Induced Genetic Male Sterility in *Nigella sativa* L. (Black Cumin)

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Summary Eight male sterile-female fertile mutants possessing a similar phenotype (semi-dwarf with dark green cup shaped pinnae), normal meiotic chromosome behavior and 100.0% sterile pollen grains were scored at M_2 (0.1% over mutagenized population; 7845 plants scored) following different mutagen treatments (0.25%, 2h EMS; 0.5%, 4h dES; 1.00%, 2h H_2O_2 ; 0.25%, 4h NaN_3 ; 0.50%, 4h NH_2OH ; 100 Gy, gamma irradiations) to dry seeds (moisture content: 19.04%) of *Nigella sativa* L. (Family: Ranunculaceae; common name: black cumin). The marked phenotype of the M_2 mutant plants did not persist in subsequent generations (M_3 and M_4). Male sterility is reported to be of the non-structural nuclear type. Segregation studies revealed that male sterility was monogenic recessive to male fertility. The male sterile plant type assessed over the generations (M_2 to M_4) was characterized morphologically, palynologically (pollen attributes studied following SEM analysis, DAPI staining and using stain tests for viability), and cytologically in relation to male fertile normal plants. The results obtained were discussed.

Key words *Nigella sativa*, Mutation, Genetic male sterility, DAPI staining, SEM analysis.

Male meiosis associated with the course of microsporogenesis plays a pivotal role in fertility, reproduction and breeding behaviour of a species (Sawhney and Shukla 1994, Budar and Pelletier 2001, Pérez-Vich *et al.* 2005, Li *et al.* 2007). The uniqueness of male meiosis is that it is genetically programmed by a set of gene(s) which are sex-, time-, site- and stage-specific (Gottschalk and Kaul 1974, Kaul 1988, Skorupska and Palmer 1990, Nirmala and Kaul 1993, Palmer 2000, Datta and Saha 2001, Bhattacharya and Datta 2011) and that the gene mutations (natural and induced) affecting the harmonious combination of the universality of male meiotic gene(s) may result in the development of male sterility due to the formation of non-functional pollen grains. Genetically controlled male sterile-female fertile plants are widely reported in angiosperms. The male sterile plants are significant for breeding endeavours (Palmer 2000, Pagliarini *et al.* 2011) as well as for their potential use in conservation of gene(s) due to outcrossing (Hockett 1988, Toker and Çağırhan 2000, Comai and Cartwright 2005, Perez-Vich *et al.* 2005, Mercier *et al.* 2008). The present communication describe the male sterile-female fertile mutant plant type (induced at M_2) morphologically, palynologically, and cytogenetically in relation to normal male fertile-female fertile plants of *Nigella sativa* L. (Family: Ranunculaceae; common name: black cumin; an important herb in Ayurvedic medicine and also a spice-yielding plant important to commerce: Datta *et al.* 2012). The objective of the work is to characterise the mutant plant type in order to facilitate its effective exploration in efficient breeding.

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Materials and methods

Screening of the male sterile plants

Eight phenotypically (0.1% over the M_2 mutagenised population, 7845 plants scored) alike (semi-dwarf, dark green pinnae of leaves and pinnae folded inwards forming cup like configuration) M_2 plants were screened in different mutagen treatments (two in 0.25%, 2 h EMS, one each in 0.5%, 4 h dES, 1.00%, 2 h H_2O_2 , 0.25%, 4 h NaN_3 , 0.50%, 4 h NH_2OH and two in 100 Gy gamma irradiations—dry seed treatments, moisture content—19.04%) during the course of cytogenetical studies. All the plants possessed 100.0% sterile pollen grains. Male sterile and normal male fertile-female fertile plants are designated as MS and MF, respectively, in the text.

Morphological analysis

Morphological parameters (quantitative traits) were assessed in both plant types over the generations (M_2 to M_4). A test of significance (t test, 54 DF) was conducted for each quantitative trait between the plant types to determine significant variations, if any.

Meiotic studies

Meiotic analysis (three plants from each plant type were assessed at M_2 , M_3 and M_4) was performed from suitable sized flower buds fixed in 1:3 acetic alcohol (v/v) overnight and stored in 70% alcohol under refrigeration ($16 \pm 1^\circ C$). PMCs were stained in 2% acetocarmine and well-scattered diplotene, metaphase I (MI) and anaphase I (AI) cells were scored. Data was pooled over the plants as well as over the generations for each plant type. Photomicrographs were taken from temporary squash preparations.

Pollen attributes

Pollen sterility in MS and MF were analysed by staining pollen grains from matured anthers in 1% acetocarmine. Fully stained pollen grains were considered fertile (Marks 1954).

Pollen grain viability (Lugol's iodine: detects presence of starch, viable pollen turn black, Bengtsson (2006); Amido black: detects presence of protein in pollen wall, viable pollen turn black, Regan and Moffatt (1990); Neutral red: detects presence of cytoplasm, viable pollen turn red, Horn and Clark (1992); Aniline blue: detects presence of callose in pollen wall, viable pollen turn blue Bengtsson [2006]), and the number of vegetative (v) and generative (g) nuclei (three nucleate stage) per pollen grain (staining with DAPI-4',6-diamidino-2-phenyl indole, Johnson and McCormick [2001]) were studied under a fluorescence microscope (Carl Zeiss Axio Fluor 900 EX, Carl Zeiss Mag Analytic 10.1), while pollen morphology (following Scanning Electron Microscopy- SEM Model-Zeiss EVO[®] HD, Germany; samples coated with 200–300 Å thick gold) was also analysed in MS and MF to assess clear differentiation between the plant types, if any.

Segregation analysis

Seeds of M_2 mutant plants formed due to open pollination were sown in M_3 in separate lines. The male sterile plants (five floral buds) were also crossed with pollen grains from a normal fertile plant (96.4% pollen fertility) and subsequently F_1 and F_2 generation were raised. M_3 and F_2 plants were used to study the inheritance pattern of the male sterile trait using χ^2 -test analysis.

Results and discussion

Morphological aspects

The MS plants induced at M_2 following different mutagen treatments were phenotypically alike, possessing semi-dwarf habit, dark green pinnae of leaves (chlorophyll *a* 2.33 mg/gm, *b*

Table 1. Cytomorphological and palynological attributes in male fertile (MF) and male sterile (MS) plant types of *N. sativa*.

Attributes	MF	MS
Morphological:		
Plant height (cm)	50.39±0.94	19.91±2.67***
Flower size (cm ²)	3.07±0.03×2.99±0.02	1.8±0.07***×1.68±0.09***
Capsules/plant	12.1±1.16	7.1±0.98***
Capsule size (cm)	1.13±0.08	1.16±0.06*
Seeds/capsule	77.4±3.49	57.5±7.82***
Seed size (mm ²)	2.55±0.02×1.42±0.02	2.29±0.02*×1.24±0.02**
Cytological:		
Mean association/cell at MI	5.51II±0.91I	5.65II±0.76I
Total PMCs scored	240	550
Equal AI chromosome segregation (%)	93.82	97.18
Total AI cells scored	81	249
Pollen fertility (%)	88.97 (1079)	0.00
Pollen stain tests (%):		
Lugol's iodine	97.06 (272)	5.58 (215)
Amido black	100.00 (304)	4.58 (371)
Neutral red	100.00 (256)	64.41 (207)
Aniline blue	99.09 (318)	3.23 (278)
DAPI association (%):		
0v+2g	12.55	9.86
1v+2g	83.02	2.03
0v+1g	2.21	27.49
1v+0g	2.21	46.17
0v+2-4g	0.00	6.34
v-dispersed	0.00	8.11

*, ** and *** significant at 0.05, 0.01 and 0.001 probability levels.

Values in parentheses are the number of pollen grains assessed.

1.24 mg/gm; MF: chlorophyll *a* 1.18 mg/gm, *b* 0.48 mg/gm) and the pinnae were folded upward to form cup-like configuration. However, the marked phenotype did not persist among the male sterile plants analysed at M₃ and M₄. Pre-anthesis floral buds of MS on bagging did not set fruit. However, fruit setting and seed formation were not affected on open pollination in the male sterile plants. Open pollination of MS plants possibly affected phenotypic expression(s) at the latter generations. Outcrossing has been found to influence male sterile phenotypes in different plant species (Hockett and Eslick 1970, Rachie *et al.* 1975, Myers and Gritton 1988, Hockett *et al.* 1989, Toker and Çağiran 2000). The identical phenotypes of the M₂ mutant plants, induced following different mutagen treatments, suggested that all the mutagens administered were possibly effective in inducing similar mutation by targeting the specific set of gene(s) in the genome.

The quantitative morphological parameters were significantly ($p < 0.05$ to < 0.001) reduced in MS than MF (Table 1). Transverse sections of ovary showed five chambers with 10 ovules in axile placentation uniformly in MF; while, MS plants were with four to five chambers possessing eight to 10 ovules mostly. Occasionally one to two degenerative ovule(s) were also noted in MS.

Meiosis

MF showed $2n=12$ chromosome always; while, MS were mostly with $2n=12$ (Fig. 1A–C) chromosomes; although, variations in chromosome number was also observed (Fig. 1D–I). The mean association of chromosomes per cell at MI was 5.51II±0.91I (5.73II±0.53I at M₂, 5.29II±1.42I at M₃, 5.51II±0.98I at M₄) in MF and 5.65II±0.76I in MS (5.18II±1.64I to 5.92II±0.16I). AI in both plant types was nearly comparable (Table 1). One out of the three MS

Fig. 1. Meiosis in MS (A–C) showing $2n=12$ chromosomes. A. 6II at diakinesis, B. 6II at MI, C. 6:6 separation of chromosome at AI. Meiosis in MS-m (D–I). D. 8II at MI, E. 6II+2I at MI, F. 12II at diakinesis, G–H. 12II at MI. I. 12:12 separation of chromosome. Scale bar=10 μ m.

plants analysed at M_4 showed aberrant meiotic behaviour (designated as MS-m). MS-m was without any phenotypic marker trait. The mean association of chromosomes per cell at MI including diakinesis in MS-m was noted to be $6.09II+0.16I$ (201 PMCs scored). Out of 201 PMCs scored, about 32 cells were hyperploids (15.92%). Eight PMCs were distinct and showed, variously, $n=7$ (12.5%) (Fig. 1F), $n=8$ (12.5%) (Fig. 1D) and $n=12$ (75.0%) (Fig. 1G–H) chromosomes. In MS-m, about 94.55% cells (55 cells estimated) at AI were cytologically balanced (6/6); while, the rest had 12/12 (Fig. 1I) separation (3.64%) of chromosome and bridge formation with an accompanying fragment (1.82%). The cytologically marked plant may be the outcome of outcrossing. Gene(s) accumulated due to outcrossing may possibly affected meiosis differentially. Protein, namely PAIR I, has been reported to affect homologous pairing at meiosis (Nonomura *et al.* 2004).

Pollen attributes

Pollen attributes are presented in Table 1. As compared to fertile pollen grains, mostly found (Fig. 2A) in MF, all the MS plants showed 100.0% sterile pollen grains which were thick-walled (Fig. 2B) and in a few cases were agglutinated and degenerative in nature (Fig. 2C). Pollen viability was much higher in MF (97.06% to 100.0%) compared to MS (3.23% to 64.41%). Pollen nuclei composition in MS was mostly abnormal (0v+1 g, 1v+0 g [Fig. 2E], 0v+2–4 g [Fig. 2F] and v dis-

Fig. 2. Pollen characteristics (A–I). A. Fertile pollen grains in MF, B. Thick walled sterile pollen grains in MS, C. MS showing agglutinated pollen grains, D–F. Nuclei composition following DAPI staining—1v+2g (D), 1v+0g (E), 0v+4g (F), G–I. Surface characteristic following SEM—MF (G), MS (H), agglutinated pollen grains in MS (I). Scale bar=20 μ m.

persed were abnormal associations, while 1g+2v [Fig. 2D] and 0v+2g were considered normal; MS-88.11%; MF-4.42%). SEM analysis revealed that MF pollen grains were very large (polar axis-PA $39.45 \mu\text{m} \pm 2.52$, equatorial diameter: ED $39.45 \mu\text{m} \pm 2.46$) spheroidal in shape (Fig. 2G) in comparison to sub-prolate grains, very large (PA $36.0 \mu\text{m} \pm 1.82$, ED $27.3 \mu\text{m} \pm 1.47$) pollen grains of MS (Fig. 2H). Agglutinated pollen grains were collapsed in nature, large (PA $36.6 \mu\text{m} \pm 2.30$, ED $24.9 \mu\text{m} \pm 1.76$) and prolate (Fig. 2I). Pollen grains in MF were tricolpate, the colpi were not fused, with spinulose exine and muricate intine, whereas MS possessed narrower colpi with depressed poles and stronger spines (Fig. 2G–H).

Segregation analysis

M_2 seeds of MS plants sown at M_3 segregated to 145 MF and 129 MS plants (plants bulked over MS lines) to a close fit of 1 : 1 ($\chi^2=0.94$, DF 1, $p>0.30<0.50$) ratio. Upon controlled pollination (MS-stigma parent and MF-pollen parent), F_1 (all male fertile) and F_2 generations were raised. F_2 plants segregated to a close fit of 3 : 1 ratio (MF-221, MS-80, total-301, $\chi^2=0.38$, DF 1, $p>0.50$).

Male sterility described in the text has been non-structural nuclear type (Gottschalk and Kaul 1974, Johns *et al.* 1981). The induced mutant plant type is significant as it possesses complete male

sterility, female fertility, and monogenic recessive inheritance of the trait. Further, male sterile lines maintained through open pollination in few generations possibly widened the gene pool thereby providing a base for selection in breeding programs of black cumin.

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