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SHORT RESEARCH ARTICLE

Emergence of micronuclei as polymorphism induced genomic instability in Fennel (*Foeniculum vulgare* Mill.).

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Abstract

Micronuclei (MN) are extra-nuclear bodies containing damaged chromosomal fragments as well as complete chromosomes that are not otherwise incorporated into the nucleus after cell division. The present experiment was conducted to assess mutagenic effects of ethyl methane sulphonate (EMS) on the meiotic behavior of pollen mother cells of fennel (*Foeniculum vulgare* Mill.). Cytological analysis revealed that micronuclei were incited at all the three concentrations of EMS. A peculiar correlation of chromosomal abnormalities, mainly bridge and laggard at anaphase I and II with this nuclear polymorphism were experienced for genomic instability. Pollen viability was additionally inspected through pollen fertility. Thus, the MN formations bring about incomplete elimination of the genome, which can be proficiently use as the light of hope inbreeding programmers to produce addition and substitution lines.

Keywords: Chromosomal abnormalities, EMS, genotoxic, Fennel, Pollen viability

In the field of cytogenetics, formation of micronuclei (MN) is a topic that has been recently escalated. MN are extra nuclear bodies that are not incorporated into the nucleus after cell division. It contains damaged chromosomal fragments or entire chromosomes and cannot move towards the spindle poles (Fenech 2002). Defects in the cell repair systems and chromosomal deviations can instigate the development of micronuclei. There are various types of physical and chemical agents which can influence the structure of the DNA accordingly prompts double-strand breaks (DSBs), which may bring about chromosome irregularity and development of micronuclei, eventually leading to DNA loss. However, the use, the micronucleus assay has become one of the most popular methods to assess genotoxicity of different chemical, physical and ionizing radiation factors (Sommer et al. 2021). Ethyl methane sulphonate (EMS) is one of potent mutagen and carcinogen (Meuth and Arrand 1982). Recently, EMS has been considered as the best mutagenic agent known to evolve new varieties of crops in higher plants through induction of useful mutations (Kumar and Rai 2007). Fennel (*Foeniculum vulgare* Mill.), a self-pollinating crop plant, generally known as Saunf, belongs to Apiaceae family. Its high economic importance, medicinal properties and pharmacological significance is well known. Evaluation of the mutagenic impact of chemical mutagen on the meiotic behavior of fennel and to study the micronuclei development at the cellular level through cytogenetic analysis is the aim of current experimental study. The genotoxicity which serves as an excellent marker of this alkylating agent (EMS) is calculated in terms of micronuclei frequency.

Inbred seeds of the fennel (*Foeniculum vulgare* Mill.) variety AF-2 were procured from National Research Center on Seed Spices (NRCSS-ICAR) Ajmer, Rajasthan. The present study on induced mutagenesis in fennel was carried out in the Plant Genetics Laboratory, Department of Botany, University of Allahabad during 2019-2020.The seeds were soaked overnight at room temperature for 24 h. Subsequently, the water was emptied and pre-soaked seeds

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were treated with various concentrations of EMS solution viz., 0.1%, 0.3% and 0.5% (v/v) prepared in buffer solution (potassium phosphate 100 mM) at pH.7.0 with time-frames of 3 hours. The treated seeds were washed thoroughly (2–3 minutes) in running tap water to eliminate the remaining impact of the EMS. One set of seeds was kept in distilled water to be used as control. These treated seeds were sown in respective pots in complete randomized block design (CRBD) along with control in three replicates to raise the generation. The young flower buds were collected (9:15–9:30 AM) and fixed in Carnoy'solution (glacial acetic acid : absolute alcohol, 1:3) for one day at room temperature and subsequently transferred to 90% alcohol for the long time preservation. Anthers were crushed in 2% acetocarmine stain and slides were prepared followed by gentle tapping. Then slides were observed under microscope (40X) and photographs were taken using PCTV software. Fifteen plants were randomly selected per treatment concentration along with control set. Mature floral buds of fennel were taken, and their pollen grains were spread over the slide and stained by using glycerol- carmine dye. The slides were Prepared and observed under microscope to estimate pollen fertility. Adequately stained, globose, nucleated pollens were marked as fertile whereas sparsely stained, shriveled, and enucleated pollens were regarded as sterile. Statistical software, SPSS 16.0 was employed for statistical assessment. The data for cytological parameters were analyzed using one way analysis of variance and Duncan's multiple range test; whereas graph was plotted using Sigma Plot 10.

Chromosome structural changes and formation of micronuclei

The cytogenetic research contains insight into the structure and number of chromosomes. In control, PMC with 11 bivalents is assembled precisely at the equator of metaphase I and anaphase I (Fig. 1B), as a proper polar migration of chromosomes. EMS treatment is well known to, have considerable influence on the chromosome morphology. By the application of different doses of EMS, a range of different abnormalities were recorded in PMCs, in which micronuclei formation was a critical concern. Frequency of micronuclei recorded was minimum (2.15 ± 0.02%) at the lowest dose (0.1%, EMS) whereas it was 3.89 ± 0.06% at intermediate dose 0.3%, EMS, but significantly increased upto 6.66 ± 0.07% at highest dose 0.5% of EMS treatment (Table 1). The representation of micronuclei in relation to number of nuclei and anaphasic abnormalities and a total of MN frequency recorded for 0.1% EMS to 0.5% EMS treatment is given in Table 2. It was found that mononucleate and binucleate conditions could be induced by all the EMS doses. Highest (2.58 ± 0.25%) frequency of mononucleate was reported at higher dose (0.5%) of EMS, whereas minimum (1.33 ± 0.16%) frequency was at lower dose, 0.1%. Binucleate frequency increased from 0.45 ± 0.23 at 0.1% to 1.66 ± 0.15% at higher dose (0.5%) of EMS. Trinucleate, tetranucleate and pentanucleate frequency were reported at intermediate as well as higher dose of EMS (Table 2). In addition to MN, a considerable frequency of laggards (Fig. 1K) and bridges (Fig. 1L) were retrieved that showed integration with MN formation. The laggards at anaphase and anaphasic bridges were absent at lower dose (0.1%), along with this per cent frequency of laggards at anaphase increased from 1.11 ± 0.55% at 0.3% to 2.22 ± 0.43% at 0.5% EMS. Anaphase bridges were also increases gradually 1.21 ± 0.63% to 1.66 ± 0.51% from intermediate dose (0.3% EMS) to higher dose (0.5%), respectively (Table 2). Partitioning and enucleation of MN from parent cell occurred via microcell, as evidenced in Fig. 1N. It was perceived that formation of microcell was seen at the highest two treatment sets i.e., 0.3% EMS (0.34 ± 0.17%) and 0.5% EMS (1.66 ± 0.15%). Pollen fertility measure was additionally pronounced to survey the effect of EMS mutagenic activity on viability of pollens.

Table 1. Micronuclei formation in *Foeniculum vulgare* Mill. induced by EMS 3h treatment

Treatment	Total number of PMCs (Mean)	Number of PMCs with MN (Mean ± S.E.)	PMCs with MN % (Mean ± S.E.)
Control	376	0	0
0.1% EMS	372	7.72 ± 0.32	2.15 ± 0.02
0.3%EMS	384	14.99 ± 0.16	3.89 ± 0.06
0.5%EMS	360	23.96 ± 0.29	6.66 ± 0.07

Table 2. Configuration of micronuclei in relation to number of nuclei along with anaphasic abnormalities

Treatment	Number of MN per PMC (Mean ± S.E.)					No. of Anaphase cells (Mean ± SE)		No. of Microcell (Mean ± SE)
	Mononucleate	Binucleate	Trinucleate	Tetranucleate	Pentanucleate	LaggardsI/II	BridgesI/II cleate	
Control	-	-	-	-	-	-	-	-
0.1%EMS	1.33 ± 0.16	0.45 ± 0.23	-	-	-	-	-	-
0.3%EMS	1.81 ± 0.16	1.11 ± 0.56	1.21 ± 0.53	0.77 ± 0.53	0.73 ± 0.23	1.11 ± 0.55	1.21 ± 0.63	0.34 ± 0.17
0.5%EMS	2.58 ± 0.25	1.66 ± 0.15	1.76 ± 0.51	1.02 ± 0.52	1.02 ± 0.51	2.22 ± 0.43	1.66 ± 0.51	1.66 ± 0.15

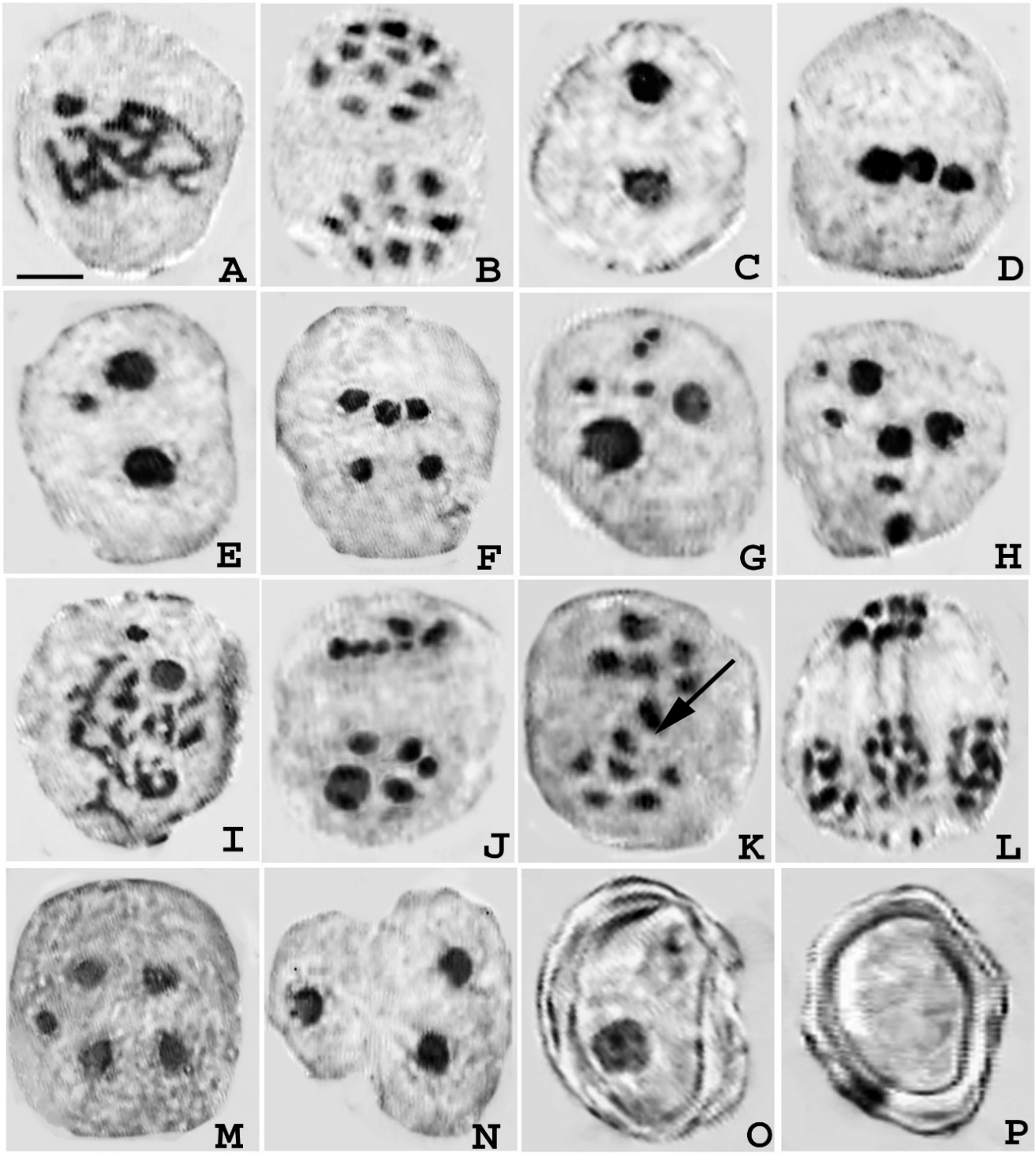

Fig. 1. Micronuclei induced by EMS treatment. A: Normal prophase; B: Normal anaphase I (2n = 11:11); C: Binucleate PMC single micronucleus; D: Nuclear budding with single micronucleus condition; E: Binucleate PMCs with one micronuclei F-H: Multinucleate condition I: One micronucleus at prophase I; J: Micronuclei at anaphase I; K: Single laggard at Anaphase I; L. Double Bridge at Anaphase I; M: Telophase I with micronuclei; N: Micronuclei with microcell; O: Fertile pollen; P: Sterile pollen Scale Bar =10.2mm

It was seen that in the event of control, fertile pollens (Fig. 10) were prominently stained globose nucleated element, while sterile pollens (Fig. 1P) were inadequately stained and contracted. In this mutagenic treatment it was found that highest (98.21 ± 0.13%) pollen fertility in control set (Fig. 2.) that went to gradually decline upto 68.21 ± 0.74% at 0.5% EMS. Fig. 2 depicts graphical representation of the Pollen fertility trend in the control as well as treated sets.

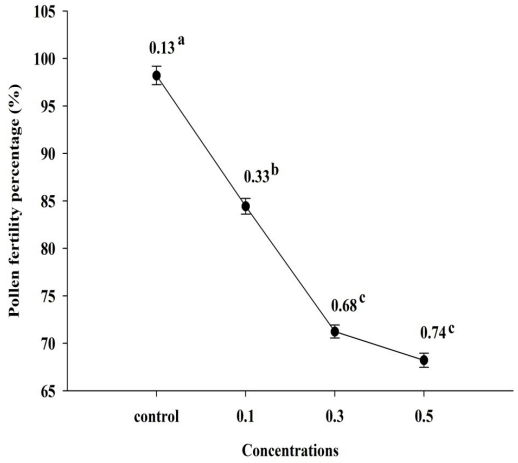

Fig. 2. Effect of EMS treatment on the pollen fertility of *Foeniculum vulgare* Mill.

Ever since the pioneering works on the concept of micronuclei (MN), consequences and repercussions, linked with it have been investigated and several theories have been thereafter forwarded. Schiffmann and De Boni (1991) previously expressed that, MN are chromatin containing structures that don't have any recognizable connections to the cell nucleus, whereas Crasta et al. (2012) explained that for micronuclei arrangement occurs, when some chromosome in cells fails to join a daughter nucleus and structure their own nuclear envelop. This nuclear envelope of micronuclei are structurally similar to primary nucleus, however it is less dynamic in terms of DNA replication, transcript and DNA repair mechanism (Terradas et al. 2016). These deformities in micronuclei bring about decrease of nuclear protein levels and furthermore influence nuclear trafficking process (Crasta et al. 2012; Hatch et al. 2013; Hoffelder et al. 2004). Genotoxic agents may likewise initiate the formation of micronuclei, as this can prompt cell death and genomic instability. Critical evaluation of recent reports asserts that MN may result due to obliteration of cell cycle via clastogenic or aneugenic effects (Walker et al. 1996). Luzhna et al. (2013) reported that clastogenic agent introduces breaks in chromosomes while aneugens influences cell division, builds chromosome mis-segregation that outcomes in the lagging of chromosome and spindle properties bringing about aneuploidy.

Greene et al. (2003) reported that, EMS synthetically modifies DNA through the change of oxygen present in nucleotide bases as well as in DNA phosphate gatherings in plants, and as a result, blending happens in which G sets with T as opposed to C and A sets with C instead of T, yielding transition mutation with the deviation of original G/C pair being supplanted by A/T pair. Kumar and Singh (2018) supported these findings with the opinion that EMS plays a key role in the breakage of chromosome at guanine rich regions and further alters the base of DNA which causes the formation of micronuclei. These MN may be related to DNA damage response (Terradas et al. 2009) and have power to presence or absence of specific micronuclear DNA content along with nuclear pore complex might be decisive factors for the transcriptional movement of MN (Hoffelder et al. 2004). Micronuclei are capable to form some other kinds of nuclear structure, such as nuclear bud which is mainly formed by the broken fragments of nucleo-plasmic bridges (Utani et al. 2010; Fenech et al. 2011). From some studies, an idea has been conceived that these nuclear buds might have been converted into micronuclei during interphase stage of the cell cycle (Shimizu et al. 1998).

Apart from micronuclei, different kinds of other chromosomal abnormalities such as laggard and bridges have also been accounted by the EMS treatment. Formation of anaphasic bridges occurs due to dicentric chromosome which is formed by telomeric fusion events and miss repair of DNA breaks (Fenech et al. 2011). The laggards formation at anaphase may be due to a delay in terminalization (Kumar and Tripathi 2007); and is also ascribed to the mis-connection of kinetochore that leads towards the laggard chromosome which produce the micronuclei in future (Vazquez-Diez et al. (2016). Moreover, formation of micronuclei can also occur by some other process which includes polyploidization. Accordingly, the formation of micronuclei originate from the elimination of exceeding DNA of the main nucleus in an attempt to restore the normal conditions of ploidy. The formation of micronuclei along with other chromosomal anomalies has great impact on pollen fertility. Baptists-Giacomelli et al. (2000) previously examined that, micronuclei created during meiosis either remains upto the tetrad stage or is removed from microspores as microcytes. These removed microcytes led to small and sterile pollen grains at maturity. Darkly stained pollens grains are judged as fertile whereas lighter stained pollens were regarded as sterile.

Formation of micronuclei is used as good markers of genotoxic exposure in plants and their frequency in PMCs have been widely used to identify potential of genotoxic substances and are also indicators of chromosomal instability. There was a significant increase in the frequency of micronuclei by the EMS treatment as compared to control, indicating strong cytogenetic damage caused by EMS. High concentration of EMS doses, results. The emergence of micronuclei during the microsporogenesis and production of pollen grains (small and vigorous) can be utilized as a tool for breeding programs and generate new agronomic characters. The investigation of micronuclei and microcells found in present study is of great importance as it may open new vistas for microcell hybrids acting as ingenious tool in partial genome transfer and gene mapping (Kumar and Singh 2018; Kumar and Mishra 2020). Moreover, this technique would reduce the number of backcrosses and

be less time consuming, due to introgression of few genes, promoting the procedures in breeding programs. It can be used in intra and interspecific crosses to explore the natural genetic pool or to generate novel agronomic traits by micro protoplast fusion in this genus.

Authors' contribution

Conceptualization of research (GK, MM); Designing of the experiments (MM); Contribution of experimental materials (NRCSS ,Ajmer, India); Execution of field/ lab experiments and data collection (GK, MM,KT); Analysis of data and interpretation (GK, MM,KT); Preparation of the manuscript (MM,KT,PO).

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