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Ionizing radiation mediated effect on morphological, biochemical and microsporogenesis behavior of *Artemisia annua* L.

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Abstract

Aim: To evaluate the effect of gamma rays on the cytological, morphological and biochemical aspects of *Artemisia annua* L.

Methodology : Fresh seeds of *Artemisia annua* L. were treated with gamma rays (100 Gy, 200 Gy, 300 Gy & 400 Gy) through a ⁶⁰Co source. Along with one set of control, the seeds were sown in triplicates to raise population for meiotic study. Mature capitula were collected for pollen study. Mutagen sensitivity was also studied using morphological (germination percentage, survival percentage, plant height and internodal length) and biochemical (chlorophyll content) parameters.

Results : Total abnormality percentage (TAB%) showed direct relationship with the increasing doses of gamma rays and different chromosomal aberrations such as scattering, precocious, laggard and bridge formation etc. were also observed. Stickiness was the main chromosomal abnormality observed in treated sets, mostly at 300Gy and 400Gy doses. The results clearly elucidate that the higher doses of gamma rays substantially affected the growth parameters as evident from the data of morphological and biochemical parameter, which were considerably reduced with the increase in dose of gamma rays. At higher doses, some chlorophyll variants were observed like chlorine, xantha and viridis etc.

Interpretation : Gamma rays had induced sufficient genetic variability in *Artemisia*, hence, induced cytological disturbance alters genetic material which can be inherited to the subsequent generation. These genetic variability creates agronomically superior mutants which will be promising materials for plant breeders in near future.

Keywords : *Artemisia annua*, Chromosomal aberrations, Gamma rays, Microsporogenesis, Total abnormality percent

To Assess the effect of Gamma rays in Inbred seeds of *Artemisia annua* L. through cytological, morphological and biochemical aspects.

Fresh seeds of *Artemisia* were treated with Gamma rays at doses 100 Gy, 200 Gy, 300 Gy & 400 Gy respectively through a ⁶⁰Co source and were sown in triplicates

Due to exposure of Gamma radiation anomalies percentage were increased while pollen fertility was decreased due to disturbed male meiosis. The morphological parameters were decreased as the doses of Gamma radiation increased but at 100 Gy, the plant height was increased. Chlorophyll content was declined as the doses were increased

Assessment of morphological markers and analysis of cytological and biochemical aspects clearly elucidated that Gamma rays are beneficial mutagen at lower doses.



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Introduction

On the earth two types of radiations are found first one is natural, coming from sun and second one is manmade radiation which are produced from industrial activity, nuclear power production and wireless communication etc. Electromagnetic radiations include gamma rays, X-rays, visible light, and UV rays (Wi *et al.*, 2005). Gamma rays are ionizing rays which contain energetically charged particles (high energy photon), these rays are a part of electromagnetic spectrum. Ionizing radiation has been recognized as a powerful technique for plant improvement such as salinity tolerance and grain yield, especially in crop and medicinal plants (Vardhan *et al.*, 2017). Gamma sources are used to irradiate a wide range of plant materials, like seeds, whole plants, plant parts, flowers, anthers, pollen grains and single cell cultures or protoplasts (Dhumal and Bolbhat, 2012). In plant breeding, radiations have been used successfully to induce useful mutations. The lower doses mutagenic treatments can enhance the biochemical components, which are used for improved economic characters (Muthusamy *et al.*, 2003). Gamma radiation interferes with the process of cell division, resulting in cytological abnormalities and in a reduced frequency of dividing cells, which is ultimately reflected in reduced seedling growth and other morphological aberrations (Amjad and Anjum 2002). Gamma rays are strongly absorbed through the biological organism and they easily localise in the cells. Gamma radiation damages the cells either directly by hitting the critical target (DNA) or indirectly by mechanism through producing free radicals (reactive oxygen species). This indirect effect of irradiation is important in vegetative cells, the cytoplasm of which contain about 80% water (Kovács and Keresztes, 2002). Consequences of such ionizing radiations are long lasting which may be responsible for inducing point-mutations in organisms.

Mutation breeding plays an important role in increasing the genetic variability for desired traits in various crop plants and this has been proved through empirical studies. Mutagenic agents have been used to induce useful phenotypic variations in plants for more than seventy decades (Anitha *et al.*, 2005). Mutants of vegetative plants are often used directly as new cultivars, so it is important and often critical for their mutagenesis not to entail undesired mutations. Thus, it is essential to make mutation breeding a more efficient, directed and reliable technology (Okamura *et al.* 2015). There are several evidences where quality, quantity and stress related traits have been improved following induced mutagenesis. Induction of dwarfness, earliness, disease and pest resistance etc. are few such examples (Basi *et al.*, 2006). Meiotic study is inevitable to generalize the background of the mutants. These information's are necessary for designing a breeding programme and to bring the induced variation from the mutants to the subsequent generation. Hence, Cytogenetical status of mutant is necessary for any successful plant breeding program. Induced mutation is

one of the best alternatives for the improvement of plant as it can help to regenerate and restore the variability, which is generally lost in the process of adaptation to various stresses. Genetic variability is the most essential prerequisite for any successful crop improvement program as it provides spectrum of variants for effective selection, which can be achieved through hybridization, recombination, mutation and selection techniques (Dhumal and Bolbhat, 2012).

Medicinal plants are nature's gift to human beings to help them pursue a disease-free healthy life, and thus play an important role in preserving health (Abad *et al.*, 2012). *Artemisia annua*, commonly known as mugwort or sweet wormwood, is a genus of small herbs and shrubs found in northern temperate regions. It belongs to family Compositae (Asteraceae). Due to large number of species, ecological and cytological diversity coupled with economic importance, the genus *Artemisia* provides good source for specific and genetic diversified studies (Badr *et al.*, 2012). The strong and aromatic smell of the plant is due to the presence of artemisinin content, present especially in leaves and flowers. Artemisinin, a sesquiterpene lactone, has been identified as anti-malarial, and artemisinin derivatives are nowadays established as anti-malarial drugs. Patil *et al.* (2018) reported the lower doses of gamma irradiation show promising effect to achieve putative mutants with high concentrations of artemisinin. Recently Koobkokkrud *et al.* (2019) elucidated that amorpha-4,11-diene synthase (ADS), a key enzyme in artemisinin biosynthesis pathway, was positively affected by the lower doses of gamma rays. Since artemisinin content in wild *A. annua* is very low, therefore, attempts to enhance these constituents has been worked upon especially for people in developing countries where malarial disease is widely spread. Reports claiming beneficial characteristics of gamma rays for enhancing the productivity are well documented (Javed *et al.*, 2000; Khatri *et al.*, 2005) and this has intrigued to plan out the following study. Response of mutagen can be effectively assessed using meiotic studies as it provides an in-depth screening and, therefore, microsporogenesis is explored.

Materials and Methods

Plant material : *Artemisia annua* L. Variety EC- 415012 was processed from The National Bureau of Plant Genetic Resources (NBPGR), Bhowali, Nainital.

Gamma ray treatment: Four different packets of fresh seeds of *Artemisia annua* L. were prepared for irradiation and were subjected to different graded doses of gamma rays *i.e.*, 100, 200, 300 and 400 Gy at a dose rate of 7.247 K Gy hr⁻¹. Co⁶⁰ was radioactive isotope source used in gamma chamber, and this irradiation was carried out at NBRI, Lucknow. Soon after irradiation, treated seeds were sown in triplicates in complete randomized block design, along with control set.

Morphological analysis: Different morphological parameters were analyzed for the effect of gamma rays on the plant morphology: germination percentage, The data was recorded after 7th day from sowing; ii. Survivability Percentage: The data was calculated on 14th day from the day of seedling emergence.

Plant height and internodal length: Plant height was measured from the tip of the shoot with a scale and internodal length was measured when plant height attained its maximum length.

Meiotic preparation: Study of microsporogenesis: For detailed microsporogenesis study, young capitula of *Artemisia annua* were fixed in Carnoy's fixative (3:1 absolute alcohol: Glacial acetic acid v/v) for 24 hrs and then preserved in 70% alcohol for meiotic study. Anthers were smeared in 2% acetocarmine stain followed by squash preparation. Slides were observed under the microscope and pollen fertility was studied using acetocarmine glycerine stainability test. Photomicrograph of chromosome were captured with Olympus PCTV Vision software. Following formulae were used for calculating abnormality percentage and pollen fertility –

$$\text{Total abnormality percentage (TAB) \%} = \frac{\text{Number of Abnormal Pollen mother cell (PMC)}}{\text{Total number of Pollen mother cell observed}} \times 100$$

$$\text{Pollen fertility (\%)} = \frac{\text{Number of fertile pollens}}{\text{Total number of pollens}} \times 100$$

Biochemical analysis: Photosynthetic pigments (Chl a, Chl b and Carotenoid content) were estimated by the method of Lichtenthaler and Welburn (1983). A 20 mg of leaf was homogenised in 80% acetone to prepare leaf extract and optical density of supernatant was read at three different wavelength (470nm, 646nm and 663nm).

Statistical analysis: All the experiments were carried out in three replicates. Statistical analysis was performed using SPSS 16.0 software. One way ANOVA and Duncan's Multiple Range Test ($P < 0.05$) was conducted for mean separation and the graph was plotted by sigma plot 10.0 software.

Results and Discussion

Effect of Gamma radiation on morphological parameters

Germination and survival percentage: Seed germination is an important parameter to estimate the effect of mutagen on plants. The inhibitory dose is one of the important factors to predict radio sensitivity level in plants (Kumar *et al.*, 2013). It quantifies a dose where 50% of seeds can survive, germinate and expose to their normal growth. In this study, gamma irradiated seeds showed a reduction germination in comparison to control (Fig. 2A). The

maximum germination percent was recorded in control (96.5), which reduced from 92.3 (100Gy) to 50.1 (400Gy) respectively. In case of survival percentage, the mean value varied in accordance with the doses of gamma radiation (Fig. 2B). At 400Gy, the lowest survivability percentage was observed 40.2 which suggests that the LD₅₀ of gamma rays that causes 50% survival reduction was 300 Gy. Further, the doses lower than LD₅₀ value showed normal morphological characters in survived plants. The decrease in the percentage of seed germination may be ascribed to the chromosomal aberrations, disturbance in DNA and auxin synthesis and to impaired cell metabolism (Kirtane and Dhupal, 2004). According to Datta (2009), the inhibition of seed germination at high doses could be due to the damaged tissues, chromosomes and subsequent mitotic retardation, and the severity of damage depend on the doses used. Kumar and Dwivedi (2015) observed germination percentages of seeds of three varieties (AA-1, AA-2, and GA-1) of *Trachyspermum ammi* (L.) Sprague (ajwain) that were gamma-irradiated by Co⁶⁰ source significantly decreased along with increasing doses of gamma rays. Reduced survival rate at higher mutagenic level has been attributed to various factors, such as chromosomal damage leading to meiotic arrest (Khursheed *et al.*, 2008) also at metabolic levels. Higher doses of gamma rays disrupts chloroplast membrane and metabolism, due to which photosynthesis is affected which ultimately reduces survivability and causes death of the plant.

Plant height and internodal length: Gamma rays caused significant decrease in plant height (Fig. 2C). However, at 100Gy the plant height was 115 which was more than the control (95.6). At 400 Gy, plant height was observed 52.3. However, the number of internodal length decreased in a dose - dependent manner. The internodal length was 7.2 cm in control and decreased to 3.8 cm in 400Gy (Fig. 2D). Gamma radiation had inhibitory effects on physiological and physical traits (Khan and Goyal 2009). Higher dosage of Gamma radiation, the more evident the expression of morphological changes is observed in the plants. Therefore, low level of radiation produced positive effects such as increase in plant height. Low levels of gamma rays induce growth stimulation signals by increasing the antioxidative ability of cells or by changing the hormonal signaling in plants (Ali *et al.*, 2016). Plants irradiated with high dose of gamma rays disturbs the hormone balance, leaf gas exchange, water exchange and enzyme activity (Kiong *et al.*, 2008), these might be the probable reason for reduction in intermodal length due to insufficient supply of water and minerals to the plants, further the growth rate is also inhibited with increase in radiation dose due to mutations in DNA that synthesize DNA at the interphase stage leading to bud disruption and interruption in cell differentiation (Ali *et al.*, 2016).

Influence of gamma radiation on chromosomal behavior: *Artemisia annua* L. exhibits chromosome complement of 2n=18. The control plants showed normal pattern of meiosis with respect

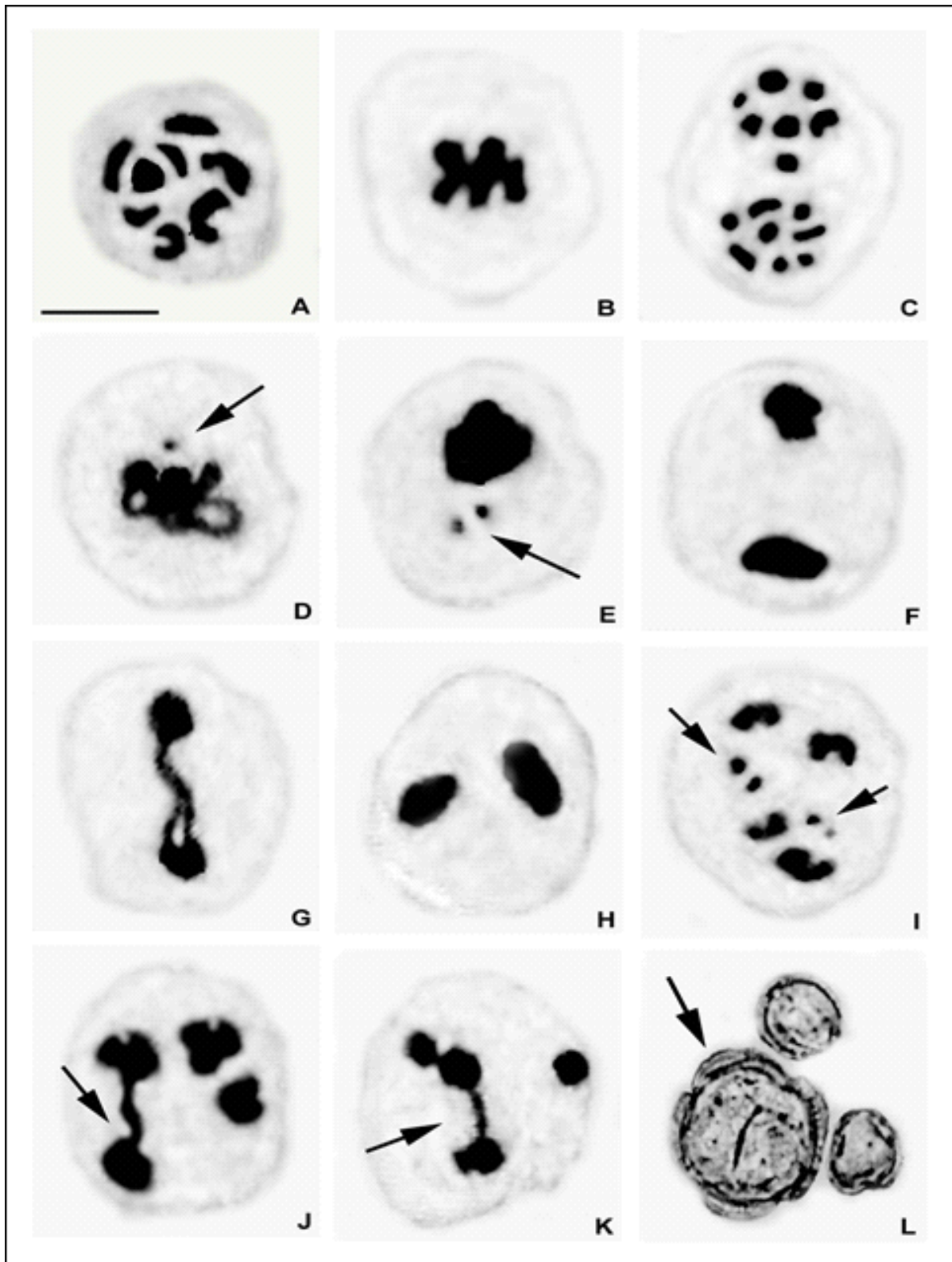


Fig. 1 : Meiotic stages of *Artemisia annua* L. -A. Diakinesis, B. Normal Metaphase I, C. Normal Anaphase I, D. One precocious chromosome at Metaphase I, E. Two precocious chromosomes at Sticky Metaphase I, F. Stickiness at Anaphase I, G. Bridge formation at Anaphase I, H. Stickiness at Metaphase II, I. Four laggards chromosomes at Anaphase II, J. bridge formation at Anaphase II, K. Disturbed polarity with bridge formation at Anaphase II, L. Fertile pollens (Scale bar-10.17 μ m).

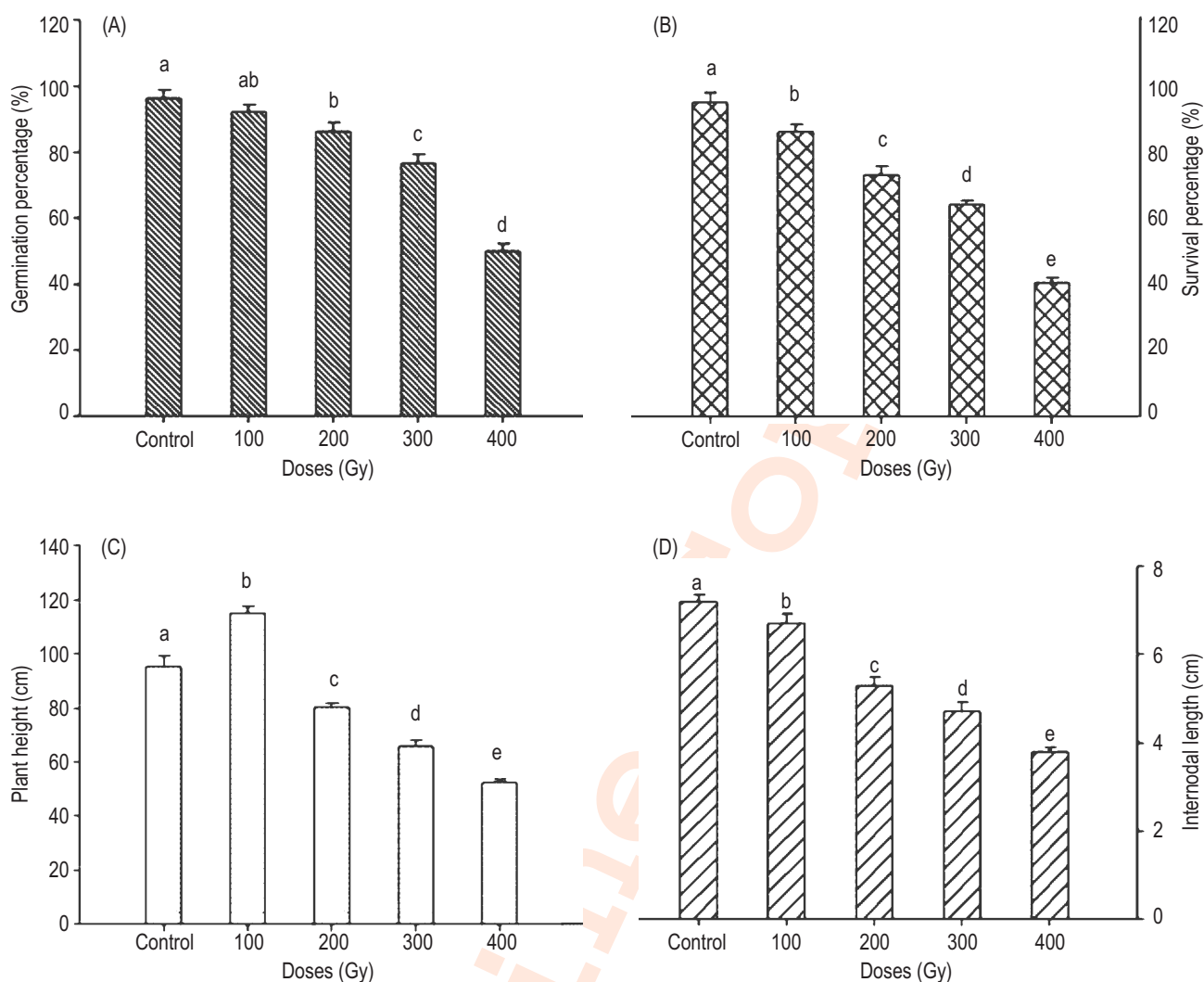


Fig. 2 : Influence of gamma radiation on morphological parameters (A) Germination percentage; (B) Survival percentage; (C) Plant height and (D) Internodal length.

to control, 9 bivalents arranged at metaphase I (Fig. 1B) and 9:9 separation at anaphase I (Fig. 1C). Exposure to gamma radiation altered the behavior of chromosomes and abnormal divisions pollen mother cells.

Effect of gamma rays on chromosomal abnormalities and pollen fertility at various doses is given in Table 1. Abnormality percentage increased with increasing doses of gamma irradiation. Different microscopic views of meiotic cells were analyzed from each treatment for scoring the chromosomal aberrations at metaphase and anaphase stages. The abnormality percent increased from 6.24 at 100Gy to 25.38 at 400Gy. The radiation can induce chromosomal breakages not only by direct hits but also indirectly by the reaction chemical products produced in water surrounding the chromosomes (Basi *et al.*, 2006). Yamaguchi *et al.* (2008) reported that nuclear DNA content

of *Chrysanthemums* decreased with increasing dose and dose rate of gamma rays, indicating that the nuclear DNA content could be used as an index of radiation damage.

Various chromosomal anomalies like scattering, stickiness, precocious movement of chromosome, unorientation, bridges, laggard and disturbed polarity etc. were detected in both metaphasic and anaphasic stages in treated plants (Fig. 1). Mitoinhibitory effects of gamma radiation at higher doses has been reported earlier in *Hordeum vulgare* (Eroglu *et al.*, 2007), *Allium cepa* (Ahirwar, 2015) and *Triticum aestivum* (Borzouei *et al.*, 2010). Stickiness (Fig. 1E, F, H) arises due to depolymerization of nucleic acid caused by mutagenic treatment (Jabee *et al.*, 2008). Loop formation (Fig. 1D) occurs due to loss of histone proteins or abnormal function of spindle, which fails to attach to kinetochore leading to improper cell division (Kumar and Bhardwaj, 2019).

Precocious movement (Fig. 1D, E) of chromosome at metaphase is due to migration of chromosomes to the poles, which can result into early chiasma terminalization in diakinesis or metaphase I (Srivastava and Kapoor, 2008) or due to disruption of spindle formation (Kumar and Dwivedi, 2015). Laggard formation (Fig. 1I) is due to delayed terminalisation, chromosomal stickiness or failure of chromosomal movement (Reddy and Munirajappa, 2012). Jackson (1988) suggested that some of the bridges (Fig. 1G, J) may occur due to a crossover between a paracentric inversion heterozygote loop and centromere and another in the loop of same bivalent. Disturbed polarity (Fig. 1K) or tripolarity might be due to spindle disfunctioning (Kumar and Dwivedi, 2012). Perhaps, chromosomal aberrations might due to interaction of ionizing particles with the protoplasm, mediated through excitation introduced by radiation that ultimately increases aberration frequency (Shukla nee Tripathi and Kumar, 2011). According to Esnault *et al.* (2010), single stand breaks (SSBs) are most abundant DNA lesions that arise from oxidative attack, while double strand breaks (DSBs) are induced by ionizing radiations, however, most DSBs repair can be error prone, which is potentially detrimental for the cell. Both of these SSBs and DSBs can be repaired by cell cycle arrest mechanism.

Pollen fertility: Pollen fertility in control plants was 94.41 whereas in treated plants it decreased in a dose dependent manner. The ionizing radiation disturbed male meiosis and its impact on viability of pollen grains. Induction of more and more chromosomal abnormalities greatly affected microsporogenesis by forming nonviable gametes, which considerably reduced pollen fertility (Kumar and Rai, 2007).

Effect of gamma rays on photosynthetic pigments: Chlorophyll *a* and chlorophyll *b* are directly associated with photosynthesis and represents mostly half of the total quantity of green and yellow pigment. Carotenoids are essential constituents of Chl-binding proteins, and also protect the plants against photoinhibitors. The decrease in photosynthetic pigments clearly reflects the mutagenic effect of gamma rays on *Artemisia*. A dose dependent decrease in photosynthetic pigments was registered in all treated sets (Fig. 3). For gamma irradiation higher frequency of chlorophyll mutation with moderate doses of mutagens was observed (Fig. 4). Chlorophyll mutations are considered as the most dependable indices for evaluating the efficiency of different mutagens in inducing genetic variability for crop improvement and are also used as genetic markers in basic and applied research (Kolar *et al.*, 2011). The green pigment in leaves differed among different chlorophyll mutants as observed at lower doses, ranging from yellow viridis (Fig. 4B), semi-chlorina (Fig. 4C) to semi-xantha (Fig. 4D) type of mutants. Among the mutants recorded, semi-chlorina type was more predominant followed by yellow viridis and semi-xantha. According to Saha and Paul (2019) chlorophyll mutations show independent responses to different doses of gamma rays as they occur random gamma irradiation breaks the bond which initiates chemical reaction and

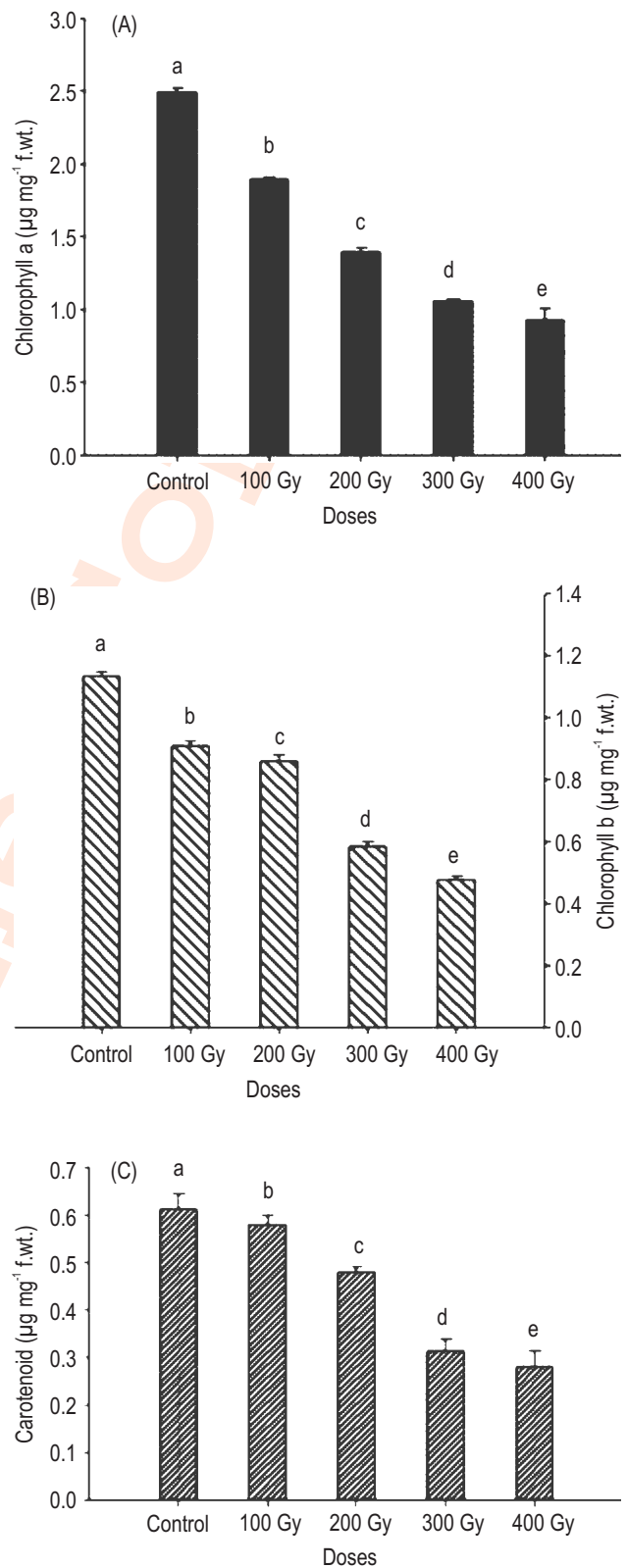


Fig. 3 : Effect of gamma radiation on the pigment content of *Artemisia annua* L.: (A) Chlorophyll *a*; (B) Chlorophyll *b* and (C) Carotenoid.



Fig. 4 : Different chlorophyll variants in *Artemisia annua*: (A) Control; (B) Yellow viridis; (C) Semi-chlorina and (D) Semi-xantha.

Table 1: Metaphasic and anaphasic abnormalities induced through gamma rays in *Artemisia annua*

Doses (Gy)	No. of PMC's observed	Metaphasic Abnormalities (%)				Anaphasic Abnormalities (%)						Oth.* (%)	TAB* (%)	Pollen fertility (%)	
		Sc*	Pm*	St*	Un*	Br*	Lg*	Un*	St*	Asy*	Dp*				
Control	350	-	-	-	-	-	-	-	-	-	-	-	-	-	94.41 ±1.46 ^a
100	310	0.53 ±0.10	0.54 ±0.11	1.08 ±0.09	0.75 ±0.19	-	-	0.54 ±0.11	1.09 ±0.03	1.09 ±0.31	0.74 ±0.26	0.64 ±0.18	6.24 ±0.07	92.37 ±1.85 ^a	
200	290	1.16 ±0.16	0.92 ±0.08	1.73 ±0.18	1.38 ±0.22	-	-	1.39 ±0.06	1.50 ±0.45	1.50 ±0.13	1.04 ±0.04	1.37 ±0.36	11.25 ±0.08	87.19 ±1.87 ^b	
300	291	2.07 ±0.72	1.72 ±0.19	3.45 ±0.40	1.26 ±0.03	1.47 ±0.48	2.27 ±0.46	1.62 ±0.45	1.49 ±0.29	1.49 ±0.30	1.26 ±0.39	1.15 ±0.57	19.59 ±0.70	70.25 ±1.59 ^c	
400	293	2.29 ±0.15	1.82 ±0.17	3.71 ±0.45	2.19 ±0.45	2.87 ±0.34	2.44 ±0.49	1.61 ±0.17	2.98 ±0.11	2.98 ±0.17	2.25 ±0.57	1.03 ±0.51	25.38 ±0.18	58.36 ±1.05 ^d	

Sc*- Scattering; Pm*- Precocious movement; St*- Stickiness; Br*- Bridge; Lg*- Laggard; Un*- Unorientation; Asy*- Asynchronous; Dp*- Disturbed polarity; Oth*- Others; TAB* – Total abnormalities

changes cellular structure and metabolism of plants like: dilation of thylakoid membranes, alteration in photosynthesis, modulation of antioxidative system and accumulation of phenolic compounds (Kim *et al.*, 2004). Radiations alter photosynthetic apparatus by damaging the photosystems complexes, but at lower doses of gamma rays these complexes allow photosynthesis by capturing light energy, protect photo-oxidative damage of chlorophyll from ROS, and release excess energy as heat (Kovacs and Keresztes, 2002; Kim *et al.*, 2004).

Rapid and harsh environment increased stresses in plants thus by producing radiation-protected mutant plants through gamma radiation will be helpful in treating infirmity engender by the production of free radicals. So assessment of morphological markers and analysis of cytological and biochemical aspects clearly elucidated that gamma rays are beneficial mutagen at lower doses.

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