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Induction of mutations for cytoplasmic male sterility and some rare phenotypes in Indian mustard (*Brassica juncea* L.)

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Abstract

Four chemical mutagens, ethyl methane sulfonate (EMS), ethidium bromide (EBr), ethyl nitroso urea (ENU) and streptomycin were used to induce mutations in *Brassica juncea*. EMS and EBr were found to be highly efficient and yielded mutants for all the traits examined. Ninety seven mutants were recovered in the M_2 generation of 800 families. Several novel mutations such as terminal flower, tri- and tetra-locular siliqua, multiple anthers and non-shattering pods were obtained and have been described. In addition, agronomically valuable mutants such as yellow seeds and cytoplasmic male sterility have been isolated. Almost all these mutations were recessive, although some of them appeared in M_1 generation itself. The results show that mutagenesis is still relevant in polyploid species. Ethidium bromide, in particular, appears to be a very potent mutagen for Brassica.

Key words: *Brassica juncea*, cytoplasmic male sterility, ethidium bromide, EMS, induced mutations

Introduction

In plant breeding, induction of mutations is usually resorted to create variability not available in the gene pool or to correct specific deficiency of an otherwise outstanding genotype. Indian mustard (*Brassica juncea*), a natural amphidiploid, is an important oilseed crop of South Asia. India has large acreage devoted to this crop and it meets 27% of nation's requirement of oilseeds. However, its productivity is low and hovers around 1000 kg ha^{-1}. Further, all the varieties of *B. juncea* contain very high levels of erucic acid and glucosinolate in their seeds. Thus both seed oil and meal quality of *B. juncea* are inferior to 'canola' quality *B. napus*.

For improving productivity, development of hybrid varieties holds promise, because intervarietal crosses show high heterosis [1]. Although a number of alloplasmic cytoplasmic male sterile lines are available in *B. juncea*, they have been mostly unusable due to problems associated with quality or stability of male sterility/fertility restoration. Cytoplasmic male sterility (CMS) arising within the species through a spontaneous/induced mutation in the mitochondrial genome has proven useful for developing commercial hybrids in several crops such as sugar beet [2], sorghum [3] and sunflower [4]. Therefore, we attempted to induce CMS through mutagenesis.

Chemical mutagen ethidium bromide (EBr) has been reported to induce CMS in pearl millet [5] and maize [6]. Similarly, streptomycin (Spt) has been found useful for inducing mutations in extra-chromosomal genetic determinants in plants [7]. Spt- induced CMS has been reported in sugar beet [8], pearl millet [9] and sunflower [10]. Ethyl methane sulphonate (EMS), the most widely used chemical mutagen, as well as ethyl nitroso urea (ENU) have also yielded extra nuclear mutants [11].

We, therefore, chose to use chemical mutagens EBr, EMS, Spt and ENU to induce CMS in *B. juncea*. In this paper we report variability induced for male sterility and other traits and describe some novel mutations not reported so far in *Brassica* or *Arabidopsis*.

Materials and methods

Preliminary experiments based on germination percentage and growth rate of the seedlings were conducted in Petri dishes to decide the dose of mutagen to be administered for large-scale treatment. For field evaluation nearly 40000 seeds of Indian mustard (*Brassica juncea* L.) cv. Pusa Jai Kisan were soaked in tap water for 16 h. The soaked seeds were divided into six nearly equal lots and each was subjected to mutagen treatment with EBr (2, 3%), EMS (0.75, 1.5%), Spt (3%) for 6 h and with ENU (0.02%) for 4h. The treated seeds were washed in running tap water for 16 h, blotted and air dried for 2h.

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The treated seeds along with control were sown at the Experimental Farm of Indian Agricultural Research Institute, New Delhi, to raise M_1 generation in October' 1997. The M_1 plants were observed regularly and those showing morphological alterations were tagged and closely watched throughout subsequent growth till harvest.

All the tagged plants and 200 randomly selected plants from each treatment were self-pollinated. The identified male sterile plants were crossed to parental line Pusa Jai Kisan. Preliminary screening for male sterility was done by visual examination. The identified male sterile plants were further verified and confirmed by their lack of seed set upon selfing and also by non- stainability of pollen by 1% acetocarmine.

About 20 M_2 progenies were raised from each randomly selected M_1 plant in one row of 3 m length. The crossed seeds obtained from M_1 male sterile plants in different combinations were also sown in one row each. Observations for morphological variations were recorded at various stages of growth and the identified variants were selfed. Selfed, crossed and open pollinated seeds were harvested separately. The recovered mutant phenotypes were advanced to M_3.

Results and discussion

In preliminary experiments, Spt and ENU exhibited little effect on germination and seedling growth. EBr at 2% also showed no adverse effect at the initial stages. At 3%, EBr caused inhibition of emergence in 15% seeds and a 21% reduction in seedling growth. EMS caused serious damage to germinability and seedling growth: at 0.75 and 1.5% concentration reduction in seedling growth was 16 and 45%, respectively. Seed germinability was reduced by 25% at 1.5% concentration of EMS. Therefore, for large-scale treatment doses chosen were: 2 and 3% for EBr, 0.75 and 1.5% for EMS, 3% for Spt and 0.02% for ENU.

M_1 generation: No adverse effect on growth was noticed in population treated either with Spt, ENU or EBr in M_1 generation. However, EMS at 0.75 and 1.5% concentration produced drastic effects on plant growth and resulted in 15 and 82% variant seedlings, respectively (Table 1). Leaf and stem alterations such as fasciation, abnormal elongation and varying degrees of chlorosis were predominant in EMS treated population. ENU, Spt and EBr (at 2%) produced very few chlorophyll mutations (2%). Treatment with 3% EBr resulted in 8% plants showing chlorophyll mutation and morphological alterations in the M_1 generation. Male sterile and determinate flower mutants were also obtained in the M_1 generation. Five out of approximately 2000 M_1 plants were identified to be male sterile and a similar number had determinate growth habit among the population treated with 3% EBr. One plant was both determinate and male sterile. Only two out of approximately 2000 EMS (1.5%) treated population were male sterile. In 0.75 and 1.5% EMS treated M_1 population, 3 and 7 plants were identified as determinate type, respectively.

Table 1. Effect of mutagen on the occurrence of phenotypic alterations in M_1

Mutagen	Concentration (%)	Number of plant screened	Plants with altered phenotype
EBr	2.0	2000	02
EBr	3.0	2000	08
EMS	0.75	2000	15
EMS	1.5	2000	82
ENU	0.02	2000	02
Spt	3.0	2000	02

M_2 generation: Normal plant growth was restored in M_2 generation and large number of mutations for almost all traits studied were identified. The number of families showing at least one morphologically detectable mutation for different treatments is given in Table 2.

Table 2. Percentage of families segregating for morphological changes in M_2 generation

Mutagen	Concentration (%)	No. of families screened	Segregating families (%)
EBr	2.0	200	16
EBr	3.0	200	50
EMS	0.75	200	24
EMS	1.5	200	30
ENU	0.02	100	0
Spt	3.0	100	2

EBr, which showed relatively fewer altered phenotypes in M_1 was found to be a very potent mutagen: 50% of the M_2 families derived from 3% EBr were found to segregate for at least one morphological trait. EMS also yielded high frequency of mutations with not much difference between the two concentrations tested. ENU was ineffective in causing mutation at the dose tested. Treatment with Spt gave only 2% variant families.

Mutation breeding in polyploid species is considered to be difficult owing to duplicity of genes. Since most mutations are recessive, raising and screening of large number of progenies is advocated to have a fair chance of recovering mutants. Alternatively, use of haploid for inducing mutation in polyploid species such as *Brassica* is suggested [12].

We have found chemical mutagens as very effective in generating diversity for almost all traits in *B. juncea*. Contrary to expectation, screening of a moderate M_2 population was enough to recover a large

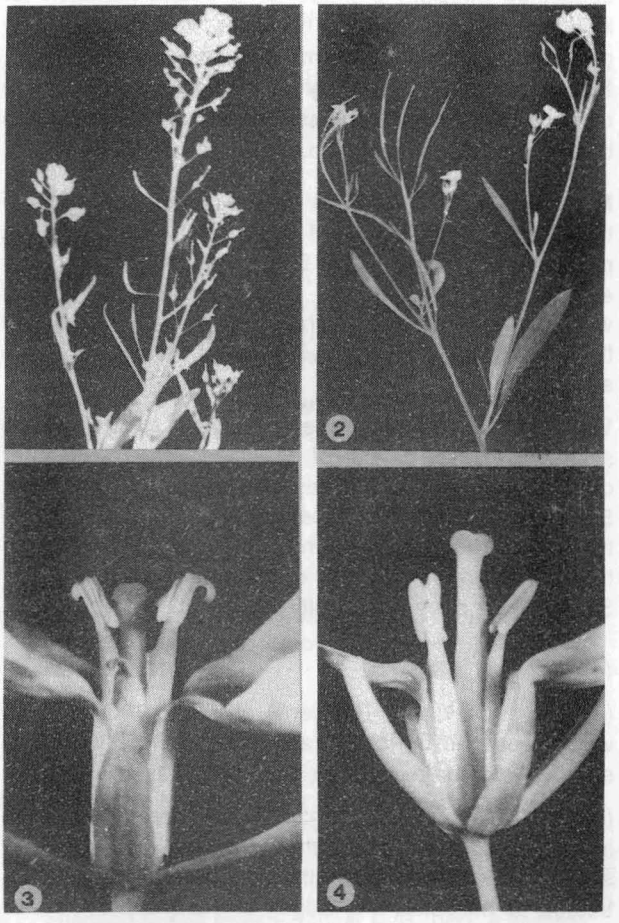
spectrum of mutants. A total of 97 mutants were recovered. For some traits many independent mutations were recovered.

EMS and EBr were very efficient in inducing mutations. While EMS is regarded as the most potent mutagen in several species, EBr has not been as widely used. Our results show that EBr has less adverse physiological effects and yields very high frequency of mutations. Some class of mutants, particularly those affecting siliqua characters were found only with EBr. EBr is an intercalating dye and causes insertional mutations [13]. EMS, an alkylating agent, is known to bring about point mutations. Thus the effect of EBr may be very general and non-specific thereby yielding higher frequency of mutations particularly at the time of replication. Two other mutagens, Spt and ENU were not found useful perhaps because of inappropriate dose chosen in our studies. Novel mutants of academic and agronomic importance are described below.

Terminal flower mutants : In *Brassica*, the inflorescence is indeterminate; both main and lateral axes continue to grow and produce flower buds (Fig.1). In terminal flower mutants, main and lateral shoots terminate in a flower (Fig. 2). Such terminal flower mutants have been reported in *Arabidopsis* [14]. We identified terminal flower mutants in both M_1 and M_2 generations. Terminal flower mutants were recovered in M_1 from EBr and EMS treatments and 22 families were found segregating for wild type and terminal flower in population treated with 3% EBr in M_2 generation. Although all mutants had determinate habit, the number of flower buds produced before the terminal flower differed. Terminal flower mutants gave rise to indeterminate progenies upon back crossing to the parent variety and produced only determinate plants upon selfing signifying recessive nature of this trait.

Leafy, terminal flower: Schultz and Haughn [15] described 'leafy' *Arabidopsis* mutants in which cauline leaves appear at the base of inflorescence and the flowers are subtended by leaf-like bracts. In 'leafy' plants petals and stamens are converted to sepals and poorly fused carpels, respectively. We obtained one plant in M_1 that showed bract at the base of flower and also had determinate habit (Fig. 2). However, the flowers of this plant were nearly normal. Weigel *et al.*, [16] reported that weak alleles of 'leafy' bear normal flowers. Thus the mutant observed in the present study appears to be a double mutant. Three other double mutants with terminal flower were also identified: two mutants, in M_1, had dark green leaves and determinate growth habit. One terminal flower mutant was also male sterile.

Occurrence of terminal flower mutant (tfl) in M_1 is surprising, so is the large number of 'tfl' mutants observed in M_2 families. In *Arabidopsis* also tfl was recovered in M_1 generation but as it turned out to be a recessive phenotype, it was said to have originated spontaneously [17]. Pusa Jai Kisan is a commercial cultivar grown over large area and we have not seen any tfl phenotype in untreated population. Although sequences homologous to *Arabidopsis thaliana TFL* gene have been identified in cultivated *Brassica* species [18], the corresponding mutant phenotypes have not been reported so far. Also determinate types are not found in any wild species of *Brassica*. Therefore, we ascribe the occurrence of 'tfl' in M_1 and M_2 due to mutagenic treatment. Mimida *et al.*, [18] using sequence information of *Arabidopsis* identified and cloned two *TFL* genes from *B. napus*. Similarly, Brunel *et al.*, [19] found two *LEAFY* genes in *B. napus*. Since *B. juncea* is an amphidiploid, it is expected to have at least two '*TFL*' loci one contributed from each diploid progenitor. Hence, occurrence of tfl in M_1 is difficult to explain.



Figs. 1-4. (1) Indeterminate inflorescence of *B. juncea* cv. Pusa Jai Kisan; (2) A mutant showing terminal flower and leafy phenotypes. Note the presence of leaf-like bracts at the base of siliquae; (3) A normal, male-fertile flower of *B. juncea* cv. Pusa Jai Kisan and (4) Male sterile flower of the mutant Bio-25.

Male sterile mutants: Seven male sterile plants were identified in M_1 (five from 3% EBr and two from 1.5% EMS). All the male sterile plants derived from EBr treatment produced male fertile progeny when crossed with the parent variety (Fig. 3). Male sterility in these lines appeared to be conditioned by nuclear, recessive genes. Of the two EMS induced male steriles, Bio-65 segregated for steriles and fertiles in BC_1 generation with male steriles outnumbering fertiles but not fitting into any genetic ratio. The other male sterile plant (Bio-25) produced only male sterile progeny (19 plants) and is CMS (Fig. 4).

Induction and isolation of mutants for organellar genomes is difficult as each cell carries multiple copies of mitochondrial and plastid genomes. Nevertheless, we have succeeded in recovering one cytoplasmic male sterile mutant from EMS treated population. This plant was male sterile in the M_1 and the back cross progenies were all male sterile. Hence it was inferred to be a maternally inherited trait. Previous reports have suggested ethidium bromide as a potent mutagen for inducing CMS [5 and 6]. However, we obtained only genic male sterile plants from EBr treatments. In contrast, EMS yielded both genic and cytoplasmic male sterile mutants. The molecular basis of the induced CMS is being currently investigated.

Mutant with extra anthers: Tetradinamous anthers are the characteristic feature of Brassica. One plant of M_2 progeny treated with 3% EBr produced flowers with eight anthers of equal size. This plant produced flowers with varying number of anthers (8-12) but no wild type flower was found (Fig. 8). This plant was male sterile also.

Variation for siliqua type: Bi-locular siliqua with a single septum is typical of *B. juncea* and most other cultivated digenomic species of genus Brassica (Figs. 5a and 6a). Only *B. campestris* has some tetralocular types. A large number of mutants varying for siliquae type occurred only in M_2 of treatment with 3% EBr. Some novel ones are briefly described below.

(i) Tri-locular siliqua

(a) Two *septa fused*: The septa originating from replum were fused and the seeds appeared in three tiers. The siliquae had three valves (Fig 6b).

(b) *Two septa independent*: The two septa remained independent and formed three locules (Fig 6c). The seeds were arranged in three compartments, hence treated as trilocular. The siliqua of this mutant had four valves. The plants bearing tri-locular siliqua also produced a few wild type siliqua with a single replum.

(ii) Tetra-locular siliqua:

Three different types of tetralocular siliquae were observed in three different M_2 families that resulted from 3% EBr treatment.

(a) *Full tetra-locular siliqua*: The two septa were fused with each other to form four locules and seeds were arranged in four tiers. The siliqua dehisced at all the four valves (Figs. 5c and 6d).

(b) *Partially tetra-locular siliqua*: The basal 5 mm portion of the siliqua was similar to wild type while the upper portion developed into four locules with seeds arranged in four tiers (Fig. 5b).

(c) *Tetra-locular siliqua enclosing a siliqua*: This type of siliqua appears to originate from development of ovary within ovary. This mutant phenotype (Bio-55-1) has four locules and the seeds are arranged in four tiers. Besides, a small rudimentary ovary is seen inside the siliqua. The siliqua dehisces along four valves (Fig. 6e). In contrast to tri-locular types, plants bearing tetra-locular siliqua produced only tetra-locular siliqua on all branches.

Long siliqua without any valve: One family was found segregating between wild type siliqua and long siliqua without any valve. Several longitudinal strips appeared. The mean length of this type of siliqua was 6.5 cm in comparison with 4.5 cm siliqua of the parental line (Fig. 7a,b). Since there was no valve the siliqua did not split upon maturity.

Variation for seed coat colour: Eleven families (4 in EMS, 7 in EBr) segregated for seed coat colour in M_2 generation. Brownish black with waxy bloom is the normal seed coat colour of the parental line (Fig. 9a). Mutant with shining yellow seed coat colour (Fig. 9b) appeared at higher frequency (8 out of 11) in comparison to other seed coat colour variations. One seed coat colour mutant had yellow seed coat but light green colour of chlorophyll of cotyledons was still present. We have classified this phenotype as greenish yellow seed coat colour mutant. In addition to yellow seeded mutants, we isolated lines with shining black seed coat colour.

Seed colour in *B. juncea* is said to be controlled by at least two genes [20]. Recovery of yellow seeded plants in several M_2 families is therefore surprising. True yellow seed colour is due to double recessive genotype while intermediate, yellow-green colour results from heterozygous condition [21]. In the light of these we seem to have recovered different mutations at these loci.

Besides these, we have identified and isolated mutants for almost all traits studied such as flowering time, oil content, glucosinolate content, seed boldness, plant height and branching habit indicating that chemical mutagenesis has been effective in generating wide spectrum of mutants at a high frequency. All the mutant

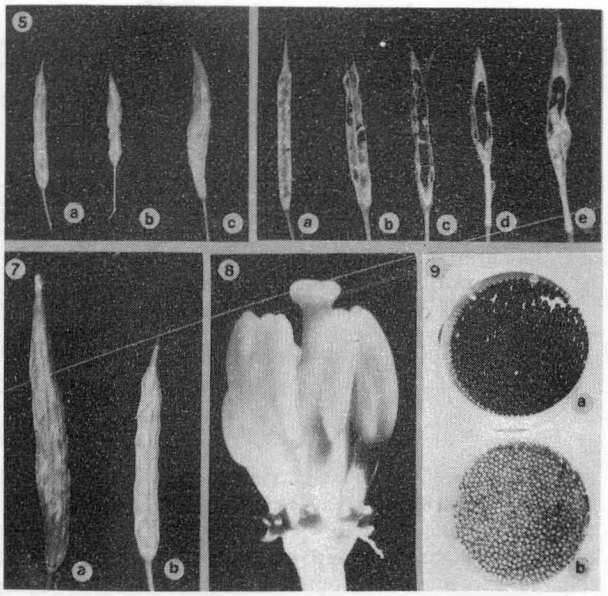


Fig. 5-9. Mutants for siliqua characters (a) Bilocular siliqua of cv. Pusa Jai Kisan, (b) partially tetralocular siliqua, (c) fully tetralocular siliqua; (6) Dissected siliqua showing septum/ locule arrangement. (a) septum of bi-locular siliqua, (b) tri-locular siliqua with three valves, (c) trilocular siliqua with two septa, (d) tetra-locular siliqua, (e) tetra-locular siliqua enclosing a rudimentary siliqua; (7) (a) Non-shattering siliqua without valve, (b) siliqua of cv. Pusa Jai Kisan; (8) Flower with eight anthers of equal size; (9) (a) Black seeds of cv. Pusa Jai Kisan, (b) yellow seed mutant

phenotypes except mutant with extra anther recovered in M_2 were found fixed in M_3.

The isolation of mutants for yellow seed colour, non-shattering pod and cytoplasmic male sterility are of great practical significance. Yellow seed colour is correlated with higher oil percentage in seeds. Indeed yellow seeded mutants showed 44% oil in seed as compared with the control which contained 38% oil. Shattering is an undesirable trait as considerable produce could be lost before harvest. In particular, yellow seed colour and non-shattering types are sought in *B. napus*. The results of our study show that mutagenesis holds promise to recover such phenotypes.

In conclusion, our results on chemical mutagenesis with *B. juncea* are highly promising. Besides the mutants described herein, we have found variants for several other traits such as oil content, glucosinolates, earliness, branching habit, seed weight etc. In particular, the efficiency of EBr as a mutagen for *Brassica* needs further exploration. The fact that with such limited population we could obtain so many mutations including those not reported so far with *Arabidopsis* prompts us to suggest EBr for *Arabidopsis* mutagenesis.

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