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RESEARCH ARTICLE

COMPARATIVE STUDY ON THE EFFECT OF EMS TREATMENT ON PRODUCTION OF SECONDARY METABOLITES IN *ANDROGRAPHIS PANICULATA* (BURM. F.) NEES

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SUMMARY Effect of 0.05% of EMS on secondary metabolite production in *Andrographis paniculata* was conducted. Calli were produced from leaf explants inoculated on Murashige and Skoog's medium supplemented with 2 mg/l NAA, 1 mg/l kinetin and 50 mg/l phenylalanine. 0.05% EMS treatment for 1 h resulted in a maximum amount of fresh weight (2.0933 g) and dry weight (0.1933 g) of callus and also the callus response (96.667). Treatment with 0.05% EMS also showed good response in secondary metabolite production when compared to control. The study also showed that concentration and treatment time of EMS influenced the secondary metabolites production in the treated samples.

Keywords: *Andrographis paniculata,* andrographolide, callus induction frequency, EMS, phenylalanine.

INTRODUCTION

Andrographis paniculata of the family Acanthaceae is one of the most popular medicinal plants widely distributed in India, China and other Southeast Asian countries. It is commonly used for the treatment of common cold, diarrhoea, fever, respiratory tract infections (Negi et al. 2008, Sareer et al. 2014, Wang et al. 2014) and possess numerous therapeutic potentials including antimalarial (Mishra et al. 2011), antioxidant (Lin et al. 2009), antibacterial (Burm et al. 2010) and anticancer activities (Subramanian et al. 2012).This herb has many vernacular names –Kalmegh in Bengali, Kiriyath in Malayalam, Nilavembu in Telugu and is commonly known as bhui-neem, because of its bitter taste as that of neem. This plant has medicinally important compounds such as diterpenoids, flavonoids and polyphenols (Chao & Lin 2010).

Irradiation and chemical mutagenesis have long been used to develop mutant plants for breeding purpose (Oehlkers 1943, Stadler 1928). The use of chemical mutagens is a very popular way to induce mutation which include ethyl methanesulphonate (EMS), colchicine, polyethylene glycol, sodium azide, 2,4- dichlorophenoxy acetic acid and acridine orange etc. EMS has become one of the best effective, reliable, powerful and frequently used chemical mutagens in plants (Brockman et al. 1984). The present study deals with the effect of EMS on callus initiation and isolation and quantifiction of the secondary metabolite content present in in vitro callus cultures.

MATERIAL AND METHODS

Healthy growing young branches with 4 or 5 nodes were collected from S. D. College, Alappuzha. Shoots were brought to the laboratory

by wrapping with a wet muslin cloth. A voucher specimen has been deposited in Kerala University Botanical Herbarium (KUBH 6031).

One wk old seedlings were used for the study. The cotton swab method adopted was that of Biswas & Bhattacharya (1971). A cotton swab dipped in 0.05% EMS solution for 1 h and 3 h was applied to the apical vegetative bud and EMS solutions were frequently added to the cotton swab by a dropper. The third leaf was taken from healthy seedlings swabbed with 70% alcohol-soaked cotton and were washed in running tap water for 20 min followed by washing with 2 drops of labolene for 5 min. After washing with distilled water they were brought in to laminar air flow. The explants were treated with 70% ethyl alcohol for 30 sec for surface sterilization and rinsed in sterile double distilled water. 0.1% mercuric chloride with different time durations were used and finally standardized the optimum concentration for sterilization. 0.1% mercuric chloride treatment for 6 min was found to be the optimum treatment time for surface sterilization.

The various concentrations of auxins alone (0.5,1and 2 mg/l NAA, 0.5,1 and 2 mg/l 2, 4-D) as well as the combined effect of auxin and cytokinin NAA (0.5,1 and 2 mg/l) and kinetin (1mg/l) were tried in this experiment. Proliferated calli from the control and treated samples were grown on the MS medium containing 2 mg/l NAA, 2 mg/l kinetin and 50 mg/l phenylalanine were used for the analysis of andrographolide after 70 d by HPLC method.

Procedure of HPLC

Chromatographic conditions:

Mobile phase:

1) Dissolve 0.14 g of anhydrous potassium dihydrogen orthophosphate in 900 ml of HPLC grade water and add 0.5 ml of orthophosphoric acid. Make up to 1000 ml with water, filter through 0.45 membrane and Degas in a sonicator for 3 min.(Solvent A)

2) Acetonitrile (Solvent B)

Standard preparation:

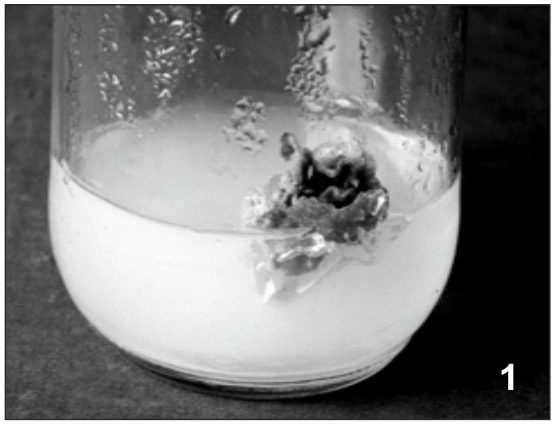
20.0 mg andrographolide was weighed to a 100 ml volumetric flask. 50 ml of HPLC grade methanol was added. Sonicated for 5–10 min and warmed on a water bath at 60–70º C for 5 min. Cooled to room temperature and volume was made up to 100 ml with methanol.

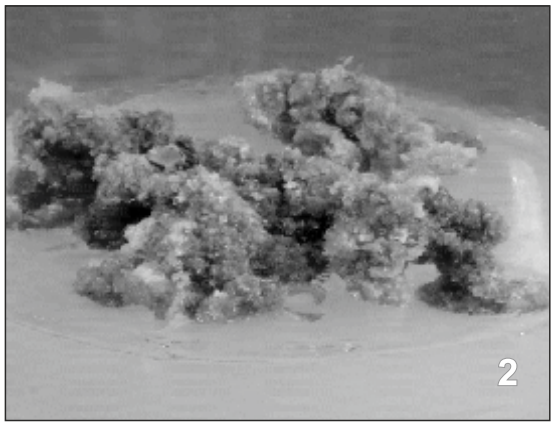
Sample preparation :

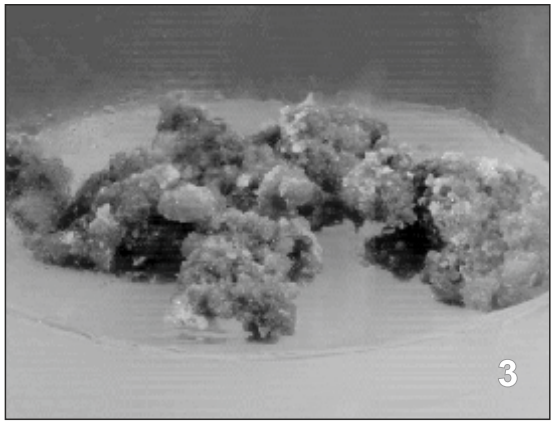
1000 mg of given material was weighed in clean, dried 250 ml beaker, 50 ml of methanol was added into a 250 ml beaker and refluxed for 10 min, cool and sonicate for 6 min. Cool and transfer to 50 ml volumetric flask, repeat the above step twice and the volume was made up to 50 ml with methanol.

OBSERVATIONS

Explants inoculated into the MS full strength medium supplemented with 2 mg/l 2, 4-D showed the high amount of fresh weight (0.5900 ± 0.011g) of the callus. But the effect of NAA at the concentration of 0.5 mg/l produced low percentage response of callus (40%) fresh weight (0.36 g) and dry weight (0.026 ± 0.0033 g) of callus. The combinations of NAA and kinetin at 2 mg/l and 1 mg/l combinations showed the maximum fresh weight (0.59 ± 0.00 g) and dry weight (0.036 ± 0.0033 g) of the callus. Different concentrations of phenylalanine (25, 50, 75 and 100 mg/l) were used here. Full strength MS medium supplemented with 2mg/l NAA,1mg/l kinetin and 50 mg/l phenylalanine showed good result in callus fresh weight (0.6103 ± 0.02 g) and largest amount of dry weight (0.0533 ± 0.0033 g) formed in this concentration. Explants treated with 0.05% EMS for 3 h produced calli in MS medium fortified with 2 mg/l NAA, 1 mg/l kinetin and 50 mg/l phenylalanine. 0.05% EMS treatment produced a higher amount of fresh







Figs 1–3: *A. paniculata.* 1. Callus in control. 2. Callus in treatment with 0.05% of EMS for 1 h. 3. Callus in treatment with 0.05% of EMS for 3 h.

weight, dry weight and percentage of the response of the callus induction than that of control. Control calli produced high amount of fresh weight (0.61 g) and dry weight (0.04 g) in MS medium containing 2 mg/l NAA, 1 mg/l kinetin and 50 mg/l phenylalanine. The maximum amount of fresh weight (2.093 g) and dry weight (0.19 g) of callus and callus response (96.6%) were found in 0.05% EMS treatment for 1 h. In this concentration callus initiation started within 11–15 d of inoculation. The sample treated with 0.05% EMS for 3 h showed an increase in fresh weight (2.04 g), dry weight (0.17 g) and callus response (96.6%) as compared to control plant. (Figs 1–3).

The calli proliferated in a significant manner and the production of andrographolide was analyzed after 70 d. Control calli produced 0.1mg/g andrographolide. The andrographolide production was 0.2 mg/g in the calli originated from the explant treated with 0.05% EMS for 1 h. Treatment of 0.05% EMS for 1 h and 3 h positively influenced the production of andrographolide. The culturing of the calli in this concentration produced increased amount of secondary metabolites as compared to the control calli. 0.05% EMS had a significant effect on andrographolide production in callus.

By HPLC analysis, control calli contain the same amount of andrographolide (0.01% w/w), neoandrographolide (0.01% w/w), 14-deoxy 11-12, didehydro andrographolide (0.01% w/w) and andrographonin (0.002% w/w). The sample treated with 0.05% EMS for 1 h showed a maximum amount of andrographolide (0.02% w/w) and andrographonin (0.004% w/w) (Figs 4–7).

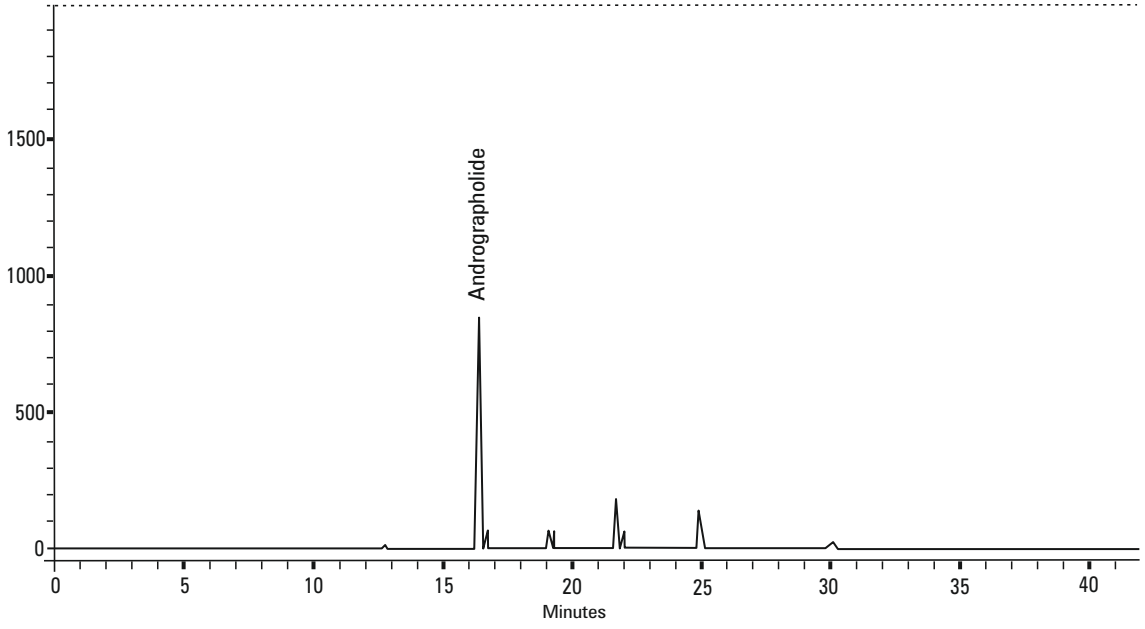


Fig. 4: HPLC chromatograms of andrographolide. Standard.

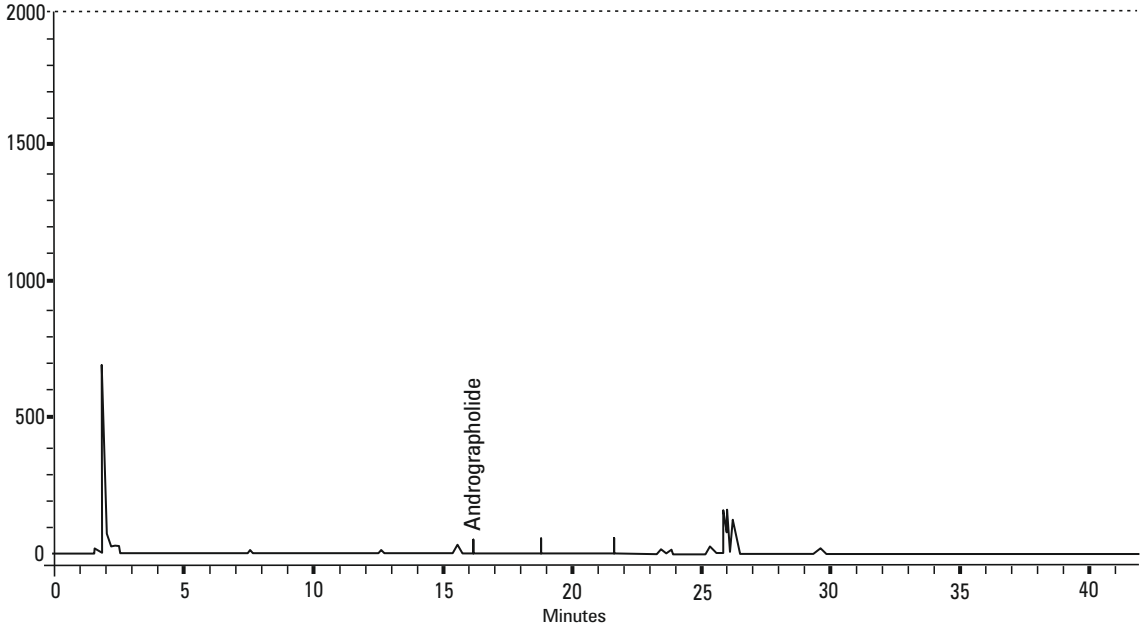


Fig. 5: HPLC chromatograms of andrographolide. Control.

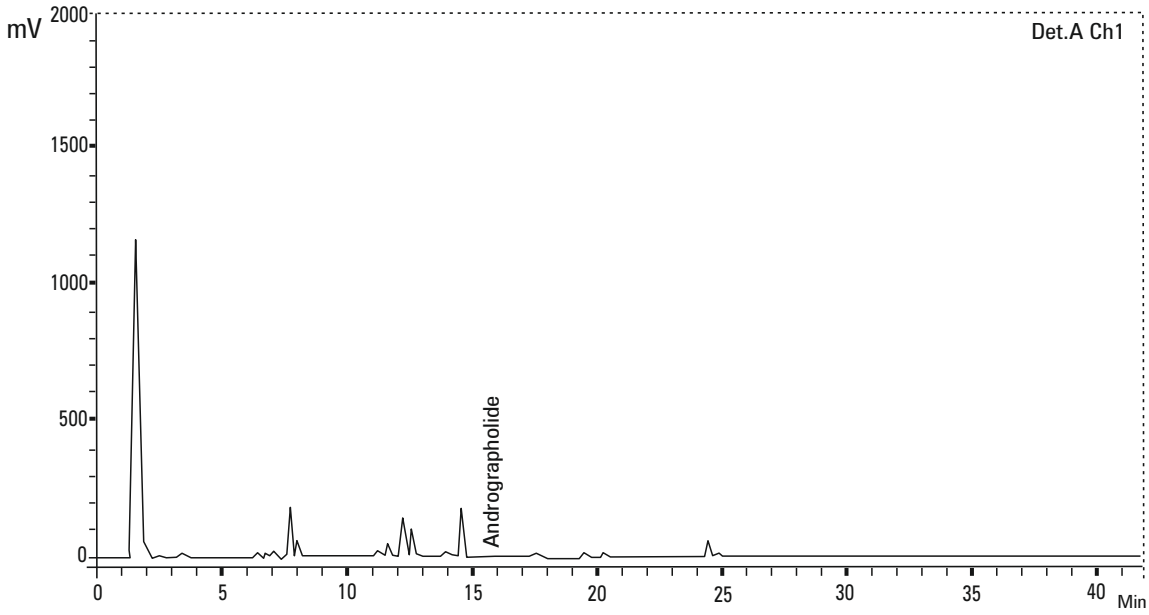


Fig. 6: HPLC chromatograms of andrographolide. 0.05% EMS for 1 h.

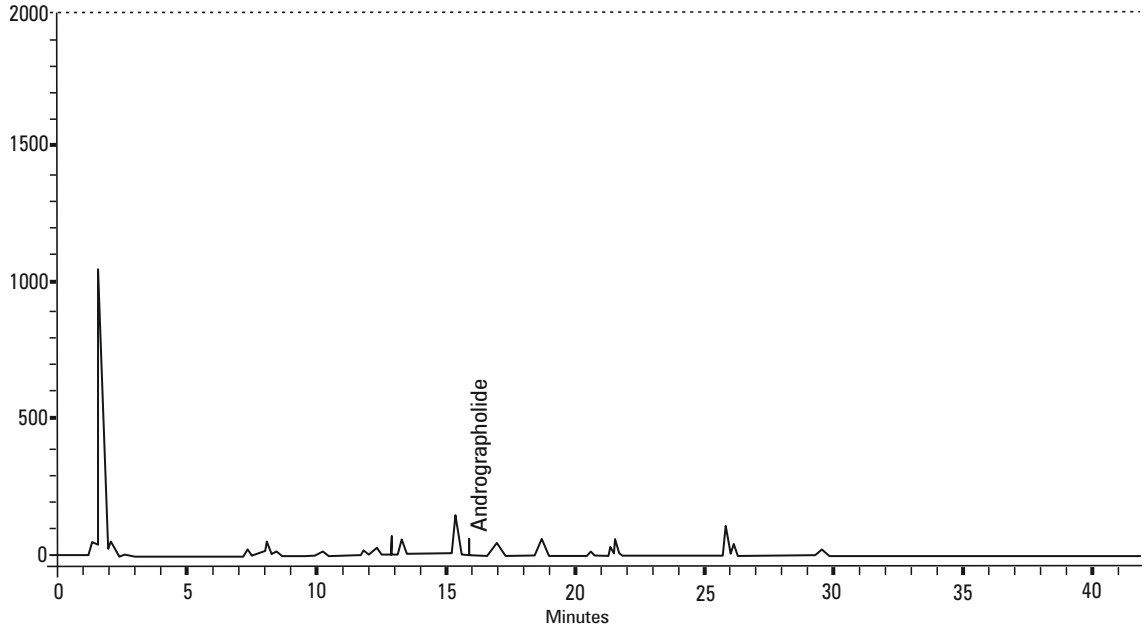


Fig. 7: HPLC chromatograms of andrographolide. 0.05% EMS for 3 h.

DISCUSSION

In this study, among different media used, full MS medium produced better results. Maximum callus initiation was found in full MS medium fortified with 2 mg/l NAA, 1 mg/l kinetin and 50 mg/l phenylalanine. MS medium was the best suitable medium as compared to other culture media viz., B_5 and Nitsch's media (Kataky & Handique 2010). In the present study, maximum andrographolide was produced in the treated samples of 0.05% EMS and a 2-fold increase in andrographolide production than the control. In rice cultivars, a decreased callus induction percentages were observed in treatments with gamma rays, EMS and sodium azide (Cheng Xiongying et al. 1987). But, up to 3-fold increase in callus growth rate was recorded after treatments with these 3 mutagenic agents.

The effect of EMS treatment on fresh weight, dry weight, callus induction frequency and secondary metabolite production on in vitro callus were recorded in this study. Lower concentration of EMS (0.05%) for 1 h was more effective in the secondary metabolite production than the lower concentration of EMS for 3 h and the control.

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RESEARCH ARTICLE

CYTOTOXIC EFFECT OF PHYSICAL AND CHEMICAL MUTAGENS IN ROOT TIP CELLS IN *RIVINIA HUMILIS* L.

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SUMMARY *Rivinia humilis* has been known to be a source of natural dye. In order to induce genetic variability, the germplasm of the plant was subjected to the mutagenic treatment of physical (gamma irradiation) and chemical mutagens (sodium azide and ethyl methanesulphonate), with the objective of improving the dye quantity and quality obtained from ripened fruits of the plant. Mutagens, basically, affect the genetic make-up of the organisms, which are reflected in modifications of various traits. Cytological analysis revealed the sensitivity of the genome to the treatments of all the three mutagens employed. It exhibited mitodepressive effects as well as affected the chromosomal entity in the form of various clastogenic and nonclastogenic changes. The frequencies of mitotic aberrant cells as well as mitotic chromosomal aberrations, in the treated root tip cells, were found to be enhanced with the increase in dose/concentrations of all the mutagens. Gamma irradiation was found to be more effective, in disturbing mitotic cell activities, and mostly inducing clastogenic chromosomal aberrations, than both the chemical mutagens. The mitotic aberrant cells were reported to contain various clastogenic and nonclastogenic chromosomal aberrations such as fragments, laggards, bridges, precocious movements, micronuclei formation at telophases, multipolar anaphases and telophases, stickiness and clumping of chromosomes with varying frequencies. Desynchronized metaphases, disoriented metaphases, anaphases and telophases along with persistent nucleolus at anaphase were also recorded.

Keywords: *Rivinia humilis*, mutagens, cytotoxic, mitodepressive, clastogenic, sensitivity, nonclastogenic.

INTRODUCTION

Rivinia humilis is a natural dye yielding plant belonging to the pokewood family Phytolaccaceae. The plant is native of tropical America and has been listed as a notorious weed in different countries around the world. Matthew (1983) mentioned the plant as an occasional weed in Sri Lanka, India and Malaysia. The plant was deliberately brought to India from Florida, strictly for the ornamental purpose and is mostly grown in gardens and greenhouses (Naik 1998). The plant is perennial and bears green coloured unriped berries. The latter turn red when ripe and yield natural red dye. The dye contains reddish pigment known as rivianin or rivinianin. It has sulphate group attached, and is very much similar to betanin, the pigment found in beet root. It contains reddish-violet betacyanin derivative,

confirmed as betanin 3'-sulphate (Imperato 1975) and orange yellow betaxanthin derivative named humilaxanthin (Strack et al. 1987). The plant has been reported to possess many medicinal properties too. Natural products obtained from different parts of *R. humilis* are traditionally used in Jamaica as antidote to poisoning, headache, cold, diarrhoea, marasmus and inflammation (Mitchell & Ahmad 2006). Salvat et al. (2001) reported the inhibitory effects of methonolic extracts of the branches of this plant against *Escherichia coli, Salmonella typhimurium, Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus* and *Enterococcus faecium*, whereas Fathima & Tilton (2012) confirmed the radical scavenging activities of leaf extracts in methanol, and suggested its potent antioxidant activity. Khan et al. (2013) evaluated the effect of berry extract of *R. humilis* on physicochemical properties and acceptability of the product, and observed the retention of 68% of the colour in *Rivinia* banana spread after 6 months of storage at 5° C, without the alteration of product quality. Joseph & Avita (2013) carried out the studies on antimicrobial activities of root and shoot against 10 bacterial and 4 fungal strains and reported the inhibitory effect against all the strains of bacteria.

Mutation breeding is the most reliable tool to modify the genetic architecture of the organism for obtaining the desirable characters. Mutations are the tools and being used to study the nature and function of genes which are the building blocks and basis of plant growth and development, thereby producing raw materials for genetic improvement of economic crops (Adamu & Aliyu 2007). The mutation, in a true sense, leads to loss or gain of function of a gene and that can be handed over to the next generation, if not auto-corrected and when passed through the germ line. These induced mutations led to the changes in genetic architecture of plants which are reflected at physiological, morphological and biochemical levels, in the first and subsequent generations (Aney 2013a, b). Mutation breeding proved to be an important tool in introducing different desirable characters of agronomic value in various plants, mostly the crop plants.

Cytological analysis with respect to mitotic and meiotic behaviour of the chromosomes is the most reliable tool to evaluate the effect and potency of various mutagens. It also provides a clue to assess sensitivity of genotypes of various plants for different mutagens including both physical and chemical mutagens. Irradiation of the seeds with physical mutagens, particularly gamma irradiation, induces gross structural changes in the chromosomes and have inhibitory effect on most of the morphological and yield attributing characters. The mutagenicity of different chemical mutagens such as sodium azide (SA) and ethyl methanesulphonate (EMS) to disturb the structural entity of chromosomes, in many plants, is also well established. The chemical mutagens, generally induce point or gene mutations, leading to the base pair substitutions and thus changing the functions of proteins without abolishing them. The mutagenic effects occurred at the chromosomal level leading to the change in genetic architecture of the plant might result in variations at morphological and physiological levels (Aney & Choudhary 2019). It helps to identify and isolate the desirable mutants for morphological, physiological and various yield attributing characters. Hence, the cytological analysis with respect to chromosomal

aberrations, either in mitosis or meiosis, is regarded as one of the most dependable criteria for estimating the effect of mutagens. Although *R. humilis* is deliberately introduced as non-native naturalized plant in India it is found best suited to Indian agronomic climate. We identified *R. humilis* as a reliable source of red natural dye that can provide an opportunity to be an alternative source of natural red dyes which are quite difficult to obtain from the underground parts of the other existing natural dye yielding plants. The present paper deals with evaluation of sensitivity and effectiveness of employed mutagens in root tip cells of *R. humilis* in order to induce the genetic variability for isolation and identification of viable high yielding mutants to exploit the plant as an alternative source of natural dye to be used in textile, cosmetics, leather, food and pharmaceutical industries.

MATERIAL AND METHODS

The seeds of *R. humilis* were procured from 5 different localities viz., Research field, Department of Botany, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur; Paradise Nursery, Nagpur, Giripeth and Shantivihar area, Nagpur and from Pauni. Healthy and uniform-sized seeds were selected and exposed to gamma rays with 50, 75, 100, 125, 150 and 200 Gy doses. Three different treatment modes viz., dry seed (DS), presoaking in water for 3 h (PSW-3H) and 6 h (PSW-6H), were used for both the chemical mutagens, SA and EMS . The seeds were treated with both the chemical mutagens for 18 h with 0.0075, 0.010, 0.020% of freshly prepared SA and 0.5, 1.0 and 1.5% of EMS. The treatment was terminated by decanting the mutagen solutions, and the treated seeds were thoroughly washed several times with distilled water to remove the traces of mutagens. Twenty seeds from each dose/concentration, along with untreated (control), were kept for germination on germination paper slots. The root tips of 1 to 1.5 cm were cut and fixed between 8 and 10 a.m. in freshly prepared Carnoy's fluid (3:1 absolute alcohol: glacial acetic acid) for 24 h, and finally preserved in 70% ethanol.

The squashes of the root tips were prepared for mitotic studies by first thoroughly washing with distilled water, and then hydrolyzing in 1N HCl for 15 min at 60^0C**.** The hydrolyzed root tips were washed twice in distilled water and kept for mordanting in 4% freshly prepared ferrous ammonium sulphate (iron alum) solution for 5 min. The traces of mordant from the root surface were removed by washing thrice with distilled water. Subsequently, the root tips were stained in 1% haematoxylin for 5–7 min and squashed in 45% acetic acid. The data on mitotic index and mitotic chromosomal abnormalities were recorded and photographed and were made permanent using different grades of butanol: acetic acid (Darlington & La Cour 1976). Mitotic indices (MI) and percentage abnormality were calculated as per the following formulae (Jabee et al. 2008, Aney et al. 2012):

$$\text{Mitotic index (MI)} = \frac{\text{Total number of cells in division}}{\text{Total number of cells scored}} \times 100$$

$$\text{\% Abnormality} = \frac{\text{Total number of aberrant cells in the root tips of treated seeds}}{\text{Total number of cells in division}} \times 100$$

OBSERVATIONS

Effect of mutagens on mitotic activities

The mutagens used in the present investigation have been proved to be effective on the mitotic cell divisions in different plants. The number of dividing cells reported to be decreased with the increase in dose/concentration of gamma rays and chemical mutagen (Tables 1–3). As evidenced from the values of MI in terms of number of dividing cells (Table 1) the mitotic chromosomal abnormalities induced in the root tip cells of the seedling of the gamma irradiated seeds were found to be significantly affected (Figs 1, 2). The lower doses (50 and 75 Gy) had slightly stimulatory effect on the mitotic process, while the same was found adversely affected at higher doses (150 and 200 Gy), where it was almost reduced to half as compared to the control (Table 1). Both the chemical mutagens, in all treatment modes, exhibited mitodepressive effect in concentration dependent manner, however, higher concentrations of both the chemical mutagens have severely affected the mitotic process than the lower concentrations (Tables 2, 3). The data on mitotic indices shown in Tables 2 and 3 clearly revealed that effectiveness of both the chemical mutagens has not only enhanced by the presoaking of seeds but also increased with the presoaking duration. Frequency of mitotic aberrant cells as well as chromosomal aberrations was colinearly increased with the increment in doses/concentration of all the 3 mutagens (Tables 1–3, Figs 1–6). Among all the mutagens, exposure of seeds to gamma rays was proved to be more effective than that of chemical mutagens, however, the treatment of seeds with SA was reported to have more adverse effect on mitotic process than the treatment with EMS (Tables 1–3).

TABLE 1: Effect of different doses of gamma rays on mitotic abnormalities in root tip cells of *R. humilis*.

Gamma rays (Gy)	No. of cells scored	No. of cells in division	MI (%)	Frequency (%)											
				Met	Ana + Tel	Br	Fr	Lg	MN	Sc	Cl	PM	MNU	PN	Abn (%)
Control	**2959**	**743**	**25.13 ± 0.51**	**10.57**	**15.66**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
50	2993	785	26.22 ± 0.64	11.04	16.62	0.15	0.46	0.45	0.77	0.30	0.15	-	-	-	2.30 ± 0.24
75	3002	820	27.33 ± 0.56	11.34	17.35	0.28	0.56	0.27	0.85	0.70	0.42	0.14	-	-	3.40 ± 0.24
100	2800	542	19.33 ± 0.37	10.03	13.47	0.54	0.79	0.29	1.12	0.84	0.79	0.24	0.46	-	5.20 ± 1.97
125	2935	525	17.90 ± 0.57	9.59	13.27	0.83	1.10	0.61	1.18	1.10	0.83	0.26	0.49	-	6.44 ± 0.49
150	2893	391	13.53 ± 0.23	9.10	12.18	1.20	1.55	0.77	1.57	1.59	1.20	0.38	0.87	-	9.15 ± 2.46
200	2755	325	11.80 ± 0.24	8.55	10.48	1.56	1.62	1.04	1.61	2.08	1.69	0.52	1.12	-	11.78 ± 1.57

Abn- Abnormality, Ana- Anaphase, Br- Bridges, Cl- Clumping of chromosomes, Fr- Fragments, Gy- Gray, Lg- Laggards, Met- Metaphase, MI-Mitotic index, MN- Micronuclei, MNU- Multinuclei, PM- Precocious movement, PN- Persistent nucleolus, Sc- Stickiness of chromosomes, ± - Standard deviation, Tel- Telophase.

TABLE 2: Effect of different concentrations of SA on mitotic abnormalities in root tip cells of *R. humilis* under variable treatment modes.

Concentrations of SA (%)	No. of cells scored	No. of cells in division	MI (%)	Frequency (%)											
				Met	Ana + Tel	Br	Fr	Lg	MN	Sc	Cl	PM	MNU	PN	Abn (%)
Control (DS)	**2698**	**686**	**25.55 ± 0.68**	**10.97**	**15.00**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	-
0.0075 (DS)	2867	643	22.39 ± 0.46	9.61	14.14	0.23	0.45	0.42	0.26	0.20	-	0.65	0.62	-	2.86 ± 0.29
0.010 (DS)	2721	574	21.11 ± 0.38	8.77	13.53	0.52	1.02	0.78	0.59	1.31	0.28	0.75	0.98	0.18	6.55 ± 0.67
0.020 (DS)	2739	516	18.82 ± 0.31	8.22	11.77	0.62	1.26	0.97	0.64	1.60	0.58	0.93	1.31	-	7.93 ± 1.26
Control (PSW-3H)	**2517**	**629**	**25.55 ± 0.27**	**11.04**	**14.88**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	-
0.0075 (PSW-3H)	2746	573	22.39 ± 0.55	10.79	13.99	0.46	0.70	0.45	0.39	0.92	0.25		0.78	-	4.47 ± 0.90
0.010 (PSW-3H)	2586	471	21.11 ± 0.33	10.47	13.59	0.60	0.89	0.66	0.59	1.15	0.56	0.56	1.19	-	6.20 ± 0.51
0.020 (PSW-3H)	3107	513	18.82 ± 0.46	9.82	12.01	0.90	1.17	0.92	0.89	1.18	0.73	0.62	1.47	-	7.76 ± 0.48
Control (PSW-6H)	**2865**	**744**	**26.06 ± 0.56**	**11.98**	**16.58**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	-
0.0075 (PSW-6H)	3058	668	21.85 ± 0.12	10.23	15.18	0.59	0.61	0.40	0.46	0.58	0.43	0.37	0.72	-	4.17 ± 1.24
0.010 (PSW-6H)	2977	518	17.38 ± 0.27	8.02	13.47	0.85	0.90	0.61	0.54	0.86	0.84	0.56	0.95	-	6.47 ± 1.55
0.020 (PSW-6H)	3057	462	15.11 ± 0.58	7.80	12.14	1.06	1.45	0.72	0.69	1.42	1.11	0.70	1.13	-	8.30 ± 2.75

Abn- Abnormality, Ana- Anaphase, Br- Bridges, Cl- Clumping of chromosomes, Fr- Fragments, Gy- Gray, Lg- Laggards, Met- Metaphase, MI-Mitotic Index, MN- Micronuclei, MNU- Multinuclei PM- Precocious movement, PN-Persistent nucleolus, Sc- Stickiness of chromosomes, ± - Standard deviation, Tel- Telophases.

TABLE 3: Effect of different concentrations of EMS on mitotic abnormalities in root tip cells of *R. humilis* under variable treatment modes.

EMS (%)	No. of cells scored	No. of cells in division	MI (%)	Frequency (%)											
				Met	Ana + Tel	Br	Fr	Lg	MN	Sc	Cl	PM	MNU	PN	Abn (%)
Control (DS)	**2698**	**686**	**25.27 ± 0.83**	**10.29**	**16.14**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	-
0.50 (DS)	2867	643	22.99 ± 0.53	9.33	14.80	0.21	0.41	-	0.39	0.42	0.21	-	0.44	-	2.52 ± 0.65
1.00 (DS)	2721	574	20.72 ± 0.29	8.82	12.02	0.54	0.81	0.27	0.52	0.82	0.30	-	0.58	-	3.89 ± 0.65
1.50 (DS)	2739	516	16.78 ±0.46	7.40	11.22	0.75	1.12	0.39	0.89	1.14	0.36	0.38	0.61	-	5.67 ± 0.96
Control (PSW-3H)	**2517**	**629**	**27.79 ± 0.21**	**10.73**	**16.24**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	
0.50 (PSW-3H)	2746	573	21.04 ± 0.61	9.65	14.33	0.47	0.69	0.22	0.50	0.70	0.45	0.23	-	-	3.71 ± 0.30
1.00 (PSW_3H)	2586	471	19.06 ± 0.42	8.48	11.39	0.64	0.97	0.66	0.64	1.01	0.63	0.31	0.33	-	5.60 ± 1.73
1.50 (PSW-3H)	3107	513	15.93 ± 0.65	8.05	10.63	0.75	1.17	0.69	0.98	1.25	0.84	0.49	0.68	-	7.80 ± 0.42
Control (PSW-6H)	**2865**	**744**	**28.02 ± 0.36**	**10.90**	**16.50**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	
0.50 (PSW-6H)	3058	668	19.35 ± 0.67	9.66	14.23	0.50	0.73	0.25	0.53	0.73	0.48	0.23	0.44	-	4.41 ± 0.35
1.00 (PSW_6H)	2977	518	16.38 ± 0.22	8.89	12.75	0.70	0.96	0.31	0.93	1.02	0.62	0.39	0.81	-	5.77 ± 2.14

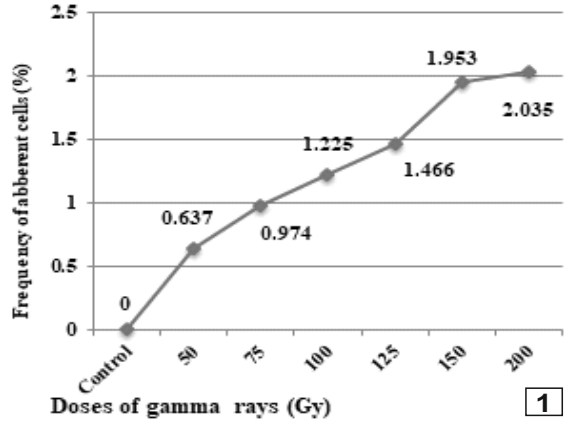
Abn- Abnormality, Ana- Anaphase, Br- Bridges, Cl- Clumping of chromosomes, Fr- Fragments, Gy- Gray, Lg- Laggards, Met- Metaphase, MI-Mitotic Index, MN- Micronuclei, MNU- Multinuclei PM- Precocious movement, PN-Persistent nucleolus, Sc- Stickiness of chromosomes, ± - Standard deviation, Tel- Telophases.

Effect of mutagens inducing chromosomal aberrations

All 3 mutagens used in the present investigation proved to be cytotoxic and were able to induce various clastogenic (structural) and nonclastogenic (physiological) chromosomal aberrations with varying frequencies. The clastogenic abnormalities recorded were chromosome fragments, laggards, single and multiple bridges at metaphases, anaphases and telophases. The major nonclastogenic aberrations observed were stickiness and clumping of chromosomes, desynchronized metaphase, disoriented chromosomes at metaphase, anaphase, telophase, etc. Multipolar anaphase and telophase, micronuclei formation were also induced by certain doses/concentration of the mutagens. Frequency of chromosomal aberrations was found to be colinearly increased with the increment in dose/concentration of all 3 mutagens (Tables 1–3, Figs 2, 4, 6). The root tip cells of the control seeds were devoid of any chromosomal abnormalities, whereas the exposure of seeds to gamma irradiation exhibited deleterious effects on the structural integrity of mitotic chromosomes and the frequency of chromosomal aberrations was found to be enhanced with the exposure rate of gamma irradiation to seeds (Fig. 2, Table 1). Similar trend of increase in frequency of chromosomal aberrations was also reported with the increase in concentration of both the chemical mutagens (Figs 4, 6, Tables 2, 3). Comparatively, all treatment modes of SA were found to be more effective than different treatment modes of EMS in inducing the chromosomal aberrations (Figs 4, 6). Deleterious effects of both the chemical mutagens on inducing chromosomal aberrations was increased with the increase in presoaking duration (Tables 2, 3, Figs 4, 6).

Various clastogenic changes in the chromosomes, such as bridges (single, double and multiple), fragments, laggards as well as micronuclei formation (Figs 7–15), and the nonclastogenic changes in the form of stickiness and clumping of chromosomes at metaphase, anaphase and telophase (Figs 20–23) were recorded from all the dose/concentration of all the mutagens and found to be enhanced with the increase in dose/concentration dependent manner. However, laggards were not reported at lower (0.50%) concentration of EMS in dry seed treatment mode (Table 3). Gamma irradiation was found to be more potent in inducing various types of chromosomal aberrations than that of both the chemical mutagens, with highest frequency observed at the higher (200 Gy) dose of gamma irradiation (Tables 1–3). Effectiveness of both the chemical mutagens, in inducing both the types of chromosomal aberrations, was found to be enhanced with the increased period of presoaking of seeds before the treatment.

Gamma irradiation induced more nonclastogenic chromosomal aberrations than both the chemical mutagens (Tables 1–3). However, in case of both the chemical mutagens, presoaking of seeds in water for 3 h and 6 h (PSW-3H and PSW-6H) increased the incidence of nonclastogenic changes than the dry seed treatment mode. Precocious movement and early separation of single and multiple chromosomes (Figs 16–19) were caused by different doses/concentrations of mutagen used, except 75 Gy dose of gamma irradiation (Table 1) and lower (0.0075%) concentration of SA in 3 h presoaking treatment mode (Table 2) as well as 0.5% and 1.00% concentrations of EMS in dry seed treatment mode (Table 3). The most prominent nonclastogenic chromosomal aberrations induced by all the mutagens were stickiness of



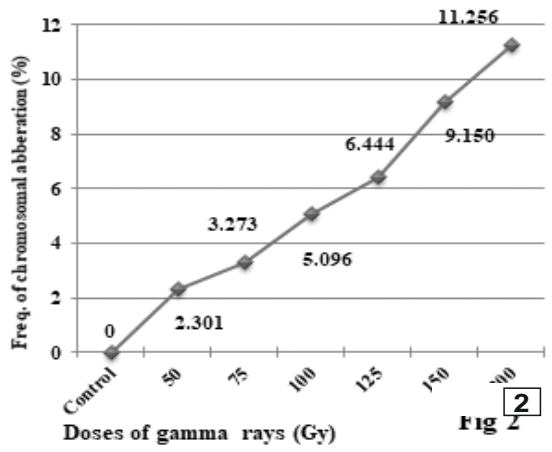
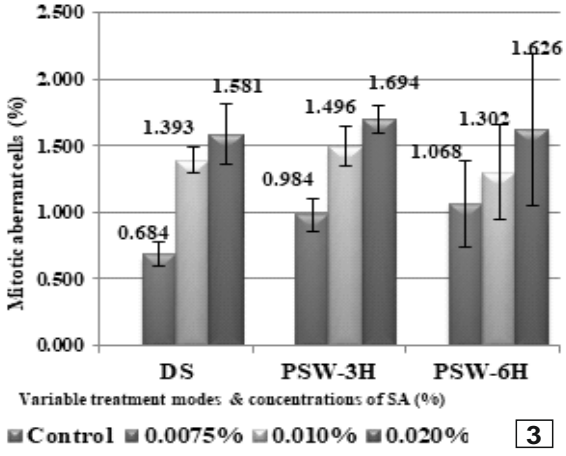
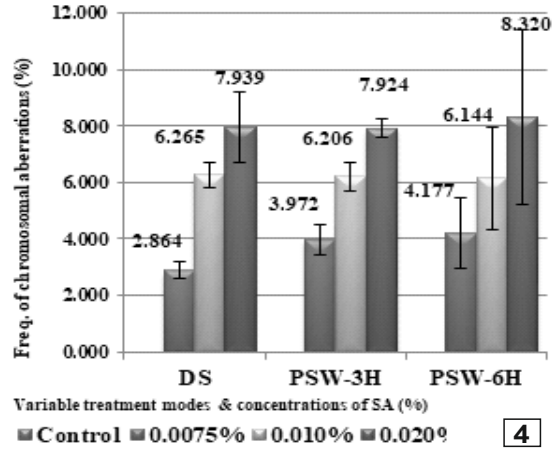
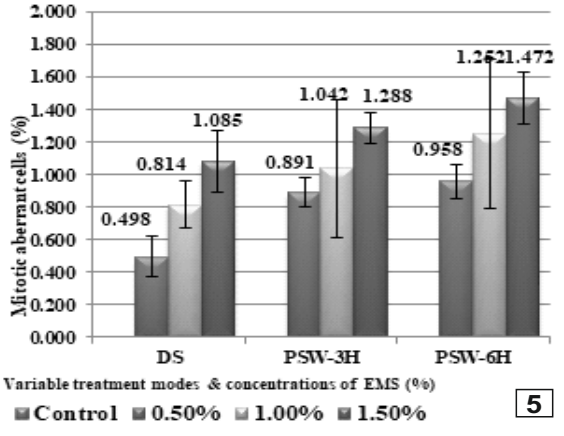
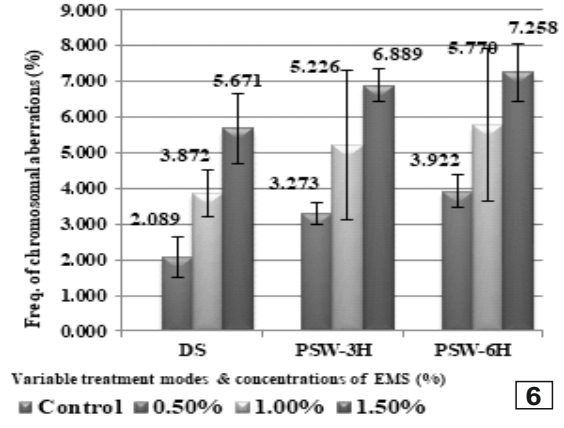


Fig 2

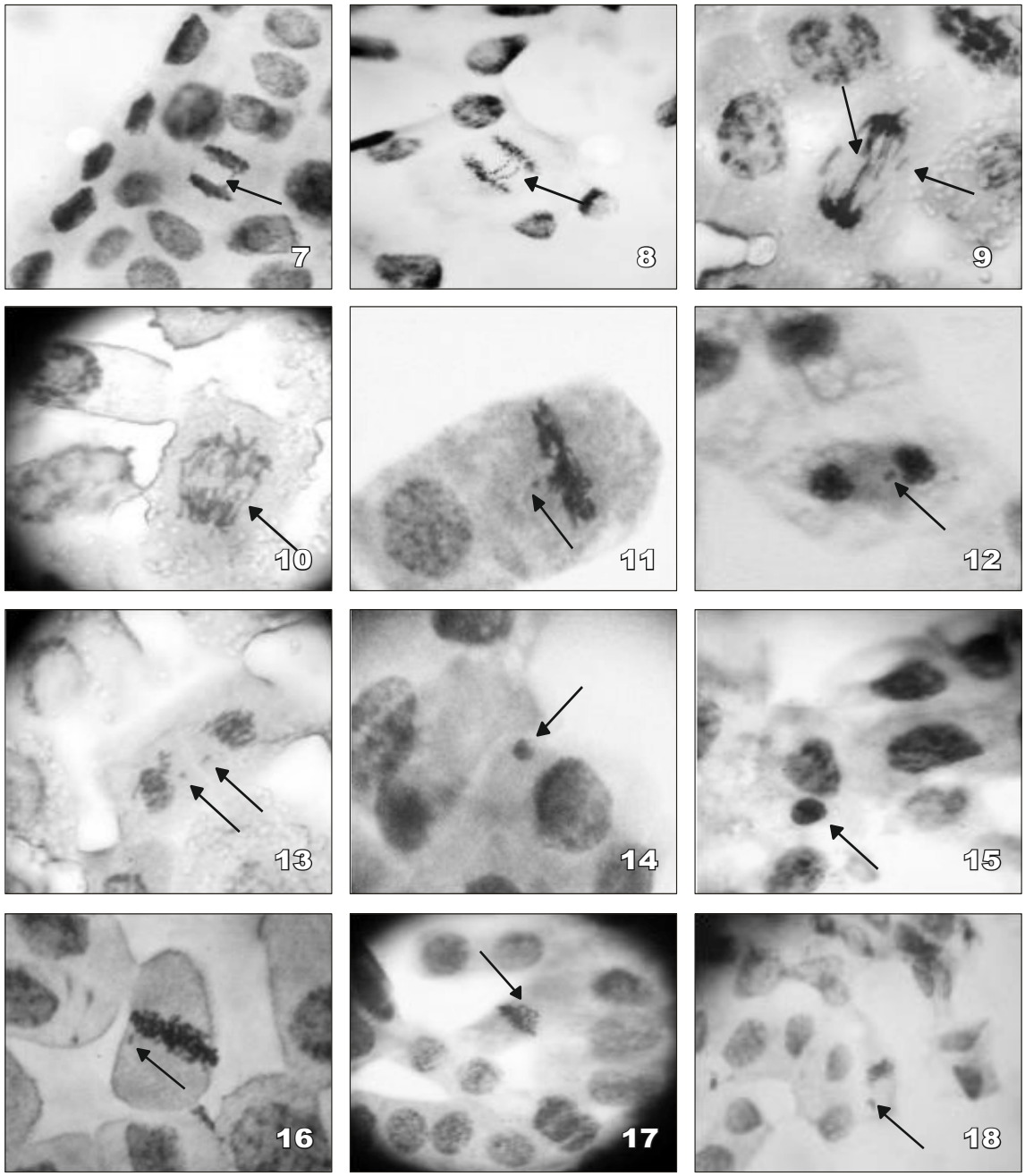




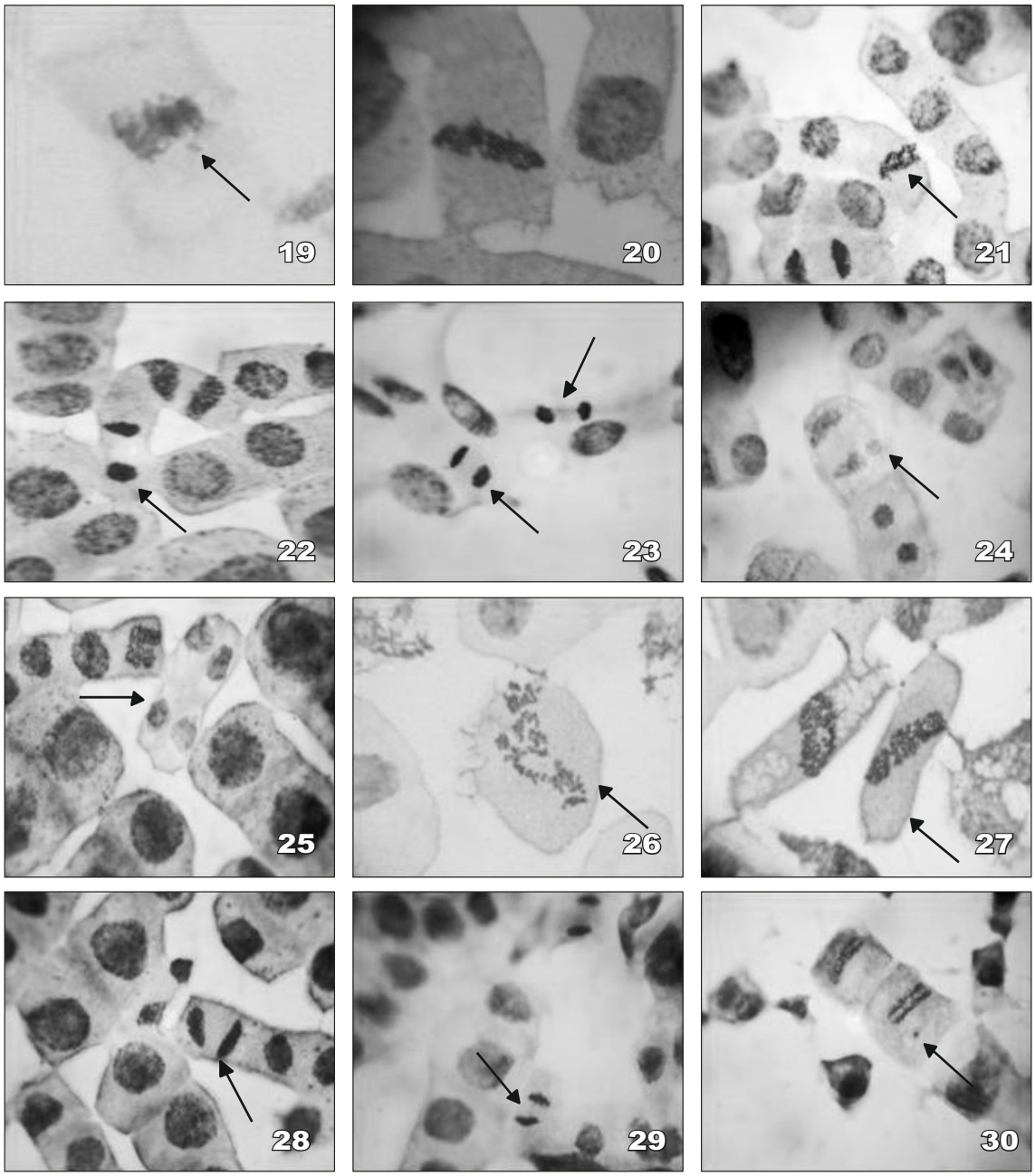




Figs 1–6: *R. humilis* 1, 2. Graphs showing effect of gamma rays on mitotic cells. 3, 4. Histograms showing effect of SA on mitotic cells. 5, 6. Histograms showing effect of EMS on mitotic cells.



Figs 7–18: *R. humilis*. Cells treated with mutagens showing various mitotic abnormalities. 7–10. Arrows point out single, double and multiple bridges with or without fragments. 11, 12. Arrows point out fragments at metaphase and anaphase. 13. Arrows point out laggards. 14,15. Arrows point out micronuclei. 16–18. Arrows point out precocious movement of chromosomes.



Figs 19–**30**: *R. humilis*. Cells treated with mutagens showing mitotic abnormalities. 19. Arrow points out early separation of chromosome. 20, 21. Cells showing stickiness of chromosomes at metaphase and early anaphase. 22, 23. Arrows point out clumping of chromosomes at anaphase and telophase.24. Arrow points out tripolar spindle. 25. Arrow points out cell with multipolar telophase. 26–29. Arrows point out disturbed and disoriented mitotic conditions. 30. Arrow points out persistent nucleolus.

chromosomes at metaphase and anaphases, where one or more chromosomes were stuck to the rest of the chromosome complement (Figs 20, 21). The frequency of stickiness of chromosome was found to be colinearly increased with the dose/concentration of all the mutagens. The highest frequency of stickiness of chromosomes was observed with 200 Gy dose of gamma rays (Table 1) followed by 0.02% and 1.50% concentrations of SA and EMS respectively, in 6 h presoaking treatment modes (Tables 2, 3). All the chromosomes of the genome stick together to form the clump and that may occur at metaphase, anaphase and telophase (Figs 22, 23). Clumping of entire chromosome complement was induced by all the doses/concentrations of mutagens used, except lower (0.0075%) concentration of SA in dry seed treatment mode. Frequency of clumping of chromosomes, observed at metaphase, anaphase and telophase was observed to be colinearly increased with increment in dose/ concentration of all the mutagens (Tables 1–3). The mutagens used in the present investigation also induced multinuclei formation resulting in the appearance of tripolar and multipolar anaphase and telophase (Figs 24, 25). It was found to be induced by all the doses/ concentrations of both the chemical mutagens, in all treatment modes, except lower (50, 75 Gy) doses of gamma rays and 0.50% concentration of EMS in 3 h presoaking treatment mode. Cells with multinuclei formation at telophase were found to be more with the treatment of SA than that of EMS and gamma rays (Tables 1–3).

Certain dose/concentration of the mutagens, used in the present investigation, also induced other types of chromosomal abnormalities which are recorded as disturbed metaphase due to nonsynchronization of chromosomes (Fig. 26), disoriented or curved metaphase (Fig. 27), disoriented anaphase and telophase (Figs 28, 29) etc. The 0.01% concentration of SA, in dry seed treatment mode, induced the nucleolar abnormality that was recorded in the form of persistent nucleolus at anaphase (Fig. 30).

DISCUSSION

Cytotoxic effects of all mutagens are evident from the cytological analysis of the data on induction of mitotic aberrant cells and the frequency of chromosomal aberrations. The dose/ concentration dependent increase in mitotic aberrant cells and the chromosomal aberrations revealed adverse effect of mutagens on root tip cells in the plant under investigation. Mitotic process and the chromosomal entity were found to be greatly affected by all mutagens. The enhanced frequency of mitotic aberrant cells and various chromosomal aberrations is an indication of cytotoxic effect of all the mutagens on the genome of plant. The genetic damage resulted by the induction of disturbances in the mitotic and meiotic cells due to the treatment of mutagens is of great importance since these genetic damages are transferred to the next generation (Kumar & Rai 2007). Sarada Mani & Seetharami Reddi (1986) stated that the treatment of mutagens induces the 'endogenous poisons' inside the cell which resulted by the breakdown of micro- and macromolecules especially enzymes and nucleoproteins in the cytoplasm. And these mitotic poisons may cause 'metabolic imbalance' which may interfere with synthesis, state and structure of nucleic acids including physiological effects and structural changes in the chromosomes during cell division. In the present investigation, the treatment of all the 3 mutagens showed deleterious effects on mitotic chromosomes which increased the abnormality of mitotic cells at anaphase, metaphase and telophase. The deleterious effect of different doses/ concent-

rations of all the employed mutagens was prominently observed in the form of various clastogenic and nonclastogenic types of chromosomal aberrations which are recorded as chromosomal fragments, laggards, single and multiple bridges, precocious movement, stickiness and clumping of chromosomes as well as persistent nucleolus.

In *R. humilis*, the mitotic chromosomes were found to be greatly affected due to the treatment of mutagens. The results recorded as linear correlations between dose rate and frequency of mitotic aberrant cells are in agreement with earlier findings by several workers. Avinash Aney et al. (2012), Bairathi & Nathawat (1979), Choudhary (1987), Choudhary & Dnyansagar (1980), D' Amato (1950), Darlington & Kollar (1947), Datta & Banerjee (1998), Kumar & Dubey (1997), More & Kothekar (1992) Nawar et al. (1970), Pal (1984), Pyatenko et al. (1970), Verma et al. (2012) and Wagner et al. (1968) have reported the occurrence of various mitotic chromosomal aberrations in different plants.

The physical and chemical mutagens are known to produce various chromosomal aberrations (Kumar & Dubey 1997). The ionizing radiations, particularly gamma radiation, reported to cause mostly the gross structural changes in the morphology of chromosomes directly (Aney 2014a) or indirectly alter one of the bases in DNA. On the contrary the chemical mutagens are known to have clastogenic effects on chromosomes via reactive oxygen-derived radicals (Ricardo & Ando 1998, Yuan & Zhang 1993). SA generally produces induced mutations leading to base pair substitution, especially GC-AT that leads to the change in amino acids resulting in the change in functions of the proteins. They can also bring about the chromosome damage as observed by Nilan & Pearson (1975) and Sander et al. (1978) in barley. The most predominant aberrations induced by SA are, translocations, lagging chromosomes, bridges, stickiness and clumping of chromosomes. EMS is also known to induce wide range of chromosomal aberrations in different plants. More effectiveness of EMS than SA in inducing chromosomal aberrations was reported by Akhtar et al. (2012) in *Linum usitatissimum.*

In the present investigation, the mutagens induced various kinds of mitotic chromosomal aberrations and that was found to be increased with the increase of dose/concentration of the mutagens. Generally, it was observed that higher the dose/concentration of the mutagen, greater is the biological damage caused. The frequency of aberrant cells can be used as an index of efficiency and effectiveness of mutagen (Kumar & Tripathi 2003). The mutagens in the present study induced chromosomal breaks and these pieces of chromosomes remained present in the form of dot-like or rod-shaped acentric fragments observed in aberrant mitotic anaphase, metaphase and telophase. Further, the lagging chromosomes and micronuclei formation were more prominently observed in aberrant anaphase and telophase respectively. Similar kinds of acentric fragments and laggards were observed by Caldecott et al. (1954) and Gaul (1965) in barley. The appearance of fragments may be due to the failure to reunite with the chromosomes (Kaur & Grover 1985), whereas, the occurrence of lagging chromosomes might be attributed to the failure to carry the chromosome to respective pole (Tarar & Dnyansagar 1980) and irregular distribution of some of the acentric fragments produced by mutagens that leads to the formation of micronuclei (Avinash Aney et al. 2012, Bhattacharya 1953). Abraham & Rajalakshmy (1989), Badr (1986) and Bhat et al. (2007)

suggested the cause for the formation of fragment and laggards as the chromosomal breakage caused due to chemical mutagens by binding at GC rich region and thus making the DNA unstable. The deformity in spindle formation and consequently chromosome segregation and failure of chromosome movement towards the respective poles during mitosis is also responsible for the origin of more lagging chromosomes. Klasterskii et al. (1976) explained the increased incidence of lagging chromosomes due to the treatment of mutagen as more lagging chromosomes are seen due to improper infoldings of the chromosomes into single chromatid and chromosome as a result of which chromatin fibers intermingle and chromosomes become attached to each other by means of subchromatid bridges. Maurya & Das (1976) suggested the linear relationship between chromosome fragments and radiation dose and stated that the presence of paired dicentric chromosome bridges and fragments was due to the monopartite behaviour of the resting chromosomes, and the fragments were the results of breakage at chromosome level rather than at chromatid level. Micronuclei, observed in the present investigation, are formed due to mutagenic effect which may lead to loss of genetic material (Aurebach 1976). The micronuclei are not formed only from the acentric fragments alone, but also by lagging chromosomes. Soren et al. (1981) observed 2 types of micronuclei i.e., heterochromatic micronuclei, formed by acentric chromosomal fragments, and euchromatic ones formed by one or few lagging chromosomes. Several workers suggested the cause for the formation and occurrence of micronuclei at telophases. It may be either due to clastogenic events in the concerned cell (Degrassi & Rizzoni 1982, Sparrow & Singlton 1953) or the result of single or group of chromosomes forming individual nuclei (Bhat et al. 2007). According to Levan (1951) the resulting acentric chromosomes which are observed as lagging chromosome or fragments during anaphase may take the shape of micronuclei at telophase if chromatin material involved is sufficiently large.

In the present study, in the treated root tip cells, the stickiness and clumping of chromosomes were prominently observed at various stages of mitosis. Beadle (1932) observed the sticky chromosomes in maize, and he attributed such irregularity to a mutation caused by a recessive gene called sticky (*st*). He referred to this as hereditary stickiness, whereas, the stickiness due to mutagen is termed as induced stickiness. The stickiness may arise due to improper clustering of chromosomes at any phase of cell cycle (McGill et al. 1974). Achkar et al. (1989) suggested the formation of sticky chromosomes as a result of breaks in the double strands of DNA and initiation of formation of intra-chromatid links during chromosome condensation, which is determined by histone protein. However, Klasterkii et al. (1976) attributed it to the entanglement of inter-chromosomal chromatin fibers that leads to sub-chromatid connection between chromosomes, whereas, Patil & Bhat (1992) considered it as a type of physical adhesion involving mainly the proteinaceous matrix of the chromatin. According to Darlington & La Cour (1940), stickiness of one or more chromosomes from the genomic complement could be a consequence of depolymerization of nucleic acids, inhibiting DNA synthesis. According to Evans (1962), the stickiness of chromosomes is due to the partial dissociation of nucleoproteins and alteration in their pattern of organization. Jayabalan & Rao (1987) suggested stickiness might be due to disturbances in balanced reaction, whereas

Gaulden (1987) hypothesized chemically induced stickiness to the action of mutagens to the failure or bad functioning of one or two types of non-histone proteins leading to improper folding of DNA and called them induced stickiness. However, according to Sax (1941) the stickiness of chromosomes is due to the change in the viscosity of cytoplasm. The excitation and/or ionization of protoplasm by the X-rays and other ionizing particles initiate a chain of events which culminates in recognizable biological effects. Avinash Aney et al. (2012) suggested the stickiness of chromosomes due to depolymerization of DNA that ultimately results in rendering more fluid on the surface of chromosomes and adhering of two or more chromosomes to each other. The clumping of chromosomes reported in the present investigation might be the consequence of stickiness of all the chromosomes in the complement (Avinash Aney et al. 2012). According to Gaul (1964), the cause of clumping can be a swelling and thickening of metaphase chromosomes which finally stick together and form a clump. More frequency of stickiness and clumping of chromosomes in the present study may be due to delay in chromosome separation caused by disturbance at cytochemical level (Sinha & Godward 1972a, b).

In the aberrant anaphase, single and double chromosome bridges with or without fragments and laggards were recorded, and these were correlated with the breakages in chromosomes. Several workers like Amer & Ali (1974), Ahmad & Yasmin (1992), Kaur & Grover (1985) and Shreekrishna (2006) postulated that the bridges induced by the mutagens might have arisen through breaks in the chromosomes followed by union of the centric fragments or due to stickiness of chromosomes at metaphase and their failure to separate at anaphase or due to reunion of chromosomes (Badr 1988, Grant 1978). The bridges at anaphase may be formed due to non-disjunction of daughter chromatids. The probable cause of bridge formation in the present study might be the fusion of two centromere bearing chromosome fragments, resulting in the formation of dicentric chromosomes. The chromosomes after effective splitting into two chromatids, which tried to migrate towards opposite poles, resulted in bridge formation. Similar observations were made by Gaul (1965) and Kumar et al. (1986). The paired bridges are thought to be formed due to the fusion of broken ends of chromosomes rather than the chromatids (Sax 1940).

Presence of aberrant metaphase and anaphase with precocious movement of one or two chromosomes towards either pole may be due to partial or complete failure of spindle mechanism (Kumar & Dubey 1997), contraction of some of the spindle fibers at early anaphase, ultimately disturbing the normal orientation of the chromosomes at anaphases (Gaul 1964), or due to spindle disfunction (Khan et al. 2005). According to Prasad (1972), the alteration of the gene controlling the spindle mechanism can disturb the biochemical pathway leading to disturbances of spindle mechanism and their coordinated mechanism (More & Kothekar 1992). Kinetochore is the important domain in the chromosome meant for the spindle microtubule-centromere association. Molecular based studies by various workers (Binarova et al. 2006, Pfarr et al. 1990, Steuer et al. 1990, Walczak et al. 1996, Wordeman & Mitchison 1995, Yao et al. 1997, Yen et al. 1992) have confirmed the role of certain proteins involved in the attachment of spindle fibers with the kinetochore and concluded that the mutations in the genes encoding these proteins led to the

disturbed spindle mechanism. The identified proteins called the centromere associated kinesin-related microtubule protein E (CENP-E) which is also essential for the chromosome congression during mitosis (Yao et al. 1997). However, according to Masoud et al. (2013) spindle polarity is essential for plant morphogenesis and the microtubule (MT) arrays play an important role in defining the mitotic polarity. The rearrangement of MTs depends on the activity of c-tubulin-containing complexes and the knockout of the c-tubulin gene was found to be lethal (Pastuglia et al. 2003), indicating that this protein is absolutely necessary for microtubule assembly in vivo (Binarova et al. 2006). Janski et al. (2012) observed the disturbed spindle polarity in *gip1* and *gip2* double mutants of *Arabidopsis* and concluded that down regulated *GIPs* are important in maintaining the spatial links between pro-spindle and MTs growing out from the spindle pole to the cortex. However, Hussey et al. (2002) and Meng et al. (2010) attributed the deformity in microtubule formation to the decreased activity of nine genes from *MAP65* family from *Arabidopsis* and from *MAP65-1c* of *Nicotiana tabacum*. The activities of these genes are under control of phosphorylation by cyclin-dependent kinases and mitogen-activated protein kinases, which decrease their ability to bundle MTs. Kawamura et al. (2006) observed the defects in spindle formation in *Arabidopsis* due to the temperature sensitive mutant allele. While, Yasuhara & Oe (2011) reported severe defects in bipolar spindle formation resulting in the appearance of multinucleated cells with variable sized nuclei due to the RNAi depletion of its tobacco homologue tobacco MT binding protein 200 called TMBP200.

The disturbed polarity observed in the present study, might be due to the induction of mutation by the mutagens that may have affected the genes either resulting in the depolymerization of microfilaments or controlling mechanism of spindle formation. Recently, the formin and augmin protein complexes have been reported to be involved in orientation and amplification of spindle microtubules (Ho et al. 2011, Hotta et al., 2012). Lee et al. (2009) in *Nicotiana benthamiana* identified Rae1 protein distributed on spindle microtubules and confirmed their MT-binding properties. They have further reported the disorganized, unfocussed and disorganized spindle due to the RNAi inhibition of NbRae1. The *Arabidopsis* genome contains four *Ran* genes (Vernoud et al. 2003) controlling spindle mechanism and the spindle targeted AtRanGAPs was confirmed by Pay et al. (2002), whereas Chen et al. (2011) observed the aberrant organization of spindle during mitosis in Osran2 knockdown rice lines.

The metabolic mechanisms are always targeted by SA. Apart from being a suppressor of cell division it also affects the polarity of spindle fibers. Fortin et al. (1990) identified the *secA* gene in *E. coli* that exhibited resistance to the mutations caused by SA. The chemical mutagens, particularly SA, are known to hamper ATP biosynthesis. The organization and movement of spindle fibers during the cell division is an ATP dependent process. Due to the reduced synthesis and availability of ATP in SA treated cells, the spindle fiber organization might have been affected, which in turn influence the organization of chromosomes at the metaphase plate and their migration during anaphase.

The occurrence of persistent nucleoli at anaphase, in the root tip cells of gamma irradiated seeds, can be assumed to be caused by the extension of heterochromatin activity up to the later stages of division. Induction of persistent

nucleoli due to gamma irradiation was also observed by Raju et al. (1989) and Ramakrishna et al. (1989) in rice.

Thus, in *R. humilis* all the chemical mutagens and gamma rays particularly, at higher doses exhibited the cytotoxic effects. The clastogenic effect of mutagens on the genome of the plant under study was clearly observed in the form of induction of various chromosomal aberrations, and the results on mitotic activities revealed that the mutagenic effectiveness increased with the increase in dose/concentration of mutagens. The genetic variability induced by these mutagens at the genomic level led to the variation in plant morphology, chlorophyll content, sterility and yield can be favourably exploited for the improvement of agronomic characters of the plant. The increase in frequency of aberrant cells and chromosomal aberration in both the presoaking treatment modes of chemical mutagens might be due to increase in permeability of cell membrane (Walles 1967) and activation of seeds at physiological level (Roychowdhary & Tah 2013).

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