phenotypic correlations of grain yield/plant were found positive and significant with all the component characters namely, plant height (0.275 & 0.208), number of branches/plant (0.494 & 0.325), number of pods/plant (0.922 & 0.687), number of seeds/plant (0.292 & 0.230) and 100-seed weight (0.415 & 0.373). Several promising interspecific derivatives were isolated for yield and its component traits. The derivatives may be used in breeding programme for improvement of chickpea.

Key words: Embryo culture, Chickpea, *Cicer arietinum*, interspecific hybrids

Introduction

Chickpea (*Cicer arietinum)* is an important post rainy season pulse crop of India. It is susceptible to a number of diseases and pests. *C. judaicum*, a wild relative of chickpea, possesses important characters like resistance to *Ascochyta* blight, *Fusarium* wilt, earliness and high branching and pod number [1, 2]. However, *C. judaicum* cannot be normally crossed to cultivated chickpea due to presence of strong post zygotic incompatibility barriers [3, 4]. Although, fertilization often occurs in crosses between these species, no fully developed seeds have been obtained [4, 5].

Efforts have been made to study barriers to inter-specific hybridization between *C. judaicum* and chickpea cultigens [4, 6]. In inter-specific crosses involving *C. judaicum*, good pollen germination, pollen tube growth and penetration of pollen tube into the ovule has been observed. However, in spite of successful fertilization, embryo usually aborts within 3-7 days after pollinations [4]. Application of *in vitro* rescue techniques proved effective in circumventing post-zygotic barriers in wide crosses of many leguminous species belonging to genara like *Cajanus* [7, 8], *Cicer* [9-14], *Medicago* [15], *Phaseolus* [16], *Lens* [5] and V*igna* [17]. The present investigation was under taken with the objective to produce interspecific hybrids between *C. arietinum* and *C. judaicum* through *in vitro* embryo rescue in order to introgress genes of economic importance in the elite chickpea cultivars.

Materials and methods

Plant materials and growing conditions

Two hundred seeds each of chickpea cultivars/genotypes, namely, L 5325, RSG 2, K 850, H 208, Pusa 408, Annegeri 1, Pusa 261, Jyoti and PDG 84-10 and 100 seeds of wild species, *Cicer judaicum* accession no. ICCW 36 were planted in field. The sowing was repeated thrice in a staggered manner at 10-day interval to have a continuous and synchronous flowering.

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Pollinations

Reciprocal crosses were made between chickpea genotypes and *C. judaicum*. In chickpea flowers, pollination takes place within 4-6 hr of anthesis. Hence, pollination was done as soon as flower opens. To prevent the pod abscission, a solution of growth regulator, gibberellic acid (50 ppm/L) was applied at the base of the flower pedicel till three days after pollination. Fertilization was regarded as normal and successful, if growth of embryo continued for not less than 10 days and pod remained green.

Ovule/ embryo culture

A regeneration protocol developed earlier [18] for immature embryo culture of wild *Cicer* spp. and chickpea genotypes was followed with modification for regenerating interspecific hybrid embryo (Table 2). Immature hybrid pods were harvested when sign of yellowing/degeneration was observed and surface sterilized for 1 min in 70% ethanol, followed by 2 min in 3% sodium hypochlorite. Ovules were carefully dissected from the sterilized pods under a dissecting microscope in sterile conditions. These ovules were immediately placed on filter paper bridges in MS liquid medium supplemented with 0.5 mg/L kinetin and 0.5 mg/L NAA (growth medium) with 60 % sucrose. After 8 days of continuous growth of ovules, embryo was dissected from ovule and sub-cultured on the same growth medium. No sterilization of embryo was done at this stage. Further subculture was done after a week on MS medium supplemented with 1.5 mg/L BAP and 1.0 mg/L NAA (proliferation medium). Regenerated shoots were rooted on MS medium supplemented with 2.0 mg/L NAA (rooting medium) (Table 2). The cultures were incubated under diffused light for 5 days, thereafter exposed to a 16/8 h photoperiod with 2000 lux illumination using cool white fluorescent lamp.

Regenerated hybrid plants were transferred to green house/poly house in pots containing a mixture of autoclaved sand, soil, vermiculite and compost in a ratio of 1: 1:1:1 and subsequently transferred to field after hardening.

Cytological and morphological characterization of interspecific hybrids

Cytological studies were carried out in interspecific hybrids and their parents. Root tips were hydrolyzed in 1N HCL solution for 20 min at 60°C, washed thoroughly with distilled water and stained in 2% Feulgen stain. Squashes were made in 2% acetocarmine solution. Somatic chromosome number were counted at metaphase I. Floral buds of appropriate stage were collected from individual hybrid plants from the field and fixed in a mixture of 95% ethanol, chloroform and propionic acid (6:3:2 v/v) for 24h and stored under refrigeration until use. Meiotic preparations were made by squashing and staining with 0.5% propionic carmine. More than 250 pollen mother cells (PMCs) were analyzed in each hybrid and chromosome configurations were recorded at first meiotic metaphase. The hybrids were morphologically characterized for different parameters. Pollen stainability of the putative hybrids was estimated (in percent) by staining the anthers with 2% acetocarmine solution after fixation of flowers in acetic alcohol just prior to anthesis.

Evaluation and selection of promising lines

The F_1 hybrids were selfed to produce F_2s and further advanced up to F_{10} generation using modified single pod descent method [19]. These advanced breeding lines were grown and evaluated in Augmented Block Design [20] during *rabi* 2006-07 in 14 blocks, where each block had 10 Advanced Breeding Lines (ABLs) along with controls *viz*., K 850 and PDG 84-10. Two rows of two meters length constituted one ABL. Row to row and plant-to-plant distance were 60 cm and 10 cm, respectively. The observations on five randomly taken plants were recorded for seven quantitative traits *viz*., days to flowering, plant height, number of branches/ plant, no. of pods/plant, seeds/pod, 100-seed weight and yield/plant. Advanced breeding lines having higher mean values for trait(s) over respective control PDG 84-10 were selected in F_{10} generation.

The estimation of magnitude of variability (variance and coefficient of variation), heritability, genetic advance and correlation coefficients were done as per the standard statistical procedures [21].

Table 1. Interspecific hybrids of *Cicer* developed through embryo rescue technique

Interspecific Cross	No. of pollinations	No. of pod set	No. of ovule	No. of embryo cultured	No. of embryo proliferated
C. arietinum x C. judaicum	1520	123	89	48	32
C. judaicum x C. arietinum	1270	88	75	38	13

Table 2. *In vitro* regeneration from immature embryo in chickpea

S.No.	Media Composition	Response
1.	MS + 0.5 mgL^{-1} NAA + 0.5 mgL^{-1} Kinetin	Growth and Induction of shoot primordial (>80%)
2.	MS +1.5 mgL^{-1} NAA + 1.0 mgL^{-1} Kinetin	Partial induction (18-25%)
3.	MS + 1.0 mgL^{-1} NAA + 0.5 mgL^{-1} Kinetin	Partial induction (31.5- 58.0%)
4.	MS + 2.0 mgL^{-1} NAA + 0.5 mgL^{-1} Kinetin	No induction of shoot primordial
5.	MS + 2.5 mgL^{-1} NAA + 1.5 mgL^{-1} Kinetin	No induction of shoot primordial
6.	MS + 1.5 mgL^{-1} BAP + 1.0 mgL^{-1} NAA	Shoot proliferation (>85.0%)
7.	MS + 1.0 mgL^{-1} BAP + 1.0 mgL^{-1} NAA	Poor shoot proliferation (<38.0%)
8.	MS + 0.5 mgL^{-1} BAP + 0.5 mgL^{-1} NAA	No shoot proliferation
9.	MS + 2.0 mgL^{-1} NAA	Rooting (Perfect rooting >80%)
10.	½MS+0.1NAA	Partial rooting (30-52%)
11.	½MS+0.1NAA	Partial rooting (<25%)
12.	MS + 0.1 mgL^{-1} IAA	No rooting
13.	MS + 0.5 mgL^{-1} IAA	No rooting
14.	MS + 0.1 mgL^{-1} IBA	No rooting
15.	MS + 0.5 mgL^{-1} IBA	No rooting
16.	MS + 1.0 mgL^{-1} IBA	No rooting

Results and discussion

Pollination, crossability barriers and ovule/embryo culture

All the nine genotypes were hand emasculated and pollinated with *C. judaicum. C. judaicum* is related species of *C. arietinum* and hence it is expected to cross easily than other species which are distantly related. However, varietal differences with respect to time required for pollen tube to reach micropyle and to ovule leading to successful fertilization were observed among the crosses. *C. judaicum* easily hybridized with PDG 84-10 and, therefore, it was selected for its best nicking ability. Hence, the selection of proper genotype of chickpea and the accession of wild species is the key to success in interspecific hybridization. Differences between pollination to fertilization time in interspecific crosses and their reciprocals have been earlier observed [22]. The time required for pollen tube to reach micropyle was almost double (26-28 hrs) when *C arietinum* was used as male parent, rather than used as female parent (16-18 hrs), in crosses with *C. judaicum*. It is, therefore, noteworthy that the time of pollination may act by preventing the fertilization in the crosses of cultivated chickpea and its wild relatives. Over 2790 pollinations were made between *C. arietinum* cv. PDG 84-10 and *C. judaicum* (ICCW 36) in straight as well as in reciprocal crosses. To promote fertilization, gibberellic acid (50 ppm /L) was applied at the base of pedicel of the flower till three days after pollination. Data on number of pollinations made, the number embryos rescued and their proliferation and pod development are presented in Table 1. In the majority of cases, no pod growth was observed and the pollinated flowers dried up and fell of the plant within 3-4 days. However, two to eight per cent pod initiation was recorded. The embryos (globular pro-embryo stage) generally aborted between 3-7 days after pollination (Fig.1a) possibly due to the degeneration of endosperm in the hybrids [23] and no mature seeds were obtained from such crosses. Ahmad and Slinkard [24] studied the extent of embryo and endosperm growth in interspecific crosses of *Cicer* spp. using histological methods and reported successful fertilization leading to zygote formation. According to them, embryo showed continued and retarded growth at different rates in various crosses, but eventually aborted at an early pro-embryo stage. They also observed reciprocal differences in early embryo growth rate and suggested that this could have implications in obtaining interspecific hybrids. In the present study 123 and 88 immature pods remained healthy in *C. arietinum* x *C. judaicum* (Cross I) and *C. judaicum* x *C. arietinum* (Cross II), respectively. A total of 89 and 75 immature ovules were recovered from the pods of cross I and cross II, respectively. These ovules were cultured on MS liquid medium with support of filter paper bridge for regeneration (Fig. 1b & Table 2). After a week, 48 hybrid immature embryos (heart/ torpedo shape) from cross I and 38 from cross II were dissected and further cultured on the modified MS medium supplemented with suitable plant growth regulators [17].

After 5-8 days, the developed embryos (cotyledon shape) were again sub-cultured on the shoot differentiation medium, which resulted into induction of multiple shoot primordia (Fig.1c). Only 32 embryos from cross I and 13 from cross II were proliferated and formed healthy shoots (Fig.1d). All the proliferated shoots were rooted successfully on rooting medium (Table 2, Fig.1e) and transferred to greenhouse/poly house and subsequently to field conditions where they grew till maturity. Successful *in vitro* regeneration from immature embryo has been earlier demonstrated in chickpea cultigens and wild species [10,12,17]. Also the development of successful inter-specific hybrids in chickpea using embryo rescue have been reported involving *C. cuneatum* [9], *C. bijugum* [14] and *C. pinnatifidum* [12]. The success in producing inter-specific hybrids depends upon the number of pollinations attempted and the specific combination involving a particular cultivar and wild species chosen, prevailing environmental conditions during crossing, genetic background and selectivity/specificity of parents keeping as female or male [25, 26]. In present study, keeping *C. arietinum* as female parent and application of growth regulator(s) to the pollinated pistils had a major effect on successful hybridization.

Morphological and cytological characterization and pollen fertility

In vitro regenerated hybrid plants were confirmed as true F_1 hybrids based on the comparative observations of their morphological characters (Table 3). All the hybrid plants were intermediate between wild and cultivated parents for growth habit (Fig. 2), seedling height, flowering, maturity and colour of seed. Somatic chromosome number in both the parents and interspecific hybrid was 2n=2x=16. Also the F_{1s} showed normal meiotic behaviour forming bivalents at Metaphase I. However, precocious disjunction of one bivalent was noticed. The F_1 hybrids were partially sterile and recorded 54 % pollen fertility, which may be ascribed to precocious separation. This could also be due to gene interactions and poor adaptation of tissue culture raised hybrid plants in open environment. Partial sterility, intermediate morphological features and normal bivalent formation during meiosis in F_1 hybrids derived from inter-specific crosses in genus *Cicer* [9, 14, 26-28] has been earlier reported.

Genetical and statistical analysis

A large magnitude of variability was observed in F_2 generation for various qualitative (growth habit, seed shape, seed surface, seed size and seed colour) and quantitative traits (Table 4) *viz*., plant height (28.0-62.67 cm), number of branches/plant (2.33-8.67), seeds/pod (1.00-1.73) and yield/plant (18.33-317.30g). The estimates of phenotypic and genotypic coefficients of variation obtained for yield/plant (64.57 & 42.97) followed by pods/plant (63.91 & 43.58) were of high order. Moderate degree of variability (both PCV and GCV) was exhibited for the number of branches/plant (31.48 & 19.28) and 100-seed weight (31.46 & 30.53). Number of seeds/pod (20.97 & 8.97) and plant height (18.93 & 13.79) showed low phenotypic and genotypic coefficient of variability. Large magnitude of variability recorded in F_2 generation for various qualitative and quantitative traits was expected due to greater diversity among parents containing different set of genes. Similar trend for higher magnitude of genetic variability in

Table 3. Characterization of $F_1^{'s}$ of cross *C. arietinum* x *C. judaicum*

S. No	Characters	*C. arietinum* (PDG 84-10)	*C. judaicum*	F_1
1.	Pollen fertility	98%	95%	54%
2.	Somatic chromosome no.	2 n = 16	2 n = 16	2 n = 16
3.	Meiotic behavior (Metaphase I)	8 II (normal chromosome segregation)	8 II (normal chromosome segregation)	(Normal meiotic association)
4.	Growth habit	Erect	Semi-erect	Intermediate
5.	Plant height(cm)	48-62	15-40	36-42
6.	Flowering (days to 50%)	90-98	80-90	70-92
7.	Maturity(days)	120-125	90-110	80-110
8.	Colour of F_1 seeds	Yellowish brown	Blackish brown	Brown

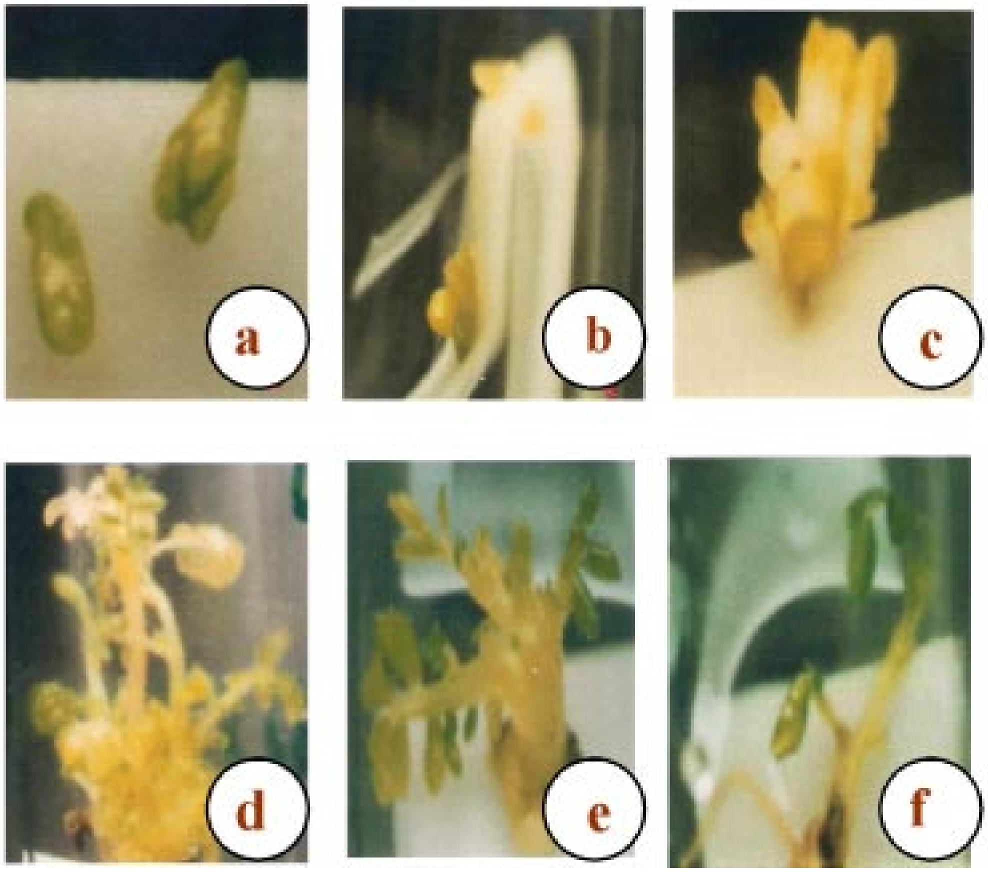

Fig. 1. Regeneration of rescued immature hybrid embryo of cross *C. arietinum* x *C. judaicum,* a) immature ovule showing sign of degeneration (yellowing) b) culture of rescued immature embryo on MS liquid medium with support of filter paper bridge, c) differentiation of immature embryo into multiple shoot primordial, d) multiple shoot proliferation, e) subculture of individual shoots on separate medium, f) rooting of shoots

Fig. 2. Morphological features of interspecific hybrids and respective parents (a) *Cicer judaicum* (b) Interspecific hybrid (c) PDG 84-10

interspecific crosses involving *C. arietinum* and *C. reticulatum* has been documented [27, 28]. The higher estimates of genotypic coefficient of variance for yield/ plant and number of pods/plant indicate a great scope for improvement in chickpea through rigorous selection. Wider gap between phenotypic and genotypic coefficient of variations for some characters found under present investigation may be due to high genotype x environment interaction.

The heritability estimates ranged from 37.6 to 94.2% for the number of branches/plant and 100-seed weight, respectively (Table 4). However, heritability for grain yield/plant (44.3%) was comparatively low to its component traits *viz*., 100-seed weight (94.2%), number seeds/pod (72.5%), plant height (53.1%) and number of pods/plant (46.5%) as expected. High estimates of genetic advance (percentage of mean) was obtained for grain yield/plant (67.9%) followed by pods/plant (60.8%) but extremely low for 100-seed weight (10.53%) and plant height (6.68%). The lowest heritability estimates was obtained for branches/plant and highest for 100-seed weight. Hence, increase in yield may be obtained through selection of component traits *viz*., 100-seed weight, seeds/pod and plant height with high

Table 4. General mean, range, variance, CV, heritability and Genetic advance for different characters in F_2 derivatives of cross *Cicer arietinum* x *C. judaicum*

Characters	General mean	Range	Variance		Coefficient of variation		Herita-bility	Genetic advance
			Genotypic	Phenotypic	Genotypic	Phenotypic		
Plant height (cm)	41.92 ±0.44	28.00-62.67	33.45	62.95	13.79	18.93	53.1	8.68
Branches/plant	05.61 ±0.114	02.33-8.67	01.17	03.12	19.28	31.48	37.60	1.37
Pods/plant	99.33 ±0.154	21.67-245.33	1873.83	4029.74	43.58	63.91	46.50	60.81
Seeds/pod	01.22 ±0.188	01.00-1.73	0.047	0.065	8.97	20.97	72.50	0.38
100-seed weight (g)	17.26 ±0.107	06.00-32.67	27.75	29.46	30.53	31.46	94.20	10.53
Yield/plant (g)	115.42 ±0.164	18.33-317.30	2459.36	5554.11	42.97	64.97	44.28	67.98

Table 5. Genotypic and Phenotypic correlation coefficient in Advance Breeding Lines (F_{10} derivatives) of cross *Cicer arietinum* x *C. Judaicum*

Characters Branches/plants	Plant height	Branches/plants	Pods/plants	Seeds/pod	100 seeds wt.
Genotypic	0.260**	0.291**	-0.149	0.185	0.275**
Phenotypic	0.145	0.231*	-0.064	0.143	0.208*
Pods/plants					
Genotypic		0.472**	0.197*	0.242	0.494**
Phenotypic		0.337	0.033	0.148	0.325**
Seeds/pod					
Genotypic			0.054	0.429**	0.922**
Phenotypic			0.010	0.384**	0.687**
100 seeds wt.					
Genotypic				0.102	0.292**
Phenotypic				0.042	0.230*
Yield/plant					
Genotypic					0.415**
Phenotypic					0.373**

*Significant at 5%; **Significant at 1%.

values of heritability and low GCV might not play major role in yield increase. Whereas, higher heritability (%) values for 100-seed weight and seeds/pod indicate low influence of environment. These trends obtained in interspecific crosses are very similar to those normally reported in intra-varietal crosses. High estimates of heritability for 100-seed weight and seeds/pod have also been reported in interspecific crosses of chickpea [27-29], pigeonpea [30] and also in intra-varietal crosses in chickpea. The characters like no. of pods/plant having high heritability and genetic advance can be used as an index for indirect selection for enhancing grain yield. These results are in agreement with the findings reported for interspecific crosses with other wild *Cicer* spp. in respect of genetic gain [27, 28].

Hundred-seed weight also showed positive and significant genotypic and phenotypic correlations with number of pods/plant (0.429 & 0.384). Similarly, pods/plant showed significant and positive genotypic and phenotypic correlation with plant height (0.291 & 0.231). If two or more desirable characters are associated, the selection becomes easier for those traits, which directly or indirectly results into the crop improvement. Significant and positive genotypic and phenotypic correlation of grain yield with plant height, number of branches/plant, number of pods/plant, number of seeds/plant and 100-seed weight indicate that increase in yield is attributed by these component traits to a large extent. The similar pattern of correlation of grain yield with component traits have been reported by earlier workers in interspecific crosses in chickpea [27, 28].

Selection of promising lines

The data on evaluation of advanced breeding lines in F_{10} generation were subjected to analysis of variance for comparing the treatments and ignoring the block effects. Since the block effect was significant, the analysis pertaining to treatment comparison eliminating block effect was only considered here. Promising lines were selected on the basis of higher mean of ABL's over control (Table 6). Thirty eight lines were found promising for the number of branches/plant, 35 for plant height, nine for yield/plant, ten for number of pods/plant, five for earliness and only three for 100-seed weight as compared to check, PDG 84-10. The selection of a good number of agronomically superior lines is an instance of translating the expressed variability getting released through recombination of favourable genes in interspecific hybridization.

Even in advance generation (F_{10}), many promising lines showed various undesirable traits such as, pod shattering, prostrate growth habit, hard seed coat, undesirable seed surface and seed color. Therefore, several lines had to be rejected due to these undesirable traits. Generally, the negative and poor correlation between phenotypes of various traits made selection of progenies very difficult in early generations. It was also observed that segregation in several characters *viz*., growth habit, flower and seed color, seed size and seed shape continued to occur even in F6/F7 due to disharmony between genes.

The superior lines isolated through intense selection in advance breeding material having high yielding traits may be used as good source of donors for high number of branches and pods/plant for enhancing productivity in chickpea improvement programme. These advance breeding lines may also be used for molecular mapping of different traits related to yield and its components.

Table 6. Promising lines isolated for various characters from ABLs population in F_{10} generation of cross *C. arietinum* x *C. judaicum*

S.N	Character	Mean value of check I (PDG 84-10)	SE±	CD	No. of superior lines over check
1.	Days to flower (d)	85.0	3.0506	6.5930	05
2.	Plant height (cm)	52.0	4.7365	10.2367	35
3.	Branches/plant	7.5	5.0140	10.8363	38
4.	Pods/plant	45.0	2.3240	15.024	10
5.	Seeds/pod	2.0	2.6976	5.8301	00
6.	100seed weight (g)	24.5	2.4649	5.3272	03
7.	Yield/plant (g)	32.8	1.9185	4.1462	09

Where, Check I, PDG 84-10
Check II, K 850 was compared for seed size (28.0g/100seeds) only

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