Nuclear Polymorphism Induced Genomic Instability in Cluster Bean [*Cyamopsis tetragonoloba* (L.) Taub.] (Fabaceae)

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Summary Several environmental and mutagenic factors lead to induce nuclear polymorphism like micronuclei (MN) and nuclear buds. MN are the disintegrated partitioned nuclear or chromosomal fragment. Their occurrence is indicator of genomic instability or chromosomal turmoil. Combined mutagenic treatment of gamma rays and ethyl methane sulphonate (EMS) was experienced to induce formation of MN and nuclear buds in cluster bean [*Cyamopsis tetragonoloba* (L.) Taub]. A considerable proportion of microcells was also encountered that display future fate of micronuclei in the cell. A peculiar correlation with this nuclear polymorphism was experienced on the presence of bridges and laggards at anaphase I and II stages. Frequency of bridges and laggards were collinear to the combined mutagenic action. Pollen viability was also examined through pollen fertility percentage to inculcate effects of the nuclear incineration on the viability of pollen and the results depicted a dose dependent decline in pollen fertility.

Key words Cluster bean, Pollen mother cell, Micronucleus, Microcell, Mutagen, Pollen fertility, Gamma ray, EMS.

Diligency in the cellular processes, specifically of the cell division, is a quintessential pre-requisite for fidelity in inheritance whereas errors may lead to alter the end-products of meiosis. Acquisition of reports for error in nucleus is a serious ground for concern, since it is the action centre for entire cell expression and is a vestibule for intrinsic hereditary factors. The significance of nucleus can be easily estimated by the fact that enucleated cells have a restricted fate and functioning. In-site nuclear study has assisted in unravelling the hidden mysteries of inheritance; with this, it has also paved the stepping stones for classical genetics. Advent of cytogenetic studies provided testament for nuclear and chromosomal behaviour and also decrypted the influence of mutagenic agents and environmental factors on integrity of these genetical entities. A prominent indicator for mutagenic errors in nucleus is the occurrence of MN that depreciates from the principle nucleus of cell. MN or the Howell-Jolly bodies, are chromatin-containing structures found in the cytoplasm of cells and are surrounded by a membrane that has no detectable link to the cell nucleus (Schiffmann and De Boni 1991). An outcome of nuclear abstraction, MN may result due to varied consequences. MN are expressed in dividing cells that either contain chromosome fragments lacking centromere and/or whole chromosomes that are unable to move

to the spindle poles (Fenech 2000). According to Schmid (1975), chromosomal damage can be easily accessed by MN measurement. With cell cycle culmination, these fragmented segments or the laggards get enclosed in a separate nuclear membrane, a structure that is MN. It resembles in structure with daughter nucleus although being way smaller in size (Sedelnikova *et al.* 2007).

Among many forwarded reasons for genesis of MN, laggards and bridges are important, and this argument requires studying the frequency of these two chromosomal aberrations for contemplating about the integration among the three abnormalities. Emanation of bridges and MN occurs through similar mechanism and henceforth, computation of bridges is of paramount significance to assess the mutagenic genotoxicity level. Nuclear buds, a nuclear polymorphism exhibited as budding or protrusions of nucleus, is another component of abnormalities which is regarded as stable marker of genotoxicity at interphase (Ruddle 1962).

Cluster bean is a splendid leguminous plant used as gum yielding, vegetable and forage crop. The infinite ethnobotanical and nutraceutical possibilities can be elevated using mutagenic strategies since the plant is primarily self-pollinating crop having narrow genetic base. Numerous works are present on the effect of single mutagen however present work is planned to combine physical and chemical mutagen together. For this, combined mutagenic action of gamma rays and EMS was optimized in the present work. Gamma rays are elec-

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tromagnetic radiations with high penetrating strength, enough to cause ionization of atoms and molecules. EMS is a monofunctional alkylating agent and is considered as a potent mutagen and carcinogen (Meuth and Arrand 1982). It has been documented in several previous findings that higher doses of gamma radiation (Verma and Khan 2016) and EMS (Kumar and Chaudhary 2015) lead to MN induction. The mutation frequency may either increase or decrease when physical and chemical mutagens act independently of each other or compete for the same sites (Srivastav and Raina 1981). Efficiency of physical or chemical mutagen by combining gamma rays and EMS has been significantly worked by Jayabalan and Rao (1988) in tomato where combination led to genetic variation. Cytological study may facilitate for encrypting and imaging the cytogenetical influences of combined mutagenic interaction.

Materials and methods

Plant system and treatment with mutagens

Seeds of cluster bean of variety RGC-1038, was procured from CAZRI, Rajasthan, India. Fresh and healthy seeds of cluster bean were selected and kept in five separate airtight plastic packets. The seeds were initially irradiated with ionizing gamma rays at 100, 200, 300, 400 and 500 Gy from a ^{60}Co source at National Botanical Research Institute, Lucknow with dose rate of 2 sGy^{-1} . After irradiation, pre-soaked seeds of each dose were immersed in 0.3% EMS in 20 mL of phosphate buffer of pH 7.0 for 3 h. Few seeds were dipped in distilled water and were regarded as control. Then, the treated seeds were thoroughly washed in distilled water and sown in pots in triplicates along with control. Higher two treatment *i.e.* 400 and 500 Gy+EMS were lethal for plants.

Analysis of meiosis in PMCs and pollen fertility

Young floral buds were fixed in Carnoy's fixative (1 part glacial acetic acid: 3 parts alcohol). After 24 h the fixative was decanted and the buds were preserved in 70% alcohol at 4°C . For meiotic study, anthers were teased on slide in a drop of alcohol. Furthermore, 2% acetocarmine was used for chromosomal staining. Slides were observed under an Olympus microscope whereas Nikon microscope was deployed for snapping images at various stages. Pollen fertility percentage was also documented using glycerol-acetocarmine stainability test (Marks 1954). Five slides for each treatment set were prepared out of which ten fields of views in each slide were recorded.

Statistical analysis

A statistical software, SPSS 16.0 was employed for statistical calibration. The data for cytological parameters were analyzed using one way analysis of variance and Duncan's multiple range test; whereas graph was

plotted using SigmaPlot 10 (Systat software Inc.).

Results

Manifestation of cytogenetic study harboured an insight to the chromosomal architecture and number. In control, PMCs with seven bivalents were precisely assembled at equator at metaphase I and anaphase I was witnessed as proper poleward migration of chromosomes (Fig. 1A and B, respectively). Combined treatment, was, however, experienced to have considerable implications on chromosomal morphology. A range of different abnormalities were recorded, of which MN formation was of serious concern. At the lowest dose (100 Gy+0.3% EMS), percent frequency of MN recorded was $2.18 \pm 0.25\%$ which was calculated to be $3.93 \pm 0.45\%$ at 200 Gy+0.3% EMS, which increased up to $7.56 \pm 0.32\%$ at highest combined treatment (300 Gy+0.3% EMS) (Table 1).

Table 2 is a representation of total MN frequency in relation to number of accessory MN. This data is representation of the total MN frequency recorded for 100 Gy+0.3% EMS to 300 Gy+0.3% EMS treatment. Mono-micronucleate condition was found at all the three treatment sets in highest frequency and was recorded to ascend from $1.42 \pm 0.27\%$ at 100 Gy+0.3% EMS to $3.14 \pm 0.19\%$ at 300 Gy+0.3% EMS set. Binucleate frequency increased from 0.76 ± 0.08 to $1.96 \pm 0.37\%$ from lower to higher treatment set. Least frequency was computed for pentanucleate condition; it was evidenced at highest treatment set only and calculated to be $0.59 \pm 0.30\%$. Besides MN, a considerable frequency of laggards (Fig. 1K) and bridges were retrieved that display integration with MN formation. Percent frequency of laggards at anaphase increased from $0.53 \pm 0.28\%$ at 100 Gy+0.3% EMS to $3.03 \pm 0.16\%$ at 300 Gy+0.3% EMS. Anaphase bridges were absent at lower combined treatment but recorded at 200 Gy+0.3% EMS ($0.91 \pm 0.17\%$) and 300 Gy+0.3% EMS treatment sets ($1.85 \pm 0.18\%$). Partitioning and enucleation of MN from parent cell occurred *via* microcell, as evidenced in Fig. 1L–O. It was perceived that formation of microcell was seen at the highest two treatment sets *i.e.*, 200 Gy+0.3% EMS ($0.43 \pm 0.08\%$) and 300 Gy+0.3% EMS ($1.53 \pm 0.36\%$). Pollen fertility criterion was also adjudged to assess the impact of combined mutagenic action on viability of pollens. It was perceived that in case of control, pollens were eminently stained globose nucleated entity, whereas sterile pollens were sparsely stained and shrunken. Highest pollen fertility was recorded in control set that went for a continuous decline with respect to each increasing treatment. It was calculated to be calculated $97.33 \pm 0.88\%$ in case of control which declined up to $70 \pm 1.15\%$ at the highest treatment set. Fig. 2 depicts graphical representation of the Pollen fertility trend in the control as well as treated sets. Fig. 1P displays sterile pollens.

Fig. 1. Meiotic cells of control and treated plants. A: normal metaphase I; B: normal anaphase I; C: prophase I with 2 MN; D: metaphase I with MN; E: anaphase I with MN; F: telophase I with MN; G: metaphase II with MN; H: nuclear budding; I: prophase with I nuclear bleb; J: multinucleate PMC with budding; K: laggards at anaphase; L: microcell at Anaphase I; M: microcell at telophase I; N: microcell; O: microcell at anaphase II; P: sterile pollens. Scale bar=9.5 μ m.

Table 1. Frequency of micronuclei induced by combined treatment of gamma rays and EMS in cluster bean.

Treatment	Total number of PMCs (Mean)	Number of PMCs with MN (Mean $\pm$ S.E.)	PMCs with MN % (Mean $\pm$ S.E.)
Control	309.33	0	0
100 Gy+0.3% EMS	303.33	6.66 $\pm$ 0.88	2.18 $\pm$ 0.25
200 Gy+0.3% EMS	300.33	11.66 $\pm$ 1.20	3.93 $\pm$ 0.45
300 Gy+0.3% EMS	286.33	21.66 $\pm$ 1.20	7.56 $\pm$ 0.32

S.E.=Standard Error.

Table 2. Configuration of MN in relation to number of nuclei alongwith anaphase abnormalities in cluster bean.

Treatment	Number of MN per PMC (Mean $\pm$ S.E.)					Number of anaphase cells (Mean $\pm$ S.E.)		Number of microcell (Mean $\pm$ S.E.)
	Mononucleate	Binucleate	Trinucleate	Tetranucleate	Pentanucleate	Laggards I/II	Bridges I/II	
Control	0	0	0	0	0	0	0	0
100 Gy+0.3% EMS	1.42 $\pm$ 0.27	0.76 $\pm$ 0.08	0	0	0	0.53 $\pm$ 0.28	0	0
200 Gy+0.3% EMS	1.92 $\pm$ 0.26	1.11 $\pm$ 0.10	0.40 $\pm$ 0.27	0.47 $\pm$ 0.24	0	1.69 $\pm$ 0.25	0.91 $\pm$ 0.17	0.43 $\pm$ 0.08
300 Gy+0.3% EMS	3.14 $\pm$ 0.19	1.96 $\pm$ 0.37	1.04 $\pm$ 0.03	0.81 $\pm$ 0.09	0.59 $\pm$ 0.30	3.03 $\pm$ 0.16	1.85 $\pm$ 0.18	1.53 $\pm$ 0.36

Fig. 2. Graphical account on pollen fertility percentage in cluster bean after combined treatment of mutagens.

Discussion

Ever since the pioneering works on the concept of MN, consequences and repercussions, linked with it have been investigated and several theories have been thereafter forwarded. 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). Clastogenic attack introduces breaks in chromosomes while aneugen affects cell division and spindle properties resulting in aneuploidy (Luzhna *et al.* 2013).

Gernand *et al.* (2005) opined that MN are products of miss-repaired or unrepaired DNA double stranded breaks. This plausibly has occurred due to the combination of two mutagens where physical and chemical mutagen complements each other. This co-action is termed as synergistic interaction. The synergism occurs firstly because of first mutagen treatment making accessible otherwise non available sites for reaction to the second mutagen; and secondly pre-mutational lesions induced by first mutagen becomes fixed due to inhibitory effect of the second mutagen on repair enzyme (Payez and Deering 1972). Patil and Bhatia (1992) reported that the localization of breaks along the chromosomes result from the affinity of EMS for guanine rich areas on the other hand the radiations can have direct effect on chromosomes. It implies that the two mutagens complement each other, where gamma rays introduce direct damage to DNA molecule and EMS advances to cause chemical modification of bases.

Fenech *et al.* (2011) cited that main mechanism of MN formation is hypomethylation of centromeric and paracentromeric satellite repeats in human lymphocyte culture. It may also be derived by processes such as polyploidization, in which they originate from the elimination of exceeding DNA of the main nucleus in an attempt to restore the normal conditions of ploidy in *A. cepa* (Fernandes *et al.* 2007). According to Terradas *et al.* (2009), MN might be involved in DNA damage responses. It is reported that MN is able to synthesize its own DNA (Kramer *et al.* 1990) for restoration of the

damage, albeit there is asynchrony in the level of progression among the parent nucleus and MN. Presence or absence of nuclear pore complex together with the specific micronuclear DNA content might be decisive factors for the transcriptional activity of MN (Hoffelder *et al.* 2004).

Among other polymorphic structures of nucleus, nuclear buds are significant that are marked as nuclear protrusions. Fig. 1H displays nuclear bud. Furthermore, remnants of broken nucleoplasmic bridges may also descend into buds (Utani *et al.* 2010). Synchronised segregation of chromosomes requires fidelity in the alliance of centromeric components with the kinetochore complex. Vázquez-Diez *et al.* (2016) attributed that misattachment of kinetochore leads to laggards that outcomes into MN formation. Mutagenic action also incidences into chromosomal errors leading to breaks and formation of bridges. Bridges originate from dicentric chromosomes that are formed through miss repair of DNA breaks and telomere fusion events (Fenech *et al.* 2011). Effect of mutagen was also witnessed on pollen fertility. Darkly stained pollens grains were judged as fertile whereas lighter stained pollens were regarded as sterile. Chromosomal alterations and abnormal meiosis compounded to cause sterility of pollens.

Disintegration of the MN from the parent nucleus occurs *via* formation of microcells. Formation of microcell has also been reported in leguminous crop, Sunn hemp (Kumar and Dwivedi 2016). It consists of a single MN and a thin rim of cytoplasm surrounded by an intact plasma membrane in animal cells (Sekiguchi *et al.* 1978). Microcell mediated chromosome transfer serve an ingenious purpose of genetic mapping and may be reciprocated for the identification of a disease-bearing chromosome (Whitney *et al.* 1995). Its creation is a process of enucleation, henceforth it can be envisioned that a microcell might single out certain intrinsic hereditary factors along with it leaving behind a deficient cell.

Human carcinogenic predictions associated with MN (Bonassi *et al.* 2007) have made it noteworthy for bio-monitoring. MN designates genomic instability whereas its occurrence denotes a passage that genome tends to undergo nuclear restitution. The underlining repercussion of MN is aneuploidy (Seoane *et al.* 2000). Microcell creation mediated enucleation may lead to paradigm shift in studying tumorigenic course and inheritance loss instilled through genetic ailments.

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