GENETIC SENSITIVITY TOWARDS MMS MUTAGENESIS ASSESSED THROUGH IN VITRO GROWTH AND CYTOLOGICAL TEST IN NIGELLA SATIVA L

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Abstract: The availability of genetic diversity is utmost important in the changing climatic condition for uninterrupted cultivation of economic crops. *Nigella sativa* L. (2n=12) belongs from family Ranunculaceae, is highly known for rich spice and medicinal value. The present experiment was aimed at inducing genetic variation for enhancement of available *Nigella sativa* L. genetic diversity. Dry and healthy seeds were treated with doses of MMS (0.1%, 0.25%, 0.5%, 0.75% and 1%), prepared in phosphate buffer at pH 7. Assessments were made on the *in vitro* seed germination and seedling growth for estimation of mutagenic sensitivity of the *Nigella sativa* L. Mutagenicity was also tested through investigation on meiotic index and pollen fertility of the young flower collected from the treated population. It was elucidated that the *Nigella sativa* L. was most sensitive towards 0.25%, 0.5% and 0.75% MMS for expression of novel phenotypes while 1% MMS was proved lethal and cannot be further considered in the present mutation breeding experiment on *Nigella sativa* L. Therefore, the implication is that 0.25%, 0.5% and 0.75% MMS doses can be employed for possible induction of desirable mutation in the agro-economical traits of *Nigella sativa* L.

Keywords:cytological aberration, *in vitro* growth and germination,MMS, *Nigella sativa* L.

Introduction:*Nigella sativa* L. (2n=12) is a small annual spice yielding herb, in the family Ranunculaceae, also considered as a miracle herb for its wide range of medicinal properties. It has been extensively used for its biological activities and therapeutic potential and shown to possess wide spectrum of activities viz., as diuretic, antihypertensive, antidiabetic, anticancer,immunodulatory, antimicrobial, anthelmintic, antiflammatory, spasmolytic, bronchodilator, antioxidant properties and so on. Thymoquinone (TQ) is the most abundant compound of the plant, responsible for many of the seed's antioxidant and anti-inflammatory effects (Abdel-Fattah, 2000) and health-promoting effects (Ramadan and Moersel 2002; Chamblee *et al.* 1997) and syrups and suspensions (Porta et al. 1997) and in perfumery also.The ever increasing market requirement for complementary and alternative medicines, demand for improved black cumin seeds raises tremendously in the recent times. Induce mutagenesis is a key area of genetic research in agricultural science to elucidate the basic aspects of life phenomenon and for profitably raising a large number of economically superior and desirable genotypes of crop plants. The magnitude of genetic variability induced by physical and/or chemical mutagens could be exploited for obtaining the desirable lines through a careful screening and selection programmes. The chief advantage of mutation breeding is its ability to improve a single feature in a variety without significantly altering the otherwise desirable make up of agronomic characters.Chemical mutagenesis as an efficient means of crop improvement is supported by many researchers (Bhosle and Kothekar, 2010; Goyal and Khan, 2010; Prabha *et al.* 2010a, 2010b; Avijeet et al.2011; Dixit et al. 2012; Laskar *et al*., 2015) due to its higher mutation rates (Kharkwal1999) and relatively greater specificity of mutation (Bhat et al. 2005) than physical mutagens.

Qualitative and quantitative properties of a plant is directly under the control of its genetic material and mutagen directly acts on it, therefore, selection of optimized mutagen doses is necessary to avoid lethal or semi-lethal mutations with serious genetic consequences especially in diploid plant. To solve the issue and increase the accuracy, different biological indices viz. germination inhibition, pollen sterility and meiotic aberration are used for selecting most suitable doses. Role of cytogenetic tests for indications of genotoxicity, genetic variation, cytotoxicity, and mutagenic potency in crop improvement through induced mutagenesis was extensively documented by Khan*et al*., (2015). Gregory (1941) first reported the somatic complement of *N. sativa* have 12 number of chromosomes (2*n*=12). The karyomorphology analysis of *N. sativa* by Ghosh and Datta (2006) showed four morphologically distinct chromosome types (L_1= very long ≥15.0 µm, L= long 13.0 to <15.0 µm, M= medium 7.0 to <13.0 µm, S= short <7.0 µm; m= metacentric, sm= sub-metacentric, t= telocentric and sc= satellites).While ethyl methane sulphonate (EMS) (Datta and Biswas 1985; Datta et al. 1986)and other chemical mutagens have been often used on *Nigella sativa* L., information about mutagenic responsiveness of methyl methane sulphonate (MMS) on this plant is meager.Since, induced mutagenesis has remarkable possibility of improving plants with regard to their quantitative

and qualitative characters; present preliminary experiment was conducted on *N. sativa* L. for understanding the sensitivity of the genotype towards the doses of MMS and to find out the efficacy of the MMS on the *N. sativa* L. The main objectives of present study were to study the effect of different concentrations of mutagenic treatment in various biological parameters such as seed germination, plant survival, pollen fertility and the meiotic behaviour of chromosomes.

Materials and methods: Dry (moisture content 10-12%) and healthy seeds of the *Nigella sativa*L. procured fromGovernment Seed Store, Aligarh, were used for mutagenic treatments of MMS. The seeds were first pre-soaked in distilled water for 9 hours and then directly transferred (25 seeds each) to the different concentrations of mutagens for 6 hours with intermittent shaking at room temperature of 25±2°C. Based on the pilot experiment (LD 50), 0.1%, 0.25%, 0.5%, 0.75% and 1.0% MMSconcentrations were found suitable and selected for treatment. The working solutions of MMS doses were prepared in phosphate buffer at pH 7.0. The pH of the solution was maintained by using buffer tablets (MERCK manufactures, Mumbai, India). After treatment, the seeds were thoroughly washed in running tap water for 30 minutes to remove the excess of mutagen. Two sets of treated seeds in five replications of 5 seeds each (25 seeds per treatment) were designed. One set of seeds was placed in petri plates containing cotton and kept in the BOD incubator at 27 ± 1°C temperatures for *in vitro* growth analysis.Another sets of Five replicates of 5 seeds each were sown for each treatment along with untreated (control) in 9" earthen pots filled with a well prepared growth media of Farm Yard Manure, soil and sand with a ratio of 1:1:1 and kept in the net house of the Department of Botany, Aligarh Muslim University, Aligarh during the rabi season of the year 2013-14 to raise M1 generation.

The assessment on seed germination was recorded in each treatment including control from the beginning of first shoot emergence. From the data recorded, percentage of seed germination was calculated by using the formula

$$\textbf{Seed germination (\%)} = \frac{\text{No.of seeds germinated}}{\text{No.of seeds sown}} \times 100$$

$$\textbf{Inhibition (\%)} = \frac{\text{Control} - \text{Treated}}{\text{Control}} \times 100$$

Cytological studies were carried out on pollen mother cell by fixing younger flower buds from each treatment as well as control. The purpose of fixation is to kill the tissue without causing any disorder of the components to be studied. It should not only increase visibility of chromosome structure but should also clarity of details of chromosomes morphology and the primary and secondary constriction. For meiotic studies, young flowers buds from 40-50 randomly selected plants were fixed in freshly prepared Carnoy's fluid (alcohol: chloroform: acetic acid in a 6:3:1 ratio), supplemented with crystals of ferric chloride for 24 hours. The material was then washed and preserved in 70% alcohol at 4°C, then the anthers were squashed in 1% propino carmine dehydrated in NBA series (50% acetic acid + 50% normal butyl alcohol and normal butyl alcohol), mounted in Canada balsam and dried at 45°C (Bhaduri and Ghosh, 1954). More than 500 dividing PMCs from each treatment, as well as control populations were studied and analysed at metaphase I/II, anaphase I/II and telophase I/II stages. The photomicrographs were taken from temporary as well as permanent slides under the aid of "Nicon" photo micrographic unit using 10X eye piece (100X objective lens.

Percent meiotic abnormalities were calculated by using the following formula:

$$\textbf{Percent meiotic abnormalities} = \frac{\text{Total number of meiotic abnormalities}}{\text{Total number of PMCs}} \times 100$$

The pollen quality was studied from the pollen fertility and viability test. The pollen fertility was estimated from the fresh pollen samples. For acetocarmine staining, they were suspended in a drop of acetocarmine (1g refluxed in 45% acetic acid for 24h and filtered), heated over a sprit flame, squashed and examined under microscope after 5-30 min. Fresh pollen (or anther loculi or germinated grains) is dispersed on a microscope slide. The stained pollen grains with a regular outline were considered as fertile, while the hyaline (empty) ones without stain with irregular shape were considered as sterile. The following formulae were used to calculate percent fertility and sterility.

$$\textbf{Pollen fertility \%} = \frac{\text{Number of fertile pollen observed}}{\text{Total number of pollen observed}} \times 100$$

Results: The present work was undertaken to assess the effect of chemical mutagens on seed germination, plant survival, pollen fertility and meiotic behavior in M_1 generation of *Nigella sativa* L.

***In vitro* seed germination and inhibition:** Percentage of seed germination was 94.00% in control. It decreased with increasing concentrations from 81.00% - 42.00% in 0.10%-1.0% MMS treatments (Table-1, Graph-1 & 2). In general, germination was affected in all the doses of MMS; however, 0.75% and 1.00% MMS was more lethal. Inhibition in germination was found to be increase from 13.82% - 55.31% in 0.10%-1.0% MMS respectively with the simultaneous decrease in germination (Table-1).

Plant survival and pollen fertility at maturity: The survival of seedlings decreased with an increase

in dose/concentration in almost all the mutagenic treatments. The survival of plants at maturity was 90.73% in control, while decreased from 75.54% - 40.71% MMS (Table-1, Fig. 1). The average pollen fertility was 89.88% in control, while it decreased from 73.56% - 41.46% in 0.10%-1.0% MMS (Table-1).

Cytological observations: The microsporogenesis in plants raised from treated seeds was highly disturbed. The meiotic studies showed different types of chromosomal aberrations at variable frequencies. The various chromosomal aberrations during microsporogenesis in M_1 generation were multivalents, stickiness, precocious separation, laggards, bridges and unequal separation disturbed polarity, micronuclei and multinucleate condition (Fig.2). The observations showed that almost all types of chromosomal aberrations were dose dependent in the mutagen.

Abnormalities at metaphase I/II stages: At metaphase stages the PMCs with multivalents, precocious separation of chromosomes, stray chromosomes, and stickiness were observed in treated populations. The number of abnormal cells at metaphase-I/II showed a dose dependent increase in treated populations and their frequencies were the highest in the highest concentration of both mutagens. Although the frequency of multivalent, precocious separation and stickiness increased with the increasing concentrations of mutagens, but the precocious separation and stickiness were more frequent in both the mutagens. The overall percentage of abnormal cells at metaphase ranged from 3.51 % to 13.20 % in 0.10-1.0% MMS (Table 2).

Abnormalities at anaphase I/II stages: The anaphasic abnormalities such as laggards, bridges and unequal separation of chromosomes were generally absent in the control plants but their collective frequency increased from 0.39 % to 9.24 % in 0.10-1.00% MMS (Table 2). The laggards and bridges were most frequent than unequal separation, which were absent in the lower concentrations of all mutagens. Moreover the laggards and bridges did not occur in lower concentration.

Abnormalities at telophase I/II stages: The chromosomal aberrations observed at telophase-I/II in different concentrations of Caffeine and MMS were the bridges, micro nucleate and multi nucleate conditions and disturbed polarity etc. The frequency of abnormal cells ranged between 0.39 % to 7.04 % cells in 0.10% -1.0% MMS (Table 2).Perusal of the results in Table 2 revealed that meiotic aberrations increased with the increase in each concentration of both mutagens. The overall frequency of meiotic aberrations at various stages of meiosis indicated that metaphase aberrations were more common followed by anaphase and telophase aberrations (Table 2).

Combine analysis of all the mutagenic indices showed continuous increase in inhibition and aberrations with increase in doses of MMS which confirmed the genetic impact of MMS doses on the *Nigella sativa* L. genotype and suggested that the doses 0.25%, 0.50% and 0.75% MMS are suitable for mutation breeding experiment the genotype while 1.0% MMS though highly mutagenic but it is of no use in breeding experiment as germination and fertility cannot be compromised up to that level.

Discussion: In the present investigation seed germination, plant survival and pollen fertility decreased with the increase in mutagenic treatments. These results are in confirmation with Wani *et al*. (2004) in chickpea, Banu*et al*. (2004) in cowpea, Kumar (2005) in *Coriandrum sativum,* Laskar and Khan (2014) in *Vicia faba* L. However, the extent of decrease differed among different mutagens. Several workers have suggested the probable causes responsible for inhibition of seed germination. Reduction in seed germination (%) in mutagenic treatments has been attributed to delay or inhibition in physiological and biological processes necessary for seed germination which include defective enzyme production (Kumar, 2005) and inhibition of mitotic process (Ananthaswamy*et al*., 1971). The decrease in the seed germination (%) after mutagenic treatments may be ascribed to the chromosomal aberrations, disturbances in DNA and auxin synthesis and to the impaired cell metabolism (Kirtane and Dhumal, 2004). Progressive decrease in the rate of survival of plants with an increase in the doses of physical and chemical mutagens has been reported by Jayabalan and Rao (1987) in *Lycopersicon esculentum* and Kumar and Dubey (1998a, b) in *Lathyrus sativus*. Decrease in seedling survival may be attributed to the series of events occurring at the cellular level which affect the vital macromolecules and bring about a physiological imbalance in the cells as a consequence of exposure to ionizing radiations and chemical mutagens. Physiological imbalance or different types of chromosomal aberrations or both may be the main cause for drastic decrease in survival (Rao, 1983).Varying degree of pollen sterility was induced in all mutagenic treatments in the present investigation. The magnitude of sterility increased with an increase in dose/concentration of mutagens in some plants. Very high pollen sterility was recorded at the higher doses of treatments. These results are in agreement with many workers who have also reported a dose dependent increase in pollen sterility following mutagenic treatments (Kumar and Dubey, 1998a and Jafri *et al*. 2011).

In this context cytological investigations appear rewarding as they deal with the primary genetic material, the chromosome, and more appropriately the DNA which controls the phenotypes. Cytological

analysis with respect to either mitotic or meiotic behavior is considered to be one of the dependable indices to estimate the potency of mutagen. Therefore, investigations or disturbances in meiotic behavior indicating mutational genetic load form an integral part of most of the mutation studies. It also provides a considerable clue to assess sensitivity of plants for different mutagens, and to ascertain the most effective mutagen for a given crop to realize maximum results. In the present investigation a vast array of meiotic aberrations were recorded in the plants raised from the seeds treated with different concentrations of MMS. Meiotic aberrations increased with an increase in concentration of the mutagens in both the mutagens. However, the frequency of chromosomal aberrations was comparatively more in MMS indicating that it is more sensitive to the *Nigella sativa*. Different types of chromosomal aberrations viz. multivalents, stickiness, precocious separation, stray bivalent (at metaphase I/II), bridges, laggards and unequal separation, cytomixis, disturbed polarity, micronuclei and multinucleate condition (at telophase I/II) as observed in the present investigation, have also been reported by many workers in different plants, viz. Anis and Wani (1997) in *Trigonella foenum-graecum* L., Kumar and Srivastava (2001) in *Plantago ovata*, Anis and Sharma (1997) in *Capsicum annum* L., Bhat *et al*. (2005a,b) in *Viciafaba* L. Jafri *et al*. (2011) in *Cichorium intybus* L., Gulfishan*et al*. (2011) in *Capsicum annuum* L. and Khan *et al*. (2009) in *Cichoriumintybus* L. Stickiness of chromosomes was one of the most common abnormalities observed in the present investigation. Chromosomes were found clumped into one, two or many groups due to stickiness at metaphase causing difficulty in normal disjunction of chromosomes. These results are in agreement with those of Bhat *et al*. (2006) in *Vicia faba*; Ganai *et al.* (2005), Srivastava and Kapoor (2008) in fenugreek and Gulfishan *et al*. (2011) in *Capsicum annuum* L. The sticky chromosomes may result from defective functioning of one or two types of specific non-histone proteins involved in chromosome organization that are needed for chromatid separation and segregation (Gaulden, 1987).Different types of multivalents associations (tri, tetra, penta, hexa, hepta, octa and chain of bivalents) were rather more frequent at higher concentrations of all the mutagens in the present investigation have also been reported in various plants like *Trigonella foenum-graecum* L. (Abbasi and Anis, 2002), *Viciafaba* L. (Bhat *et al*., 2006a) and chick pea (Ganai*et al*., 2005; Sharma and Kumar, 2004). The multivalent formations have been attributed to pairing due to translocations and inversion. Occurrence of multivalent association is a common feature in treated plants arising mainly due to the presence of more than 2 homologous chromosomes (Srivastava and Srivastava, 2004). Precocious movement of chromosomes at metaphase I was also a dominant abnormality in all the mutagenic treatments. Precocious separation was observed by several workers in different plants (Umar and Singh, 2003) in barley, Ganai*et al*. (2005) in chickpea, Abbasi and Anis (2002) in fenugreek, Srivastava and Kapoor (2008) in fenugreek. Absence of chiasmata, which are responsible for maintenance of bivalents permitting normal chromosome segregation, is one of the reasons that can be ascribed for the precocious migration of univalents. Such disturbances in meiosis in highly heterogenous and out crossing species perhaps indicate the hybrid nature of genomes, resulting in cell cycle variations, meiotic disturbances and chromosome bridges (Bajpai, 2006). Precocious movements are possibly due to the effect of chemicals in breaking the protein moiety of the nucleoprotein backbone (Kumar and Rai, 2007).Laggards may be explained on the basis of abnormal spindle formation and chromosomal breakage. According to Tarar and Dnyansagar (1980), unsynchronized bivalents or laggards might be due to the discrepancies in spindle formation. Laggards may arise by breakage or faulty spindle resulting into imbalanced daughter nuclei and micronuclei (Singh and Chaudhary, 2005). Presence of laggards may be attributed to the inability of multivalents to separate properly (Ganai *et al*., 2005). Laggards at anaphase have also been observed by Abbasi and Anis (2002) in fenugreek, Srivastava and Kapoor (2008) in fenugreek and Gulfishan *et al*. (2011) in *Capsicum*.Bridges with or without fragments at anaphase stages were frequently observed in the present investigation. Bridges with or without fragment were also observed in many other plants like fenugreek (Abbasi and Anis, 2002, Srivastava and Kapoor, 2008), chickpea (Ganai *et al*., 2005), chilli (Singh and Chaudhary, 2005), maize (Kumar and Rai, 2007), Jafri *et al*. (2011) in *Cichorium* and Gulfishan *et al*. (2011) in *Capsicum*. The presence of chromatin bridges without fragments may be due to restitution or the fragments getting entangled or attached with normal chromatids of chromosomes (Tarar and Dnyansagar, 1980). Bridges seem to be the result of non-separation of chiasma due to stickiness.

According to Kumar and Singh, (2003) random movement of univalents to any one of the poles leads to unequal separation of chromosomes. Movement of bivalents towards the poles at anaphase I due to non-disjunction of homologous chromosomes at metaphase as observed in the present investigation may be due to the stickiness of chromosomes and could result in unequal distribution of chromosomes in the daughter nuclei (Anis and Wani, 1997). It is also reported in chickpea (Sharma and Kumar, 2004 and Ganai *et al*., 2005), fenugreek (Srivastava and

Kapoor, 2008).Generally, laggards and non-oriented bivalents may produce micronuclei at telophase if they fail to reach the poles in time to be included in the main nucleus or irregular chromosome segregation at meiosis I and II could be the result of the non-oriented bivalents formed due to spindle disc functioning or may be due to the formation of univalents at diakinesis or metaphase I, which shows an inability to congregate on the equatorial plate resulting in the formation of micronuclei and abnormal pollen grains (Kodura and Rao, 1981). The formation of micronuclei may be due to adhesion of fragments or due to lagging chromosomes (Anis *et al*., 1999). Micronuclei formation has also been reported by Kumar and Kumar (2000), Ganai *et al*. (2005) and Abbasi and Anis (2002). Multinucleate condition has been explained to a particular genotypic change suppressing the organizing capacity of nucleolar chromosome and induces the formation of adventitious nucleoli. Raina *et al*. (1994) reported that multipolar condition is mainly due to spindle dysfunction. Such conditions resulted in the unequal distribution of chromatin material in the gametes. Most of these gametes were deficient which either does not take part in fertilization or they produce aneuploids. Disturbed polarity at anaphase and telophase stages could be due to spindle disturbance. Disturbed polarity was also reported by Ganai *et al*. (2005) in chickpea, Bhat *et al*. (2006a) in *Vicia faba* L., Kumar and Rai (2007) in maize, Abbasi and Anis (2002), Wani and Anis (1997) and Srivastava and Kapoor (2008) in fenugreek.

In brief, the induced cytological disturbances are of huge importance, since it creates alteration in the genetic material which can be inherited to the subsequent generation resulting into desirable mutations, therefore, the present study revealed that the lower and moderate concentrations of MMS proved to be efficient in increasing the genetic variability in *Nigella sativa* L. Thus the genetic variability induced by mutagenesis may be effectively exploited in future generations for promising performance of the improved *Nigella sativa mutants*.

Conclusion: The estimation of mutagenic sensitivity of the crop towards the doses of the mutagen is imperative to set up any mutation breeding program. It is concluded from the present study that the *Nigella sativa* L. genotypic variation can be enhanced by using lower and intermediate doses of MMS mutagenesis, as it is quite clear from the result of all the biological indices considered. To delve the mutagenic potency for selection of doses in mutation breeding, the present study proposes the in vitro germination and PMC meiotic behavior of the treated population are the best indices. Also, the mutagenic potency of MMS on worked out in the study could be compared with the reports of other mutagens on *Nigella sativa* L. for extensive research on this economically important crop in future references.

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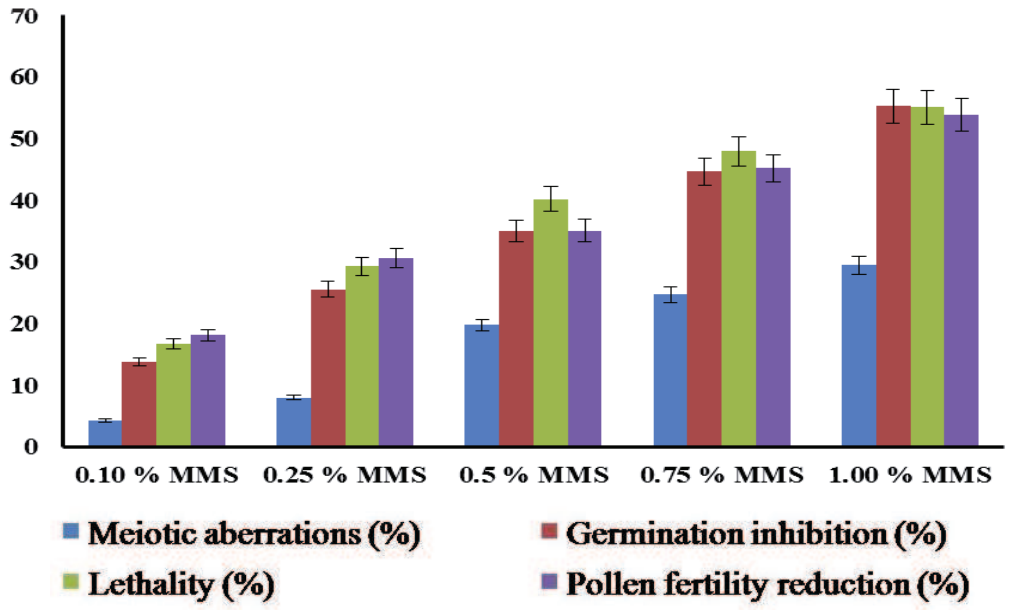
Table 1: Effect of MMS on seed germination, plant survival and pollen fertility in *Nigella sativa* L. (M_1 generation).

Treatments	Germination (%)	Inhibition (%)	Plant survival (%)	Lethality (%)	Pollen fertility (%)	Reduction (%)
Control	94.00	-	90.73	-	89.88	-
0.10% MMS	81.00	13.82	75.54	16.74	73.56	18.15
0.25% MMS	70.00	25.53	64.17	29.27	62.38	30.59
0.50% MMS	61.00	35.10	54.25	40.20	58.32	35.11
0.75% MMS	52.00	44.68	47.19	47.98	49.24	45.21
1.0% MMS	42.00	55.31	40.71	55.13	41.46	53.87

Figure 1. Comparative inhibitory effect of different MMS doses on various biological parameters of *Nigella sativa* L.



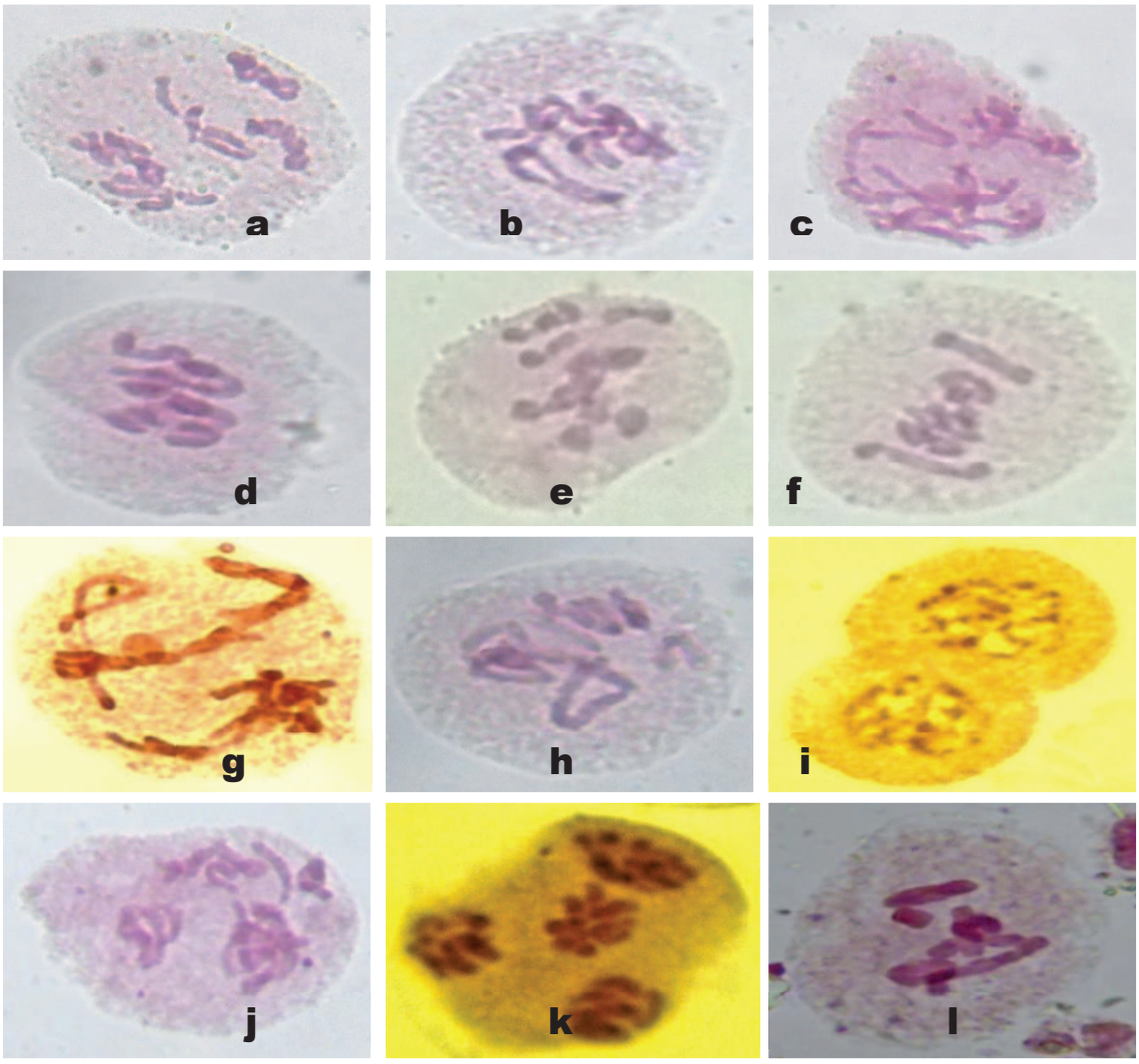


Figure 2. Representative micrograph of PMC with meiotic aberrations induced by MMS doses in *Nigella sativa* L. a. PMC showing bivalent and multivalents with abnormal arrangement. b. PMC showing non

synchronous distributions. c. PMC showing unequal distribution and disturbed polarity. d. PMC showing stickiness at Metaphase. e. PMC showing chromosome clumping and micronuclei. f. PMC showing irregular chromosome arrangement with bridge at early anaphase. g. PMC showing chromosome bridge at Anaphase-I. h. PMC showing precocious separation of chromosome at Metaphase. i. PMC showing cytomixis. j. PMC showing bridge and fragment of broken bridge at Telophase. k. PMC showing disturbed polarity at Telophase-II. l. PMC showing chromosome clumping and fragment.

Table 2.Percentage of meiotic aberrations induced by MMS doses at different Stages of meiosis in the M_1 generation of *Nigella sativa* L.

Concentrations		Observed		Metaphase-I/II (%)						Anaphase-I/II (%)				Telophase-I/II (%)					
		Total No. of PMCs	Total Abnormal PMCs	Stickiness	Precocious separations	Stray bivalents	Fragments	Disturbed Metaphase	TOTAL (%)	Laggards	Bridges	Unequal separation	TOTAL (%)	Micronuclei	Multinucleate conditions	Disturbed polarity	Cytomixis	TOTAL (%)	No. of abnormal PMCs (%)
Control		245	--	--	--	--	--	--		--	--	--		--	--	--	--		--
MMS	**0.10**	256	11	(3)1.17	(2)0.78	(4)1.56	--	--	**3.51**	-	(1)0.39	-	**0.39**	--	(1)0.39	--	--	**0.39**	**4.29**
	0.25	262	21	(5)1.91	(2)0.76	(5)1.91	(3)1.15	--	**5.73**	(1)0.38	(2)0.76	(1)0.38	**1.52**	(2)0.76	-	--	--	**0.76**	**8.01**
	0.50	248	46	(7)2.82	(3)1.21	(5)2.02	(4)1.61	(6)2.42	**10.08**	(4)1.61	(4)1.61	(5)2.02	**5.24**	(3)1.21	(2)0.80	(3)1.21	(3)1.21	**4.43**	**19.75**
	0.75	234	58	(9)3.84	(4)1.70	(6)2.56	(4)1.70	(6)2.56	**12.36**	(5)2.13	(4)1.70	(3)1.28	**5.11**	(4)1.70	(3)1.28	(3)1.28	(7)2.99	**7.25**	**24.72**
	1.00	227	67	(8)3.52	(5)2.20	(6)2.64	(7)3.08	(4)1.76	**13.20**	(6)2.64	(8)3.52	(7)3.08	**9.24**	(3)1.32	(6)2.64	(4)1.76	(3)1.32	**7.04**	**29.48**

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