Indian J. Genet., 80(3) 318-323 (2020)
DOI: 10.31742/IJGPB.80.3.11

Micronuclei assay as biomarker for ethyl methane sulphonate (EMS) genotoxicity in *Eclipta alba* (L.) Hassk.

Girjesh Kumar and Radha Mishra*

Plant Genetics Laboratory, Department of Botany, University of Allahabad, Prayagraj 211 002

(Received: May 2020; Revised: August 2020; Accepted: August 2020)

Abstract

The eukaryotic cells are complemented with a single nucleus. However, several cells show abnormal small nuclei known as micronuclei, with addition to the main nucleus. These anomalous small nuclei along with main nucleus which may be the result of exposure of cell to cytotoxic agents (Chemicals/physical mutagens). These mutagens also cause structural or numerical chromosomal aberrations. The treatment of EMS, a chemical mutagen, induces micronuclei formation and nuclear as well as chromosomal peculiarities in *Eclipta alba*. The effect of micronuclei is discussed in terms of cytotoxicity of genotoxins and several evidences of nuclear polymorphism illustrated due to the mutagenic effect of EMS. Pollen viability was also examined through pollen fertility. Micronuclei causes the genomic instability and elimination of genome which is effectively put to use in breeding programmes for the creation of aneuploids and also for haploid lines.

Key words: MN (Micronuclei), EMS (Ethyl methane sulphonate), instability, pollen fertility, genotoxins

Introduction

Micronuclei are disintegrated nuclear or chromosomal fragments. These are chromatin containing structures found in cytoplasm of the cell and surrounded by a membrane that has no detectable link to the cell nucleus (Schiffmann and De Boni 1991). Their presence is an indicator of genomic instability. Micronuclei (MN) are small extra-nuclear bodies, also called as Howell-Jolly bodies, first reported by William Howell and Justin Jolly in precursors of red blood cells (Luzhna et al. 2013). At that time they were expressed as remnants of nuclei of red blood cells (Sears and Udden 2012). Micronuclei in plants were reported by Marshall and Bianchi (1983) resulting from physical mutagens (Nuti Ronchi et al. 1986) and chemical mutagens (Verhoeven et al. 1991). Micronuclei originate either from acentric chromosomal fragment or sometimes whole chromosome left behind at anaphase which remain unable to incorporate into nucleus after the cell division (Fenech 1997). It is well recognized that micronuclei are either configuration of or a fragment of chromosomes (Gisselsson et al. 2008; Fenech 2007). Such kind of micronuclei appear due to genotoxic stress such as clastogen or aneugen (Terradas et al. 2010). Aneugenic agents create the disturbance in the formation of spindle apparatus during mitosis and form micronuclei in which a whole chromosome lag behind at anaphase, which later on gets surrounded by the nuclear envelop however in case of clastogenic agents micronuclei are produced by stimulating breakage of chromosome which result into acentric fragments, those fragments are incapable of adhering to the spindle fibres and integrating into daughter nuclei (Kumar and Rai 2007; Terradas et al. 2010). The formation of micronuclei is assessed to be a result of deformity in the cell restoration machinery, nucleic acid damages and anomalous chromosomal behaviour. Now a days, ethyl methane sulphonate (EMS) has grasped much attention worldwide as the most potent mutagenic agent known to create variations in higher plants (Luzhna et al. 2013). EMS provides a good scope for selection, as a tool for alteration in the genotype to enhance the variability of characters. In the era of complementary and alternative medicine, *Eclipta alba* (L.) Hassk. (2n=22) (common name: Bhringraj) has gained importance due to its ability to provide a multitudes of health benefits. Bhringraj has been significant part of traditional medicinal system since antiquity. Major portion of this

*Corresponding author's e-mail: radhauniv2015@gmail.com

Published by the Indian Society of Genetics & Plant Breeding, A-Block, F2, First Floor, NASC Complex, IARI P.O., Pusa Campus, New Delhi 110 012; Online management by www.isgpb.org; indianjournals.com

plant is used to prepare herbal medicines which have no side effects on human and hence, considered as an important source of primary health care for majority of world population. Bhringraj is a reservoir of medicinal properties as it contains several phytochemicals which are used as remedy for several diseases such as liver ailments, cirrhosis, infective hepatitis and other conditions involving hepatic enlargement. Since Vedic period it is well known plant for its splendid medicinal values. Bhringraj occupies an integral role in Ayurvedic therapies and it is a self-pollinating plant so there is need to generate variations with the help of mutagens to improve qualitative and quantitative traits. The current study was undertaken to assess the mutagenic potential of an alkylating agent EMS on the genetic constitution of *Eclipta alba* (L.) Hassk. The malignancy of gene due to EMS was measured in terms of micronuclei frequency. This discussion presents a comprehensive account of cytological effects of EMS on Bhringraj cultivars for mutagenic exploitation of the herb. Therefore the work is focused delebrations mainly on the investigation of micronuclei and its several advantages. This approach allows to examine the genotoxicity and pointing out some of the potential pitfalls and limitations of this phenomenon.

Materials and methods

Seeds of *Eclipta alba* (L.) Hassk. were obtained from Central Council For Regional Ayurveda Sciences (CCRAS), Jhansi, India. Healthy and fresh seeds were treated with various concentrations of freshly prepared EMS solution, *viz.,* 0.1%, 0.3% and 0.5%, prepared in a phosphate buffer with pH 7.0 for 5 h. The treated seeds were then meticulously rinsed in running tap water for 30 min. to remove excess traces of the mutagen remained over the seed coat. Intervening period of time, one set of seeds were kept without treatment to act as control. The control seeds as well as treated seeds were sown immediately in the field in replicates of three for each dose adopting the complete randomized block design (CRBD) to raise the M1 population.

For meiotic studies, flower buds of adequate size were fixed in Carnoy's fluid (1 part glacial acetic acid: 3 parts ethyl alcohol). After 24 hour of fixation, buds were transferred to 70% alcohol for longer use. 2% acetocarmine squash technique was used to examine the pollen mother cells (PMCs) for scoring meiotic anomalies. Pollen viability was analysed by using the acetocarmine glycerine stainability test. The snapshots of the pollen mother cells (PMC_S) were recorded by pinnacle PCTV capture device.

Statistical analysis was performed using the SPSS 16.0 software. Analysis of variance (ANOVA) and Duncan's multiple range test (DMRT, $P < 0.05$) was subjected for comparison of the mean and the graphical illustrations of data were plotted by using sigma plot 10.0 software.

Result and discussion

Cytogenetic study provides insight about chromosomal structure and number. Meiotic studies in the control population revealed no irregularities in the structure and behavior of chromosome and exhibited 11 normal bivalents at metaphase I (Figs. 1B and C).

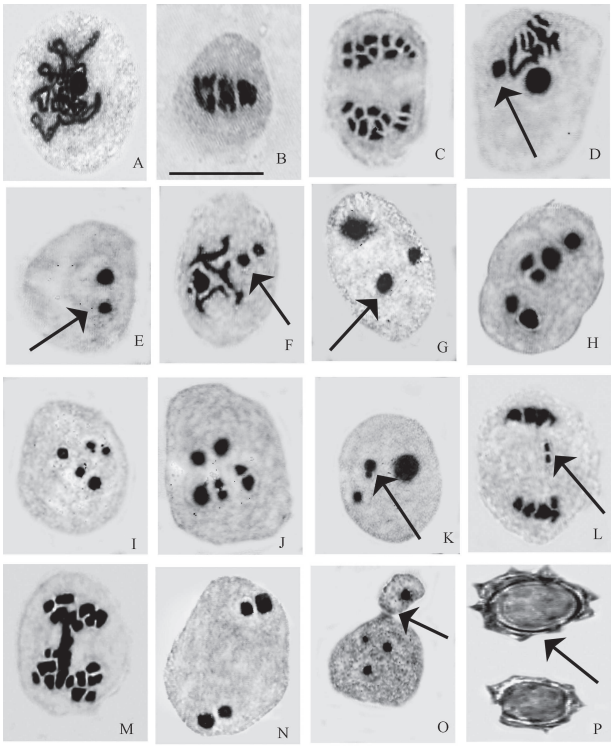

Fig. 1. Micronuclei induced by EMS treatment. A: Normal prophase; B: Normal metaphase I (n= 11); C: Normal anaphase (2n = 11:11), D: One micronucleus at prophase I; E: Binucleate PMC single micronucleus; F: Two micronucleus at prophase I; G: Trinucleate condition with 2 micronuclei; H-J: Multinucleate condition; K: Nuclear budding; L: Double laggard at Anaphase I; M. Bridge at Anaphase I; N:Telophase I with micronuclei; O: Micronuclei with Microcell; P: Fertile (Arrow) and sterile Pollen; Scale Bar- 9.73µm

However, in EMS treated plants, PMCs showed various chromosomal disturbances during meiosis. A range of different abnormalities were recorded, among which micronuclei formation was specific one. At the lowest concentration i.e. 0.1% EMS, per cent frequency of micronuclei recorded was 3.79±0.024% which was calculated to be 6.71±0.20% at 0.3% EMS, and increased to 10.29±0.21% at highest concentration of EMS (Table 1).

Table 1. Micronuclei formation in *Eclipta alba* (L.) Hassk. induced by EMS treatment

Treatment	Concen-tration	Total no. of PMC's scored	No. of PMC's with MN	% of PMC's with MN
EMS	Control	235	-	-
	0.1%	274	9.33±0.04	3.79±0.02
	0.3%	289	18.00±0.12	6.71±0.20
	0.5%	265	29.66±0.45	10.29±0.21

PMC's = Pollen mother cells, MN= Micronuclei

Table 2 is a depiction of total micronuclei frequency in to number of additional micro nuclei. This data is a depiction of total micronuclei frequency recorded for 0.1% EMS to 0.3% EMS treatment. Mono-micronucleate condition was found dominant in all the three treatment sets in highest frequency and was recorded to increase from 1.82±0.17 at 0.1% EMS to 3.03±0.30 at 0.5 % EMS set.

Binucleate frequency ascend from 0.97±0.10 to 2.39±0.13% from lowest to highest treatment set. Least frequency was calculated for pentanucleate condition; it was observed in 0.3 and 0.5 % treatment sets only and calculated to be 0.11±0.11 and 0.50±0.12, respectively. Besides micronuclei, a considerable frequency of laggards (Fig. 1) and bridges, was observed that exhibited integration with micronuclei formation. These micronuclei formation give rise aneuploidy (Seoane et al. 2000) which observed during the meiosis process of *E. alba*.

Per cent frequency of laggards at anaphase increased from 0.58±0.31% at 0.1% EMS to 1.09±0.10% at 0.3% EMS treatment sets while at 0.5% EMS, it was calculated to be 1.14±0.25%. Splitting and enucleation of micro nuclei from parent cell occurred via microcell, as evident in some stages (Fig. O). It was discernible that formation of microcell was seen at the highest two treatment sets at *i.e.* at 0.3% (0.22±0.11%) and 0.5% (0.37±0.10%).

The maximum pollen fertility was recorded in the control set with mean per cent value observed as 97.17±1.15% meanwhile in the treated sets, the pollen fertility shows a gradual decline along with increasing concentrations of EMS as shown in Fig. 2, which

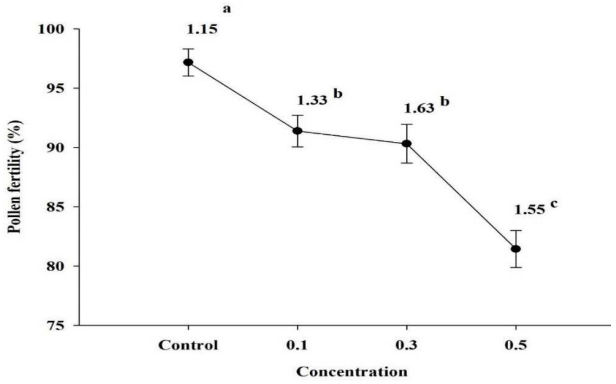

Fig. 2. Effect of EMS treatment on the pollen fertility of *Eclipta alba* (L.) Hassk.

deciphered graphical representation of the pollen fertility trend in the control as well as treated sets. Figure 1P displayed sterile pollens.

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

	Number of MN per PMC (Mean±S.E.)					No. of Anaphase cells (Mean±SE)		No. of Microcell (Mean±SE)
	Mononucleate	Binucleate	Trinucleate	Tetranucleate	Pentanu-cleate	LaggardsI/II	BridgesI/II	
Control	0	0	0	0	0	0	0	0
0.1% EMS	1.82±0.17	0.97±0.10	0.37±0.1	0	0	0.58±0.31	0	0
0.3% EMS	1.86±0.35	1.48±0.39	1.15±0.08	0.23±0.20	0.11±0.11	1.09±0.10	0	0.22±0.11
0.5% EMS	3.03±0.30	2.39±0.13	1.37±0.0.09	0.76±0.23	0.50±0.12	1.14±0.25	0.62±0.11	0.37±0.10

There are many studies done previously on regular or irregular mitosis as well as meiosis which suggest the abnormal behaviour of chromosome either naturally (Thakur and Singh 1968; Trivedi and Kumar 1984) or induced by physical and chemical mutagens (Kumar and Tiwary 2014; Kumar and Mishra 2020). These studies give a clear picture about the anomalies found in *E. alba*. However, the result suggests entrapment of chromosome within micronuclei which induces chromothripsis (shattering of chromosome), accompanied by the random re-arrangement and ligation of the fragments leading to the chromoanagenesis (formation of abnormal chromosome) which is the reason for genomic instability and aneuploids. Genomic instability has been suggested to lead nuclear pleomorphism and nuclear deformities (Bignold 2003). As EMS stimulates abnormal segregation of chromosomes or chromatid which renders the poleward movement of chromosomes during anaphase and get manifested as laggards which cease to assemble in daughter cells at telophase. Confinement of these laggards within a nuclear encasement culminates in micronuclei. Appearance of micronuclei acts as a marker of genotoxic damage (Kamboj et al. 2007) and these micronuclei assay is an important tool to assess the level genotoxicity of different doses of mutagens.

The earlier exhaustive works on the concept of micronuclei, and consequences associated with it have been discovered and various effective speculations have been proposed. The censorious study of various recent reports suggest that micronuclei may result due to disturbance of cell cycle by way of clastogenecity which invades chromosomal breakage while aneugen affects cell division and spindle properties resulting in aneuploidy and micronucleus formation, respectively (Luzhna et al. 2013). As clastogenic and aneugenic mechanisms of physical and chemical mutagens (Walker et al. 1996) cumulatively or separately renders the genetic architecture of the cell's nucleus. The formation of mononucleate and binucleate (Fig. 1D and E) PMCs are indicator of clastogenic effects (Leme and Marin – Morales 2008) whereas micronuclei formed due to breakage of chromosome strands show multinucleate micronuclei bearing PMCs (Figs. 1H-J) are consequences of aneugenic effect resulting from chromosome loss.

Micronuclei assays have been considered, as the most effective and simplest way to explore the mutagenic effect aided by chemicals due to the fact that micronuclei result from damages, rather than wrongly repaired, in the parental cells (Ribeiro 2003). Growing evidences of micronuclei and its involvement in chromatin/chromosome status regulation, gene expression; and genetics have an equal influence on the development of genomic instability (Aypar et al. 2011).

Chaudhary et al. (2015) opined that mutagenic effect of EMS could be related to its ability to induce nuclear alteration in the form of nuclear buds, micronuclei, irregular nuclei, etc. EMS causes the breakage of chromosome at guanine rich areas and also modifies the bases of DNA which cause the micronuclei formation (Kumar and Singh 2018). Hoffelder et al. (2004) suggested that the presence or absence of nuclear pore complex together with the specific micronuclear DNA content might be one of the decisive factors for the transcriptional activity of micronuclei. Similarly other diverse structures of nucleus, such as nuclear buds which are produced by the remnants of broken nucleoplasmic bridges (Utani et al. 2010; Fenech et al. 2011). They are originated from dicentric chromosome, by breakage of nucleoplasmic bridges during cytokinesis resulting into a micronucleus formation. From some studies, an idea has been conceived that these nuclear buds might have converted into micronuclei during interphase stage of the cell cycle (Shimizu et al. 1998) submissively, micronuclei paved a clue to understand the mechanism underlying the nucleus reconstruction after mitosis or meiosis. Simultaneously micronuclei and nuclear buds are indicator for genomic instability. Chromosome losses from disorderly arrangement of chromosomes in the cell's cytoplasm which triggers the induction of micro cells (Fig. 1O). Few authors believe that if the synthesis of DNA in a micronuclei composed of an entire chromosome, it is synchronic to nuclear DNA synthesis, the lost chromosome could be integrated into the nuclear genome (Gustavino et al. 1994). Evidence was complementary to the main nucleus, then the cell might lose a certain gene dosages (Terradas et al. 2010) even resulting in cell death (Fernandes et al. 2007). Another reason for micronuclei formation as reported by Vazquez-Diez et al. (2016), is attributed to the mis-attachment of kinetochore which leads towards the laggard chromosome which produce the micronuclei in future. The formation of micronuclei along with other chromosomal anomalies has great impact on pollen fertility. Darkly stained pollen grains were judged as fertile whereas lightly stained pollens were recorded as sterile pollen. Abnormal meiosis compounded to cause sterility of pollens. Disintegration

of the micronuclei from the parent nucleus occur via formation of microcell which has also been reported in some crops, such as sunnhemp and cluster bean (Kumar and Dwivedi 2016; Kumar and Singh 2018) and formation of micronuclei is considered to be a result of defects in the cell repair machinery, nucleic acid damages and anomalous chromosomal behaviour.

The cytological illustration of ultrastructural data induced by EMS indicate an increased condensation amount of micronucleated chromatins which divulges a connection between the size of micronuclei and the proportion of euchromatin and heterochromatin. Large micronuclei always contains a large quantity of euchromatin, where as small micronuclei are almost heterochromatin. These appealing observations of variability in ultrastructure of chromatins shows gradual disintegration of micronuclei from a structure having larger amount of euchromatin into a structure having smaller amount of heterochromatin (Gernand et al. 2005).

Briefly summarizing the process of micronuclei formation, it is important to emphasize the major cause of micronuclei containing acentric chromatids or chromosomes is the result of unpaired or misrepaired DNA breaks, whereas micronuclei with whole chromatid/chromosomes are formed due to hypomethylation of satellite centromeric/ paracentromeric sequences, Kinetochore defects, dysfunctional spindle and mutation in anaphase checkpoint genes. These are the major responsible factors for inducing micronuclei (Luzhna et al. 2013). The detailed study provides valuable data on offspring's behaviour towards genotoxic agents in relation to micronuclei formation. Due to easy detection of micronuclei these can be used as an efficient biomarker to study the chromosomal aberration induced by mutagenic agents. Micronuclei studies are the leading field in mutation research. Micronuclei formation leads to genome elimination which is an important tool in breeding programmes for producing addition and substitution lines. 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 (Ege and Ringertz 1974; Ege et al. 1977; Fournier 1982). It may also lead to several physiological effects and useful in interspecific breeding for improvised traits. The above information would be highly supportive in planning an effective tool as breeding program for developing new traits with desirable characters for human welfare.

Authors' contribution

Conceptualization of research (GK, RM); Designing of the experiments (RM); Contribution of experimental materials (CCRAS, Jhansi, India); Execution of field/ lab experiments and data collection (GK, RM); Analysis of data and interpretation (GK, RM); Preparation of the manuscript (RM).

Declaration

The authors declare no conflict of interest.

Acknowledgements

The authors express deep sense of gratitude towards CCRAS, Jhansi Uttar Pradesh for providing seeds of *Eclipta alba* (L.) Hassk. and also would like to thank the Department of Botany, Allahabad for providing space for experiment. Corresponding author extends gratitude to University Grant Commission for financial assistance.

References

Aypar U., Morgan W. F. and Baulch J. E. 2011. Radiation-induced epigenetic alterations after low and high LET irradiations. Mutat Res. Fund. Mol. M., **707**: 24-33.

Bhat T. A., Clastogenic effect of EMS and MMS in *Vicia faba* L. 2005. J. Cytol. Genet., **6**: 117-122.

Bignold L. P. 2003. Pathogenetic mechanisms of nuclear pleomorphism of tumour cells based on the mutator phenotype theory of Carcinogenesis. Histol. Histopathol., **18**: 657-664.

Ege T. and Ringertz N. R. 1974. Preparation of microcells by enucleation of micronucleate cells. Exp. Cell Res., **87**: 378-382.

Fenech M. 2007. Cytokinesis-block micronucleus cytome assay. Nat protc., **2**: 1084.

Fenech M., Kirsch-Volders M., Natarajan A. T., Surralles J., Crott J. W., Parry J., Norppa H., Eastmond D. A., Tucker J. D. and Thomas P. 2011. Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. Mutagenesis, **26**: 125-132.

Fenech M. 1997. The advantages and disadvantages of the cytokinesis-block micronucleus method. Mutat Res., **392**: 11-18.

Fernandes T. C. C., Mazzeo D. E. C. and Marin-Morales M. A. 2007. Mechanism of micronuclei formation in polyploidizated cells of *Allium cepa* exposed to trifluralin herbicid. Pestic Biochem. Physiol., **88**: 252-259.

Gernand D., Rutten T., Varshney A., Rubtsova M., Prodanovic S., Bruss C., Kumlehn J., Matzk F. and Houben A. 2005. Uniparental chromosome elimination at mitosis and interphase in wheat and pearl millet crosses involves micronucleus formation, progressive heterochromatinization, and DNA fragmentation. Plant Cell, **17**: 2431-2438.

Gisselsson D. 2008. Classification of chromosome segregation errors in cancer. Chromosoma, **117**: 511-519.

Gustavino B., Degrassi F., Filipponi R., Modesti D., Tanzarella C. and Rizzoni M. 1994. Mitotic indirect non-disjunction in phyto hemagglutinine stimulated human lymphocytes. Mutagenesis, **9**: 17-21.

Hoffelder D. R., Luo L., Burke N. A., Watkins S. C., Gollin S. M. and Saunders W. S. 2004. Resolution of anaphase bridges in cancer cells. Chromosoma, **112**: 389-397.

Kamboj M. and Mahajan S. 2007. Micronucleus—an upcoming marker of genotoxic damage. Clin Oral Investing., **11**: 121-126.

Kumar G. and Chaudhary N. 2015. Nuclear Blebs Assay for Genome Instability in *Phaseolus vulgaris* L. Cytologia, **80**: 505-512.

Kumar G. and Dwivedi S. 2016. Induced nuclear pleomorphism in pollen mother cells of *Crotalaria juncea* L. (sunnhemp). Cytologia, **81**: 323-327.

Kumar G. and Mishra R. 2020. Modulated UV-B radiation invoking cytomorphological and biochemical aspects of a medicinal plant, Bhringraj (*Eclipta alba* (L.) Hassk.). J. Environ. Biol., **41**: 623-630.

Kumar G. and Rai P. K. 2007. EMS induced karyomorphological variations in maize (*Zea mays* L.) inbreds. Turk. J. Biol., **31**: 187-195.

Kumar G. and Singh S. 2018. Nuclear polymorphism induced genomic instability in cluster bean [*Cyamopsis tetragonoloba* (L.) Taub.] (Fabaceae). Cytologia, **83**: 137-141.

Kumar R. and Tiwary R. K. 2014. Effect of X-ray Treatment in Somatic Chromosomes of *Eclipta alba* (L.) Hassk. Cytologia, **79**: 63-68.

Leme D. M. and Marin-Morales M. A. 2008. Chromosome aberration and micronucleus frequencies in Allium cepa cells exposed to petroleum polluted water—a case study, Mutat Res/GenTox En., **650**: 80-86.

Luzhna L., Kathiria P. and Kovalchuk O. 2013. Micronuclei in genotoxicity assessment: from genetics to epigenetics and beyond. Front. Genet., **4**: 131.

Marshall I. and Bianchi M. 1983. Micronucleus Induction in *Vicia faba* Roots: Part 1 Absence of Dose-rate, Fractionation and Oxygen Effect at Low Doses of Low LET Radiations. Int. J. Radiat. Biol., **44:** 151-162.

Ribeiro L. R. 2003. Teste do micronúcleoemmedulaóssea de roedores *in vivo*. *Mutagênese Ambiental.* ULBRA, Canoas, 173-178.

Ronchi V. N., Bonatti S., Durante M. and Turchi G. 1986. Preferential localization of chemically induced breaks in heterochromatic regions of *Vicia faba* and *Allium cepa* chromosomes—II. 4-epoxyethyl-1, 2-epoxy-cyclohexane interacts specifically with highly repetitive sequences of DNA in *Allium cepa*. Environ. Exp. Bot., **26**: 127-135.

Schiffmann D. and De Boni U. 1991. Dislocation of chromatin elements in prophase induced by diethylstilbestrol: A novel mechanism by which micronuclei can arise. Mutat Res. – Found. Mol. M., **246**: 113-122.

Sears D. A. and Udden M. M. 2012. Howell-Jolly bodies: A brief historical review. Am. J. Med. Sci., **343**: 407-409.

Shimizu N., Itoh N., Utiyama H. and Wahl G. M. 1998. Selective Entrapment of Extra chromosomally Amplified DNA by Nuclear Budding and Micronucleation during S-phase. J. Cell Biol., **140**: 1307-1320.

Thakur V. and Singh N. P. 1968. Cytology of *Eclipta alba* Hassk., J. Ind. Bot. Soc., **26**: 206-210.

Trivedi R. N. and Kumar R. 1984. Cytomorphological studies in natural population of *Eclipta alba* (L.) Hasak. Cytologia, **49**: 731-738.

Utani K. I., Kohno Y., Okamoto A. and Shimizu N. 2010 Emergence of micronuclei and their effects on the fate of cells under replication stress. PloS One, **5**: e 10089.

Vázquez-Diez C., Yamagata K., Trivedi S., Haverfield J. and Fitz Harris G. 2016. Micronucleus formation causes perpetual unilateral chromosome inheritance in mouse embryos. Proc. Natl. Acad. Sci., **113**: 626-631.

Verhoeven H. A., Ramulu K. S., Gilissen L. J. W., Famelaer I., Dijkhuis P. and Blaas J. 1991. Partial genome transfer through micronuclei in plants. Acta Bot. Neerl., **40**: 97-113.

Verth G., Terradas J. and Goossens M. 2010. Observational evidence of resonantly damped propagating kink waves in the solar corona. Astrophys. J. Lett., **718**: 102.

Walker J. A., Boreham D. R., Unrau P. and Duncan A. M. 1996. Chromosome content and ultrastructure of radiation-induced micronuclei, Mutagenesis, **11**: 419-424.