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Genetic variability in the progeny of androgenic dihaploid plants and selection of high agronomic performing lines in *Brassica juncea*

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Abstract

Androgenic lines of *Brassica juncea* cv. PR-45 raised by anther culture, were screen for genetic variation. 393 androgenic plants were transferred to pots to study the R_0 generation. These plants showed substantial variation for different characters. Seed progenies of 27 lines of R_0 plants were sown in the field to study R_1 generation. Androgenic plants within lines were significantly homogeneous for the various agronomic characters studied. Two lines were shorter (18 - 20 %) than the control plants, with a remarkable feature of early maturation. Three lines showed 27 - 31 % higher yield than the parent cultivar.

Additional key words: androgenesis, doubled haploid, gametoclonal variation, Indian mustard.

Introduction

Since the first report of *in vitro* androgenesis in *Datura innoxia* (Guha and Maheshwari 1964), androgenic haploids of many crop plants could be raised (Bhojwani and Rajdan 1996). Androgenesis has been accomplished successfully in most of *Brassica* species and the technique is being routinely applied in the breeding of *B. napus* and *B. oleracea* (Cao *et al*. 1995). Anther culture has been reported in the *B. juncea* (George and Rao 1982, Sharma and Bhojwani 1985, Agarwal and Bhojwani 1993). However, no effort has been made to field test the dihaploid plants of *B. juncea* for variations in agronomic traits. Since *B. juncea* is an important edible oil crop of the Indian subcontinent, the present study was undertaken to screen the field grown pollen plants of *B. juncea* for gametoclonal variation.

Materials and methods

The seeds of *Brassica juncea* cv. PR-45 were obtained from the Indian Agriculture Research Institute, New Delhi, India. The seeds were sown in the Botanical Garden of the University of Delhi in November. For anther culture 2 - 3 mm flower buds at uninucleate stage of microspore were collected in the morning. The buds were pre-washed with diluted solution of *Tween* 20, and sterilized by treating with 0.1 % solution of $HgCl_2$ for 5 min. After three rinses in sterilized distilled water, the anthers were dissected out and cultured as previously reported (Agarwal and Bhojwani 1993). After 4 weeks, the healthy and normal looking embryos were individually picked and transferred for germination to B_5 medium (Gamborg *et al*. 1968) supplemented either with 0.28 μM gibberellic acid (GA_3) and subjected to 4 °C for 6 d.

During the off-season (February to October) healthy plumular shoots from germinated pollen embryos, were chopped into small 4 - 5 nodal segments and cultured on MS (Murashige and Skoog 1962) medium supplemented with BAP (0.2 μM). Shoots are multiplied by axillary shoot proliferation by every 3 - 4 weekly subculturing.

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Abbreviations: BAP - benzylaminopurine; NAA - naphthaleneacetic acid.
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In the first week of August four nodal segments from micropropagated shoots were treated with 1 % colchicine colchicine solution to induce diploidization. Sterilized cotton swabs soaked in colchicine solution were placed on the axillary buds. After 24 h the cotton swabs were removed and single node segments were cultured on MS + 02 μM BAP for axillary shoot development.

Healthy 3 - 4 cm long shoots from, both colchicine treated and untreated axillary buds were cultured on MS medium supplemented with NAA (0.1 μM) to induce rooting.

After 7 d on the rooting medium, plants were removed from culture tubes, their roots washed gently under running tap water to remove the adhering medium, and planted in acid washed sand in 7 × 7 cm^2 plastic pots. The plants were irrigated with 1/4 MS macro and micro salts for the initial 7 d and thereafter with tap water. Initially, the plants were covered with transparent polythene bags to maintain them under high humidity, and kept under 16-h photoperiod at irradiance of 15 μmol m^{-2} s^{-1} and temperature of 25 ± 2 °C. After 2 - 3 d the polythene bags were removed for approximately 30 min. The exposure time was gradually increased and after 10 d the bags were completely removed. The 3-week-old acclimatized plants were transplanted to clay pots containing garden soil and organic manure (3:1). The plants were irrigated with tap water at intervals of 2 d. These R_0 plants were grown in pots until maturity.

R_0 plants were transferred to pots in two batches. In order to assess the spontaneous diploidization, plants from 1st batch were transferred with and without colchicine treatment, whereas in 2nd batch only colchicine treated plants were transferred. The plants were bagged to collect the self-seeds for the analysis of their R_1 generation. The plants with 1 or 2 seeded pods showed embryo abortion before maturation. In such cases full plants could be obtained by culturing excised immature embryos on MS + 0.2 μM BAP. The quantitative data of R_0 plants were recorded for different agronomic traits such as plant height, number of nodes, number of primary branches, number of secondary branches, total number of pods, pod length with beak, pod length without beak, seeds per pods, seeds mass, total seed mass, biomass (total dry mass of the plant), and harvest index (seed mass divided by biomass and then multiplied by 100).

Ploidy status was determined in fast growing root tips from androgenic plantlets and the seedlings, fixed in 4 % chilled formaldehyde for 2 h and transferred to acetic acid:ethanol solution (1:3) after thorough washing with water. Fixed roots tip were stained in Feulgen solutionand squashed in glycerol. DNA was estimated at 565 nm using a *Leitz MPV* (Wetzlar, Germany) scanning microdensitometer.

For cytological analysis the young flower buds from R_0 plants were fixed in alcohol, chloroform, and acetic acid (6:3:1) solution for 24 h and stored in 70 % ethanol. The anthers were squashed in 1 % acetocarmine and 20 - 25 microspore mother cells (MMCs) at metaphase-1 and anaphase-1 stages were considered for counting chromosome number. The sample cells were photographed using a *Nikon Optiphot Microscope* (Tokyo, Japan)

Selfed seeds of R_0 plants were sown in the subsequent season in the experimental plots of the Indian Agriculture Research Institute (IARI), New Delhi, India. Seeds of R_1 progeny of 20 gametoclones of 1st batch (data not included) and 27 gametoclones of 2nd batch were sown in a randomized block design in two replicates. Before sowing the seeds, experimental plots were fertilized with 60 kg ha^{-1} nitrogen, 40 kg ha^{-1} phosphorous and 40 kg ha^{-1} potash. Each row was 3 m long and distance between the rows was 45 cm. Approximately 20 - 25 plants were raised in each row. After every 15 lines 2 rows of control plants were sown. Two plants from each end of the row were discarded for data analysis to avoid edge effect. In the R_1 progeny five more traits, length of main raceme, circumference of main branch, pods on the main raceme, days to flowering and days to maturity were also recorded. In this paper, the data for 9 agronomic traits directly related to yield, time to flowering and time to maturation are presented.

The statistical method used for the analysis of data were as follows: *1*) study of mean performance and variability within each line as given by the standard error of the mean; *2*) study of variability between means of lines in relation to within lines by the analysis of a completely randomized design for each replication and pooling the variation over the two replications. This was done to provide evidence for genetic variability, if any, between lines; *3*) study of the mean value of lines over the two replications in order to detect significantly better performers in relation to the control and also those which were statistically at par with the best, *i.e.* lines giving the highest mean value. For this the analysis of randomized complete block design was used. The methodology has been adopted mainly from Steel and Torrie (1960) and Snedecor and Chochran (1968).

Results

Colchicine treated axillary buds did not grow or exhibited very slow growth. The first one or two leaves on the lateral shoots developed from colchicine treated buds were vitrified but later the shoots developed normally in 47 % cultures. The axillary shoots formed by treated and untreated buds were sub-cultured on MS medium

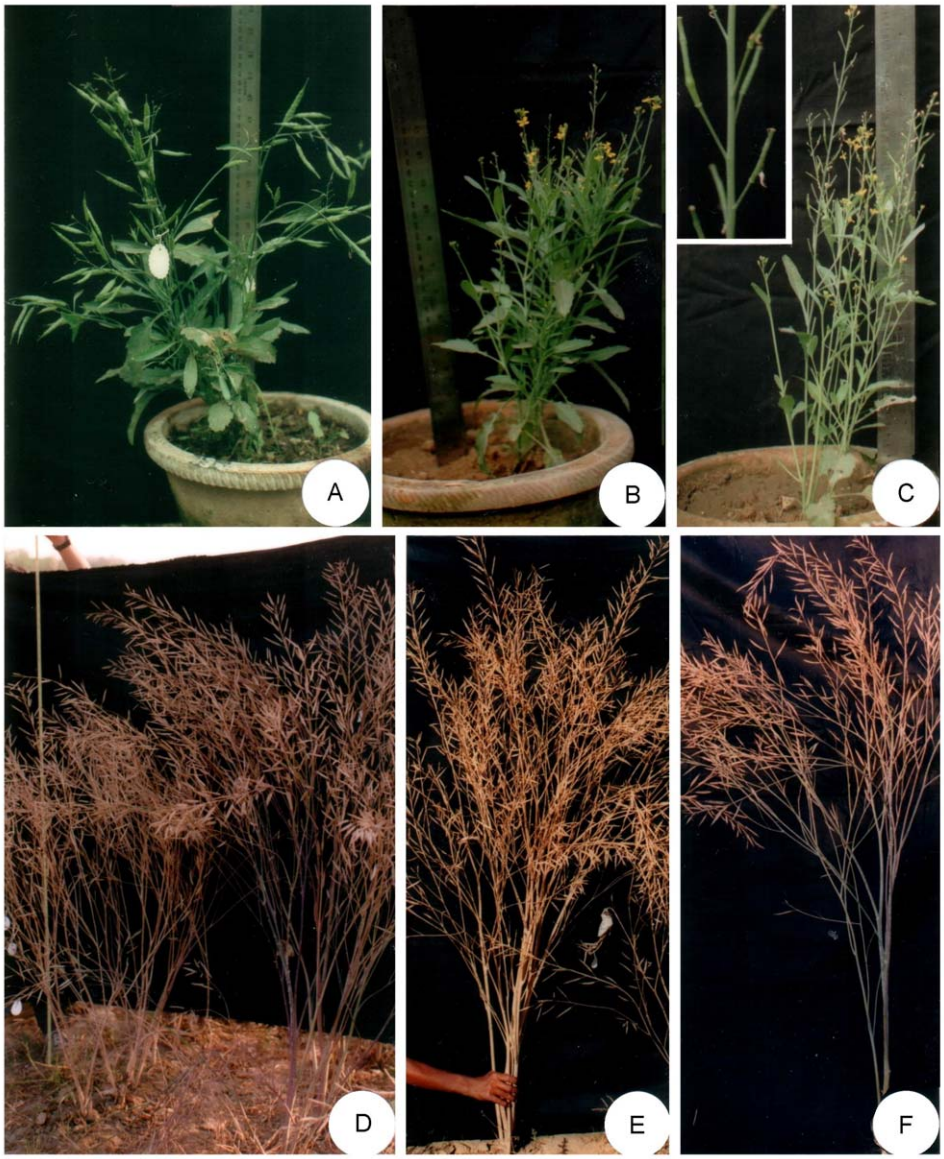

Fig. 1. *A* - a diploid fully fertile plant in R_0 generation, showed short height; this plant perpetuated the same trait in R_1 generation; *B* - fully sterile bushy plant without seeds; *C* - a sterile plant without seeds but some pods were having one or two seeds (see *inset*); *D* - two different lines at R_1 generation. Right line represents control lane and left line is the shortest line achieved after diploidization, this line is derived from the plant shown in Fig. 1*A* (note the highly homogenous nature of plants in the line); *E* - a R_1 diploid plant of high yielding line (Table 1, line 24) showing large number of secondary and tertiary branches and also high number of pods; *F* - control plant of *B. juncea* cv. PR-45.

supplemented with 0.2 μM BAP for elongation. Meiotic studies of the R_0 plants revealed 43.2 % diploidization. Spontaneous diploidization was 18.7 %.

The roots developed in the rooting medium were tiny and healthy. Both colchicine treated and untreated plants showed 76 % survival during transplantation to small plastic pots. Later on, when plants transferred from plastic pots to clay pots, colchicine treated survived to 91.3 % whereas untreated only 41 %. The lower survival of untreated plants in the later stage is due to haploid nature of the plants.

The R_0 plants (312) derived from different pollen embryos exhibited substantial variation in terms of morphology, pollen viability and seed set. One class of the plants was morphologically comparable to the parent plant attained height between 81 - 150 cm (Fig. 1*A*) and set proper seeds. The second class of plants was shorter ranging from 25 - 80 cm and showed bushy growth with lanceolate leaves (Fig. 1*B*,*C*). In general the pods of these plants were smaller (1.6 - 3.7 cm) as compared to fertile

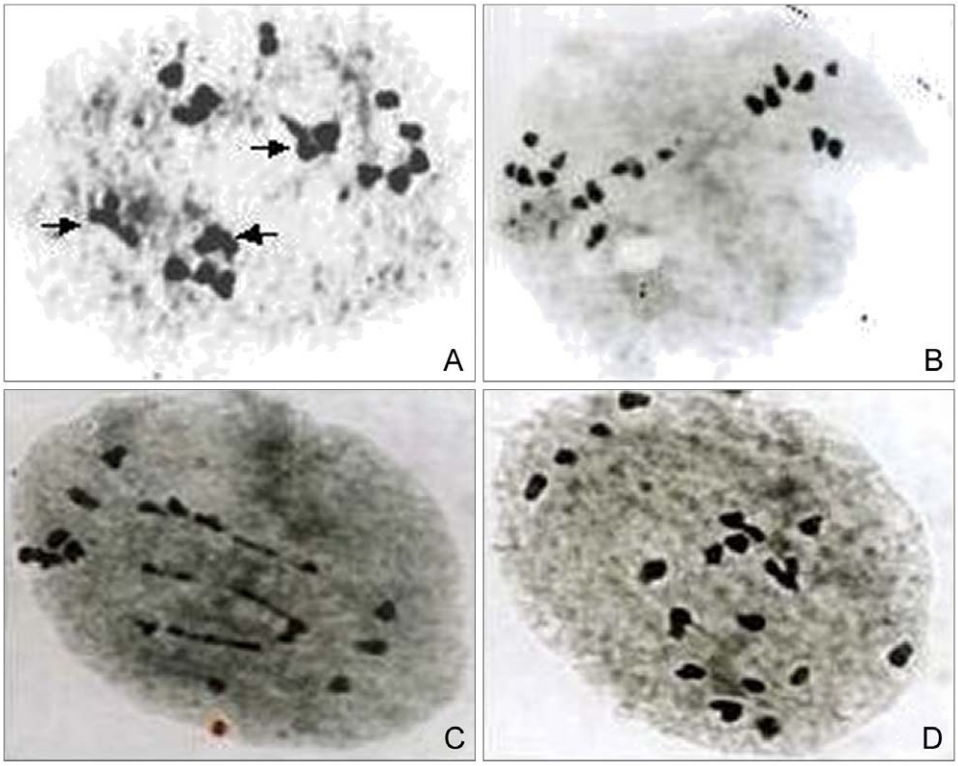

Fig. 2. *A* - microspore mother cell (MMC) of diploid plant at early metaphase stage showing 18 bivalents, *arrow* shows the merging of two bivalents at three positions; *B* - MMC of haploid plant at late anaphase stage showing 18 univalents; *C* - another MMC of haploid plant with 18 chromosomes, showing chromosome bridges and unequal distribution of chromosomes; *D* - MMC at anaphase stage showing unequal distribution of chromosomes.

plants (2.6 - 5.3 cm) and did not have seeds (Fig. 1*B*), but occasionally one or two seed were seen (Fig. 1*C*). In later case seeds showed precocious embryo germination at the early dicotyledonous stage (vivipary) which eventually died due to lack of proper growth condition. However, excised early dicotyledonous embryos from such plants germinated within two weeks on MS basal medium, and the resulting plantlet developed into fertile plants showing normal seed-set.

The cytological examination of the colchicine treated R_0 plants revealed 42.3 % diploid [2n=36, Fig. 2*A* (18 bivalents)] and 56.7 % haploid. Among the untreated plants 18.7 % were diploid and 81.3 % were haploid. The haploid plants showed 18 univalents (Fig. 2*B-D*). At anaphase-I the MMCs often showed unequal distribution of the univalents to the two poles (Fig. 2*C*,*D*). Occasionally chromosome bridges and fragments were also observed. The plants, which produced only one or two abortive seeds per pod, were haploid. Ploidy status was also confirmed by DNA estimation and pollen viability. DNA estimation revealed approximately 1.81 - 2.9 ± 0.02 pg of DNA in haploid plants while 5.58 ± 0.02 to 5.65 ± 0.2 pg in control and diploidized plants, respectively.

The dihaploid (DH) lines showed a wide range of variations for different characters. The variability between the lines was highly significant (at 0.1 % probability level, Table 2). However, the variability within the lines was not significant for most of the traits (Table 3). Two lines were significantly shorter than control plant (Fig. 1*D*). Being short in height these lines possessed less number of nodes, primary branches, biomass and thus low yield. However, it was interesting to note their early flowering and maturation characteristics. 9 DH lines (3, 6, 8, 10, 12, 16, 18, 24 and 27) were significantly taller than the control plants. None of the lines showed a significantly larger circumference of main stem, but three lines showed thickest stem (3, 8 and 24). Number of nodes was significantly higher in lines 3, 9, 10, and 18.

Number of seeds per pod, which is an important character, was significantly higher in 6 lines (4, 6, 7, 16, 21 and 24). None of the lines showed significantly higher 100-seed mass or total seed mass. However, three lines (6, 7 and 24) yielded 27 - 31 % more seed weight then control line (Table 1). Line 24 (Fig. 1*E*) was considered to be outstanding since it was either at par with the highest performer or significantly better than the control (Fig. 1*F*) for 6 of the characters. These lines showed consistently higher yield in their next generation.

Table 1. Means ± SE for different agronomic traits of various androgenic lines (** - significantly different from the control, * - at par with the control, $^{+}$ - lines performing better over control).

Line number	Plant height [cm]	Time to flowering [d]	Time to maturity [d]	Pod number [plant^{-1}]	Seed number [pod^{-1}]	Seed mass [mg]	Total seed mass [g plant^{-1}]	Biomass [g]	Harvest index
Control	157.8±3.2	54.2±1.20	148.9±0.32	751.2± 77.4	11.0±0.51	6.7±0.2	41.0±6.4	151.9±21.1	26.3±1.0
1	147.9±2.0	49.4±1.71	138.6±1.22	497.0± 48.2	11.2±0.5	6.2±0.1	21.7±2.0	85.1± 6.6	25.4±1.3
2	168.2±2.5	57.9±1.05	139.1±1.12	1207.1±136.1^{+}	11.5±0.3	6.1±0.2	47.6±5.4	174.8±16.8	26.8±0.7
3	186.9±2.0**	63.1±0.69	147.5±1.08	853.5±111.2	12.3±0.2*	6.3±0.1	40.7±4.2	144.6±15.1	28.2±0.7
4	124.3±2.7**	45.5±0.97*	133.3±0.96*	458.6± 59.2	13.6±0.3**	6.9±0.2	25.7±2.6	96.0± 8.3	26.8±0.6
5	167.0±2.8	51.9±1.46	144.0±1.25	866.8±150.0	11.5±0.5	6.8±0.2	43.1±5.7	155.0±17.9	32.1±1.2^{+}
6	186.4±2.6**	62.1±0.66	148.6±0.29	850.7± 63.6	13.0±0.3**	6.2±0.1	52.2±4.2^{+}	175.3±13.8^{+}	29.8±0.6
7	162.7±3.4	54.3±2.20	146.7±1.93	864.2±112.7	12.9±0.2**	6.5±0.2	53.0±8.8^{+}	186.6±28.9^{+}	28.0±0.7
8	185.5±2.1**	61.6±1.44	146.0±0.63	954.8± 83.0	11.8±0.2	6.3±0.1	47.0±4.2	163.9±13.5	28.3±0.9
9	169.5±2.2	57.8±0.83	145.9±0.56	786.6± 88.8	11.1±0.3	6.9±0.1	42.4±4.7	137.3±13.3	30.2±1.2
10	180.2±2.0**	61.8±1.88	148.5±0.61	682.4± 98.2	10.5±0.3	7.1±0.2^{+}	39.0±5.9	137.0±20.4	28.1±0.9
11	171.3±2.9	54.8±0.99	145.9±1.20	816.0±111.3	11.1±0.4	7.1±0.2	45.7±6.2	151.3±21.8	30.4±1.4^{+}
12	179.1±2.7**	56.2±0.66	142.7±1.17	905.2±149.9	12.2±0.2	6.5±0.2	47.1±5.8	173.0±22.9	28.0±1.0
13	177.9±1.5	60.7±1.08	149.2±0.32	930.5±142.3	12.0±0.4	7.1±0.1^{+}	44.0±5.2	157.8±17.7	27.7±0.5
14	174.6±1.8	54.6±0.97	146.7±0.85	650.7± 70.6	11.4±0.2	6.6±0.2	38.4±5.2	133.8±15.3	28.4±0.9
15	170.9±1.3	61.3±1.19	148.3±0.46	645.4± 46.1	10.8±0.3	6.7±0.2	39.6±2.1	130.1± 7.1	30.8±1.0^{+}
16	179.2±2.3**	56.3±1.30	146.2±0.85	757.6± 60.4	12.4±0.3**	6.5±0.2	43.8±3.8	145.5± 9.5	29.5±1.0
17	177.2±2.9	59.4±1.70	146.5±0.74	680.9± 61.2	12.2±0.3	6.6±0.1	44.3±3.8	154.6±11.5	28.4±0.7
18	182.4±2.8**	56.8±1.46	146.7±0.94	938.5±145.2	11.1±0.2	7.0±0.2	52.0±6.8	174.0±23.6	28.5±0.5
19	178.3±2.6	58.5±1.58	145.6±1.23	782.8± 64.3	9.7±0.6	7.0±0.2	36.0±3.0	153.0±13.0	25.1±1.3
20	155.7±2.9	52.8±1.43	145.0±1.37	1076.9±316.6^{+}	11.7±0.4	6.4±0.2	30.8±4.7	156.8±38.1	27.0±0.8
21	128.3±2.5**	43.7±1.08*	129.4±0.57*	646.8± 79.0	13.4±0.5**	6.6±0.2	26.8±2.9	97.0± 7.6	27.3±1.4
22	177.9±2.1	57.3±1.14	147.1±0.55	712.9± 60.0	12.0±0.2	7.0±0.1	46.2±3.7	160.4±11.3	28.1±0.8
23	153.5±2.1	49.4±0.51	139.6±1.31	369.0± 28.5	12.1±0.3	5.9±0.2	18.8±1.4	82.9± 5.3	22.9±1.2
24	192.0±2.4**	56.2±4.20	149.3±0.74	966.9± 78.1	12.5±0.3**	6.2±0.2	53.9±4.9^{+}	190.3±16.0^{+}	28.1±1.6
25	165.5±3.9	54.1±1.20	143.8±1.07	994.7±140.3^{+}	12.1±0.4	6.5±0.2	45.4±4.5	158.6±15.0	28.3±1.0
26	157.3±3.0	50.3±0.85	142.1±1.63	624.7± 86.8	12.3±0.3*	6.2±0.1	32.3±4.9	126.8±15.7	25.1±1.0
27	184.0±2.4**	58.4±0.82	147.4±0.45	858.3±153.9	11.5±0.2	7.4±0.2^{+}	51.1±8.2	169.3±24.6	27.0±0.9

Discussion

The R_0 plants exhibited a considerable amount of variability for several morphological characters (plant height, degree of branching and pod maturity). It may be because of the recombination and segregation during meiosis, or consequence of altered physiology of the plants induced during culture or due to non-uniform effect of colchicine treatment for diploidization. In R_0 generation fully fertile, fully sterile and partly fertile plants were achieved. The partly fertile plants were found to possess one or two seeds in pods in early stages, later these embryos aborted within the seeds due to lack of proper nutrition. The ploidy level of such plants was haploid. Therefore it is a possibility that embryo formation would have occurred by the nuclear restitution.

The lanceolate leaf shape and highly branching pattern in R_0 plants were a consequence of epigenetic

Table 2. Analysis of variance of different agronomical traits among 27 androgenic lines to representing the variation within lines to between lines (** - significant at 1 % level).

Characters	Between lines DF	Between lines MS	Within lines DF	Within lines MS	*F* value
Plant height	54	2637.57	408	105.28	25.02**
Total pods	54	644224	408	215786.2	2.99**
Seeds per pods	54	7.49	408	1.87	3.99**
Seed mass	54	0.02	408	0.01	3.49**
Total seed mass	54	1046.2	408	391.46	2.67**
Biomass	54	11866.93	408	4792.73	2.48**
Harvest index	54	43.77	408	17.0	2.57**
Days to flowering	54	259.98	408	31.09	8.36**
Days to maturity	54	239.58	408	15.08	15.88**

variation, as it was not perpetuated in R_1 generation. R_1 progeny of DH lines showed high degree homogeneity within lines. Similarly, Kubba *et al*. (1989) observed less variation within DH lines of Brussels sprouts (*Brassica oleracea* var. *gemmifera*) compared to Single Seed Descent (SSD) lines. During the present study three DH lines showed 27 - 31 % higher yield then control line. One of these lines (L - 24) was better for 6 characters, which presumably contributed to the higher yield in this line. DH lines exceeding the agronomic performance over the source cultivar have been recorded in rice, wheat and tobacco (Hu and Zeng 1984). In addition to high yield 40 % lines exhibited poorer yield performance in wheat (Snape *et al*. 1988).

Two lines were 18 - 20 % shorter and less vigour, but these lines exhibited earlier flowering and maturation by 15 d. The dwarf character and reduced vigour may by due to differential DNA replication as in tobacco DH line (De Paepe *et al*. 1981). The DH lines consistently showed a similar phenotype in R_2 generation. During the present study androgenesis proved a preferential route to generate homozygous lines. The selection of high yielding lines is a major achievement, which will hopefully lead to the release of a new cultivar in near future shortening the time period compared with conventional breeding.

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