RESEARCH REPORT

Effect of physical and chemical mutagens on meiotic chromosomes in pollen mother cells (PMCs) during microsporogenesis and pollen fertility in *Rivinia humilis* L.

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ABSTRACT

The seeds of *Rivinia humilis* L. was subjected to the mutagenic treatment of three mutagnes i.e. gamma rays, sodium azide and ethylmethane sulphonate for the enhancement in dye content obtained from ripened berries. Effect of all the three mutagens on chromosomal aberrations induced in PMCs during microsporogenesis and effect on pollen viability was analysed. Different types of chromosomal aberrations from aberrant prophases, metaphases, anaphases and telophases of both the meiotic divisions in the PMCs from the mutagenized population were recorded. Aberrant phases of both the meiotic divisions have exhibited adverse effect on chromosomal entity by all the mutagens in dose/concentration dependent manner. Chromosomal aberrations in gamma irradiated plants was ranged between 2.74-4.19 % frequencies, whereas, aberrant PMCs ranged between 16.49 to 24.69 %. Both, frequency of chromosomal aberrations and aberrant PMCs have linearly correlated with the concentrations of both the chemical mutagens in all treatment modes. SA induced the chromosomal aberrations, in all treatment mode, in the range of 1.71 to 2.51%, 2.32 to 2.92% and 2.95 to 3.14 % in dry seed, 3h presoaking and 6h presoaking treatment modes, respectively, whereas, the same was induced by EMS in the range of 2.11 to 2.89 % in dry seed, 2.39 to 2.79 % in 3h presoaking and 2.64 to 3.10 % in 6h presoaking treatment mode. Treatment of gamma irradiation was found to be more effective than both the chemical mutagens, however, SA have more pronounced effect than EMS in inducing the chromosomal aberrations in PMCs and the aberrant cells. Effectiveness of both the chemical mutagens have increased with increase in presoaking of seeds in water for 3 and 6h. Precocious separation, single and multiple bridges, laggards and disturbed polarity were the frequently observed chromosomal aberrations. All the mutagens had adverse effect on pollen viability which was reduced to 50% at certain intances. Comparatively, gamma irradiation had more effectiveness than both the chemical mutagens, however, in case of chemical mutagens, SA was observed to be more effective than EMS. Increased period of pre-soaking of seeds in water, before the treatment of chemical mutagens, has enhanced effect on pollen viability. Potent effect of all the applied mutagens on meiotic chromosomes and the pollen viability clearly revealed that the genotype of the plant is highly sensitive to all the employed mutagens.

Keywords: Meiosis, aberrations, microsporogenesis, PMCs, sensitivity, mutagnes, *Rivinia humilis*

INTRODUCTION

Rivinia humilis L. (Family Phytolaccaceae), a perennial herb, is a native of tropical America. It is growing as an occasional weed in Sri Lanka, Malaysia and India (Matthew, 1982) and has been listed as a notorious weed in several countries. The plant was deliberately introduced to India from Florida, purely for ornamental purpose and now mostly grown in gardens and greenhouses (Naik, 1998). It has green coloured unripped fruits called berries which turn red when ripped and yield red natural dye. Imperato (1975) isolated the dye producing bioactive compound and named it rivianin or rivinianin which is structurally very much similar to betanin, the pigment obtained from beet root. It contains red-violet betacyanin derivative, confirmed as betanin 3'-sulphate by Imperato (1975) and orange yellow betaxanthin derivative named as humilaxanthin by Strack *et al.* (1987).

R. humilis has reported to possess many medicinal and useful properties too. The natural products obtained from different parts of the plants are traditionally used in Jamaica as antidote to poisoning, headache, cold, diarrhea, marasmus and inflammation (Mitchell and Ahmad, 2006). Salvat *et al*., (2001) tested methonolic extracts of the branches and reported to have inhibitory effects against *Escherichia coli, Salmonella typhimurium, Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus and Enterococcus faecium*. Methonolic extract of the leaves have been confirmed to posses radical scavenging and antioxidant activities (Fathima and Tilton, 2012), whereas, Joseph and Avita (2013) carried out the studies on antimicrobial activities of root and shoot against 10 bacterial and 4 fungal strains and reported the inhibitory effects against all the strains of bacteria. Berry extract of *R. humilis* has been evaluated by Khan *et al*. (2013) 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^{o} C, without the alteration of product quality.

The genetic architecture of the organism can be altered by using various physical and chemical mutagens and hence mutation breeding is considered as the most reliable tool to obtain the desirable characters. Induced 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 various economic crops (Adamu and Aliyu, 2007). In a true sense, mutation may be spontaneous or induced, leads to loss or gain of function of a gene and if not auto-corrected and when passed to the next generation through the germ line can leads to the changes in genetic architecture which are reflected at physiological, morphological and biochemical levels, in the first and subsequent generations (Aney 2013a, b). All over the world, various physical and chemical mutagens have been successfully used for the introduction of different desirable characters of agronomic value in different plant species, mostly the crop plants.

The effect and potency of various physical and chemical mutagens can be evaluated by using the cytological analysis with respect to mitotic and meiotic behaviour of the chromosomes. Cytological analysis also provides a clue to assess sensitivity and effectiveness of genotypes of various plants. Germplasm treated with physical mutagens, particularly gamma rays, reported to disturb the chromosomal entity by inducing gross structural changes which have inhibitory effect on most of the morphological and yield attributing characters via various biochemical processes. Chemical mutagens such as Sodium azide (SA) and Ethyl methanesulphonate (EMS) have also proved their mutagenicity in various plants. These chemical mutagens, generally induce point or gene mutations leading to the base pair substitutions thereby changing the functions of proteins without abolishing them. Aney and Choudhary (2019) mentionned that the mutagenic effects at the chromosomal or gene level lead to the alteration in gentic architecture of the plant that might results in inducing variations at morphological and physiological level. The transmitted variations help to isolate the mutants with desirable morphological, physiological and yield attributing characters. Hnece, the cytological analysis with respect to chromosomal aberrations, either during mitosis or meiosis, is regarded as one of the most dependable criteria for estimating the effect of employed mutagens.

R. humilis is deliberately introduced in India as non-native naturalized plant, however, its rapid growth has revealed that it is best suited in Indian agronomic climate. Natural dyes are not only eco-friendly but also not causing ill-effects on health of human and other live stocks. 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

investigation deals with evaluation of effectiveness of applied mutagens and sensitivity of plant genome which was used in order to induce the genetic variability for the identification and isolation of viable high yielding mutants to bring the plant under cultivation as a cash crop and exploit its dye yielding potential as an alternative source of the natural dye to be used in textile, cosmetics, leather, food an pharmaceutical industries.

MATERIALS 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, Dist. Bhandara. Healthy and uniform sized seeds were selected and exposed to gamma rays with 50, 75, 100, 125, 150 and 200Gy doses. Three different treatment modes viz. dry seed (DS), presoaking in water for 3h (PSW-3H) and 6h (PSW-6H), were used for both the chemical mutagens. The seeds were treated with both the chemical mutagens for 18h with 0.0075, 0.010, 0.020% of freshly prepared SA and 0.5, 1.0 and 1.5% concentrations 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 mutagen. M_1 generation was raised from the treated seeds in greenhouse, along with control plants. Effect of all the mutagens on meiosis in PMCs during microsporogenisis and pollen viability was studied in the plants grown from treated seeds and control plants in M_1 generation.

Meiotic studies

Meiosis was studied in PMCs of the unopened flower buds. The inflorescence having flowers of all stages of development were collected during 8.00 to 10.00 am, from the M_1 plants grown in the greenhouse and fixed in carnoy's fluid for 24 hours and then preserved in 70% ethanol. After determining the appropriate developmental stage, the anthers from the unopened flower buds were dissected out and stained with 2% acetocarmine for 20 minutes. The smear was gently warmed and the used acetocarmine stain was blotted with blotting paper. Again 3-4 drops of fresh stain was added and the extra stain was blotted out with the help of blotting paper and then the cover slip was sealed with wax. Chromosomal aberrations were recorded and photographed from the dividing PMCs from the temporary micropreparation.

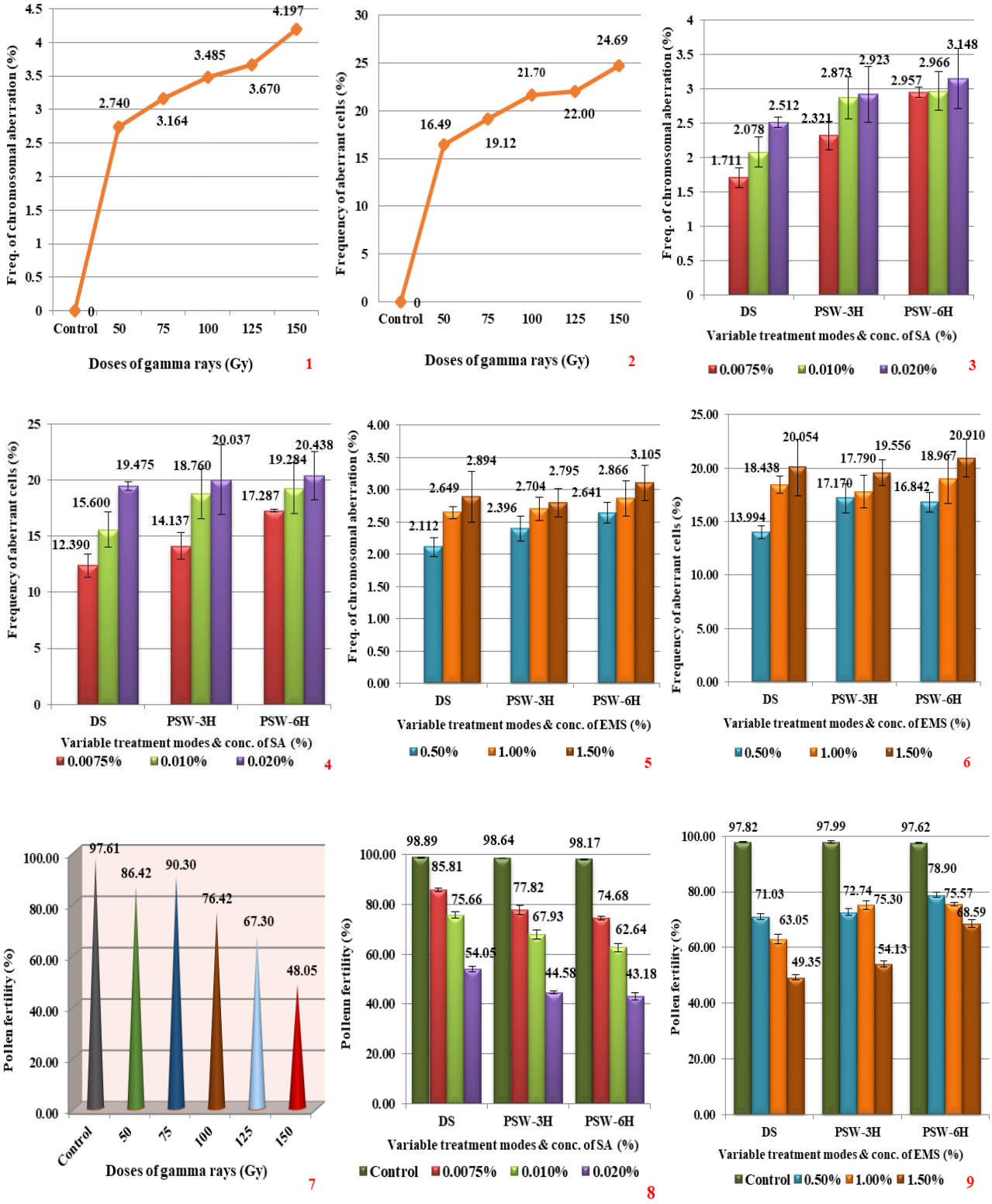
Pollen viability

Inflorescence with fully matured flowers containing matured anthers of randomly selected 25 plants from each dose/concentration of the mutagens were collected. Three anthers from each flowers, before dehiscence, were dissected out in order to determine pollen production and pollen viability. Smears of these anthers were prepared in 1:1 mixture of glycerin and 4% acetocarmine stain (Alexander, 1969). Pollen stainability as an index of viability/fertility was considered to record pollen viability. Shrunken, unstained and partially stained pollen grains were considered as sterile, while well stained, well-filled and normal sized pollen grains were considered as fertile or viable. Data was represented as percentage frequency of pollen viability.

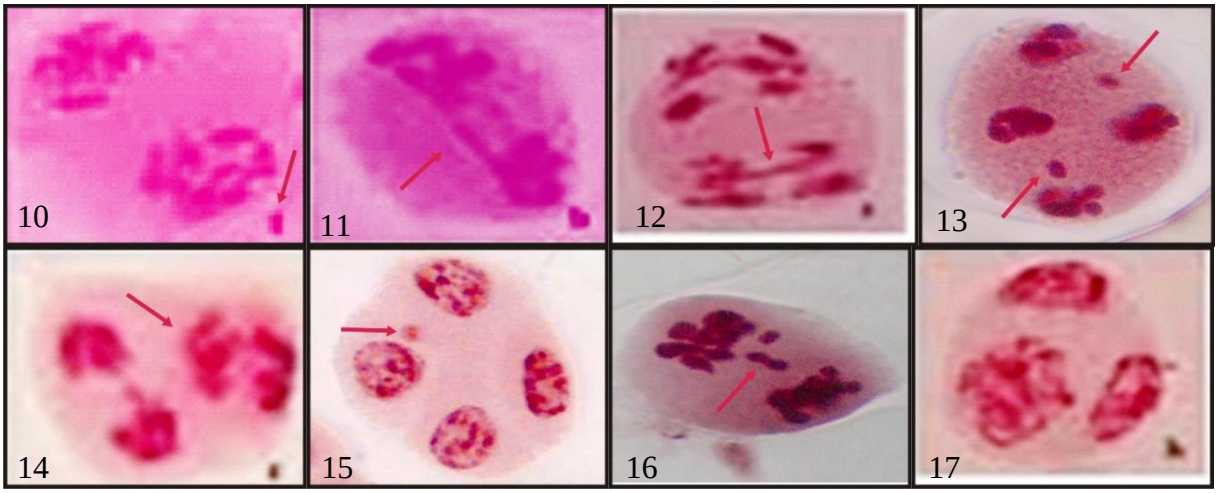
RESULTS AND DISCUSSION

Effect of mutagens on meiosis and meiotic chromosomes in PMCs:

Primary injury to plant material due to the treatment of physical and chemical mutagens is a physiological damage which is mainly restrictred to M_1 generation (Aney, 2014). Chromosomal irregularities as a result of treatment of pesticides in tobacco plant was first observed by Kostoff (1934) whereas, Grant (1978) related these irregularities with reduction in fertility. Perusal of the results presented in figs. 1 to 6 clearely reveals the differential effects of different mutagens on the meiotic activities and the chromosomal behaviour in PMCs during microsporogenesis which consequently resulted in increase number of aberrant PMCs. The PMCs of the control plant showed normal meiotic process, without the notice of any abnormalities, however, a dose/concentration dependent enhancement in chromosomal aberrations was noticed with all the three mutagens used. Various meiotic chromosomal aberrations were scored from the aberrant metaphases, anaphases and telophases, in the PMCs of plants raised from the seeds treated with varying doses/ concentrations of the employed mutagens, however, higher dose/concentration of the mutagens caused maximum chromosomal aberrations, consequently resulted in highest frequency of aberrant PMCs. Precocious separation of chromosomes, laggards, bridges and disturbed polarity (Figs. 10-17) were the most frequent chromosomal aberrations noticed. Increased in meiotic chromosomal abnormalities, at structural and physiological level, in different plants, due to various mutagens were reported by Prasad and



Figs: 1-9: *R. humilis* 1, 2. Graphs showing effect of gamma rays on meiotic chromosomal aberrations and aberrant PMCs, 3, 4. Histograms showing effect of different concentrations of SA on meiotic chromosomal aberrations and aberrant PMCs, under variable treatment modes, 5,6. Histograms showing effect of EMS on meiotic chromosomal aberrations and aberrant PMCs under variable treatment modes, 7. Histogram showing effect of gamma rays on pollen viability, 8. Histogram showing effect of SA on pollen viability, and 9. Histogram showing effect of EMS on pollen viability.



Figures: 10-17: In *R. humilis*: PMCs showing different meiotic chromosomal aberration. 10. Arrow point out precocious movement at Anaphase I, 11. Arrow point out chromosomal bridge at Anaphase I, 12. Arrow point out chromosomal bridges at Anaphase II, 13. Arrows point out chromosomal fragments at Anaphase II, 14. Arrow point out disoriented Anaphase II with chromosomal fragment, 15. Arrow point out micronuclei formation at Telophase II, 16. Arrow point out fragments and laggards and stickiness of chromosomes at Anaphase I, 17. Tripolar Anaphase I with disoriented chromosomes.

Godward (1968), Roy *et al*. (1971), SaradaMani and Seetharami Reddi (1985), Reddy *et al*. (1992), Ramakrishna *et al*. (1989), Shashikumar and Abraham (1993), Kumar and Srivastava (2001), Kumar and Rai (2007), Azad (2011), Husain *et al.* (2013), Asthana *et al*. (2014) and Prabakaran and Jayakumar (2014). In case of gamma irradiation, both frequency of meiotic aberrant cells and chromosomal aberrations was found to be correspondingly increased with the increase in dose rate of gamma rays. Both percent frequencies of chromosomal aberrations and aberrant PMCs ranged between 2.74-4.19% (Fig. 1) and 16.49-24.69% (Fig. 2), respectively.

Both the chemical mutagens also exhibited the same pattern of concentration dependent induction of chromosomal aberrations, in all treatment modes. Frequency of chromosomal aberration in dry seeds, 3h and 6h pre-soaking treatment modes of SA, respectively ranged between 1.71-2.51%, 2.32-2.92% and 2.95-3.14% (Fig. 3). Consequently, number of aberrant PMCs were increased and ranged between 12.39-19.47% for dry seeds, 14.13-20.03% for 3h presoaking and 17.28-20.43% for 6h pre-soaking treatment modes of SA (Fig. 4). Maximum effect of SA on inducting chromosomal aberrations and induction of aberrant PMCs was recorded at higher (0.20%) concentrations of SA, in 6h pre-soaking treatment mode. Data depicted in figures 3 and 4 clearly revealed that pre-soaking of seeds in water for 3 and 6h, before the treatment of mutagens, has pronounced effect of mutagen than dry seed treatment mode, in disturbing structural and physiological integrity of meiotic chromosomes.

Concentration dependent increase in both frequency of chromosomal aberrations and aberrant PMCs observed in all treatment modes of another chemical mutagen, EMS (Figs. 5 and 6). Percent frequency of chromosomal aberrations in dry seeds treatment mode of EMS ranged between 2.11-2.89%, 2.39-2.79% in 3h pre-soaking and 2.64-3.10% in 6h pre-soaking treatment modes. However, percent frequency of aberrant PMCs ranged between 13.99-20.05%, 17.17-19.55% and 16.82-20.91% in dry seeds, 3h and 6h pre-soaking treatment modes, respectively. Maximum induction of both chromosomal aberrations and aberrant PMCs was observed with higher (1.50%) concentration of EMS in all treatment modes. Effectiveness of mutagens has been reported to be enhanced in presoaking of seeds in water before the treatment of EMS than the dry seeds treatment.

Mutation, the heritable change in the genetic make-up of an individual, occurs naturally or can be induced by using external mutagenic agents. These changes, at the gene or the chromosomal level, are not the result of the common phenomenon of genetic segregation or genetic recombination but are able to cause relatively large

effects on the phenotype of the organisms. In mutation breeding, radiations and chemical agents have played a major role in altering the genetic architecture of the plant and the development of superior crop varieties translating into a tremendous economic impact on agriculture and food production. Induced mutations serves as a complimentary approach in genetic improvement of plants. Considering the potentiality of induced mutations, it has been frequently used to induce genetic variability for several desired characters. The main advantage of the mutational breeding is the possibility of improving one or two characters without changing the rest of the genotype.

In *R. humilis*, chromosomal aberrations in PMCs was found to be increased and linearly correlated with dose/concentration of the mutagens, with maximum induction of aberrations recorded at higher dose/concentration of all the mutagens. Stickiness and clumping of chromosomes were predominant at metaphases I and II, whereas, bridges and laggards were present at anaphases I and II (Figs. 12, 16). Aberrant telophases showed dominance of bridges, and disturbed polarities (Figs. 14, 17). The first case of recessive gene governing meiotic stickiness was observed by Beadle (1932). However, Anderson (1947) reported it to be caused by external agents. Several reasons can be attributed to the stickiness and clumping of chromosomes reported in the present study (Figs. 16). These could be due to i. either by depolymerization of nucleic acids caused by mutagen treatment or because of partial dissociation of nucleo-protein and alteration in their pattern of organization (Evans, 1962; Bhat *et al*., 2007), ii. disturbances in cytochemically balanced reactions (Jayabalan and Rao, 1987), iii. incorrect coding of non-histone proteins, involved in chromosome organization, due to mutation in gene (Gaulden, 1987), and iv. improper folding of the chromosome fiber into single chromatids and chromosomes (McGill *et al*., 1974 and Klasterskii *et al*., 1976). However, Kiihl *et al*. (2011) and Kumar and Dwivedi (2013) have suggested that stickiness may under genetic control, or rather, it may be controlled by a single or two pair of genes or by the interaction of several genes which may be recessive or dominant. According to Grant (1978) the chromosome fragments (Figs. 13, 16) could be correlated with multiple breaks of the chromosomes in which the integrity of chromosome is lost. The induction of chromosome fragments, chromatid exchange, and dicentric chromosomes are generally considered as unstable aberrations where chromosome breakage is thought to involve DNA molecule responsible for linear continuity of chromosomes. Evans and Sparrow (1961) have mentioned that all these aberrations are the result of unfinished repair or misrepair of DNA.

The laggards and bridges were prominently observed at aberrant anaphases and telophases I and II. Chromosome bridges were found accompanied with fragments (Figs. 11, 12, 14), although their occurrence was mostly independent of each other. According to Sax (1960) and Saylor and Smith (1966), the formation of bridge might be due to the failure of chiasmata in a bivalent to terminalize and the chromosomes stretched between the poles. Whereas, Bhattacharjee (1953) opined that interlocking of bivalent chromosomes results in the formation of bridges at anaphase I, however, Sinha and Godward (1972a) suggested that the paracentric inversions may leads to the formation of chromatin bridges. Singh and Khanna (1988) opined that the joining of sticky ends of the broken chromosome with the other chromosome as well as the unequal exchange or dicentric chromosomes may also thought to be responsible for the formation of bridge, and the number of bridges (Fig. 12) depends on the number of chromosomes taking part in the exchange. Dixit and Dubey (1986) suggested the formation of single or double bridges due to asymmetrical length of chromatids or chromosome interchanges. The laggards observed (Fig. 16), in the present investigation, might be due to the formation of acentric chromosomes resulted by the chromosomal breakage (Klasterskii *et al*. (1976), delayed terminalization, stickiness of chromosomal ends or because of the failure of the chromosomal movement towards the poles in due time (Bhat *et al*., 2007a). The acentric chromosomes failed to migrate towards the respective poles (Utsunomiya *et al*., 2002) before the chromosomes relaxed to uncoil to form daughter nuclei or they disintegrate to form micronuclei (Figs. 13, 15) at telophases (Koduru and Rao, 1981; Kumar and Rai, 2006 and Basi, *et al*., 2006). These may generate unbalanced gametes, and the randomly distributed laggards at either poles at anaphase I or II results into aneuploidy (Amer and Ali, 1988 and Haiba *et al*., 2011).
Chiasmata are responsible for the maintenance of bivalents which permit normal segregation of chromosomes. The precocious movement of chromosomes observed (Fig. 10) at aberrant metaphases and anaphases may be due to various reasons. According to Pagliarini (1990), Pagliarini and Pereira (1992), Defani-Scoarize *et al*. (1992 and 1995), Consolaro *et al*. (1996), precocious migration of the

chromosomes to the poles might be the result of univalent chromosomes, formed at the end of prophase I or precocious chiasma terminalization at diakenesis or metaphase I. They further stated that univalents may originate from the absence of crossing over at pachytene or from synaptic mutants. This view was also supported by Kumar and Yadav (2010). However, Bharathi *et al*. (2014) relate the precocious movement of the chromosomes to the deformity or inactivation of spindle mechanism that results in disturbed homology of the chromosomes and finally leads to the early movement of one or few chromosomes towards the poles from the equatorial plate of metaphases. The precocious movement of bivalents towards one pole results into unequal distribution of chromosomes or loss of complete bivalent at metaphase stage (Gandhi *et al*., 2013).

The aberrant PMCs also exhibited the presence of fragments (Figs. 13, 14, 16). The appearance of fragements during metaphases, anaphases and telophases may be due to breakage of the chromosomes by mutagenic action and failure to reunite with the chromosomes (Kaur and Grover, 1985), whereas, the occurance of lagging chromosomes could be attributed to the failure to carry the chromosomes to respective poles by spindle fibers (Tarar and Dnyansagar, 1980) and irregular distribution of some of the acentric fragments produced by the mutagens that leads to the formation of micronucleus (Bhattacharjee, 1953; Aney *et al*., 2012). Badr (1986), Abrham and Rajalkshmy (1989) 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 meiosis is also responsible for the origin of more lagging chromosomes. The increased incidence of lagging chromosomes due to the treatment of mutagen was explained by Klasterskii *et al*. (1976). According to them more lagging chromosomes are seen due to the 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 and Das (1976) suggested the linear relationship between chromosome fragments and radiation dose. They further 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.

Some of the aberrant cells showed disturbed polarities at anaphases and telophases (Figs. 14, 17). The unoriented and scattering of chromosomes could be either due to inhibition of spindle formation or the destruction of spindle fibers formed (Koduru and Rao, 1981; Kumar and Rai, 2006; Kumar and Yadav, 2010; Salam and Thoppil, 2010). Kaul and Murthy (1985) expressed that the mutation in the genes controlling the normal spindle formation and function may lead to the improper separation of univalents and bivalents and uneven segregation of the chromosomes to the opposite poles. Finally, it results in the formation of multipolar telophases or even disoriented chromosomes (Fig. 17) (Sjodin, 1970). In case of ionizing radiation, the improper functioning of microtubules may be caused due to the direct effect of stored ionizing energy that could be attributed to improper flux of binding microfilaments during orientation of chromosomes (Kumar and Dwivedi, 2013). Hence, in *R. humilis* the occurance of various types of chromosomal aberrations (Figs. 10-17) in PMCs might be due to the direct hits of gamma rays and induction of mutations controlling spindle mechanism and stickiness of chromosomes. The failure of chiasmata, delayed terminalization, paracentric inversions and disturbed homology of the chromosomes, etc. might be responsible for induction of meiotic abnormalities.

Effect of mutagens on pollen fertility

The effect of gamma rays, EMS and SA on reproductive potential, particularly, in terms of pollen fertility, of *Rivinia humilis* L. was studied in M_1 generation. Pollen stainability as an index of pollen fertility was considered to record the pollen fertility by using acetocarmine staining technique. Data on pollen fertility revealed the pollen fertility in M_1 plants decreased by all the mutagens used, in the present investigation (Figs.7-9). At some instances, the pollen fertility was reduced to even 50% due to mutagens. In case of gamma rays treatment, the control plants exhibited 97.61% pollen fertility, whereas it is ranged between 90.30-48.05% in plants raised from seeds exposed to different doses of gamma rays. 75Gy dose showed slightly moer pollen fertility over to the lower (50Gy) dose, while it it was affected in dose dependent manner at rest of the doses of gamma rays (Fig. 7).

Pollen fertility was also drastically affected by both the chemical mutagens. However, SA was found to be more effective than EMS. The effect of both the chemical mutagens on pollen fertility was observed to be varied in different treatment modes. Pollen fertility was found to be reduced with the increment in concentrations of both the chemical mutagnes, in all treatment modes. Untreated plants in dry seeds showed 98.89%, whereas, it was 98.64% in 3h pre-soaking and 98.17% in 6h pre-soaking treatment mode. The treatment of SA exhibited pollen fertility ranged between 85.81-54.05%, 77.82-44.58% and 74.88-43.18% respectively, in DS, PSW-3H and PSW-6H treatment modes (Fig. 8). Maximum effect of mutagens on pollen fertility was noted at higher (0.020%) concentration of SA, in all treatment modes, at which it was reduced to more than 50% in both pre-soaking treatment modes. The effectiveness of the mutagen was observed to be enhanced not only due to pre-soaking but also with the increase in period of pre-soaking of seeds before the treatment of mutagen (Fig. 8). Pollen fertility was aslo found to be affected by another chemical mutagen, EMS but comparatively less than the treatment of SA (Fig. 8, 9). Treatment of EMS reduced the pollen fertility in concentration dependent manner but was found to be differentially affected by different concentrations, and varied in different treatment modes (Fig. 9). Dry seed treatment of EMS has severely affected the pollen fertility as compared to both pre-soaking treatment modes (Fig. 9). The control plants, in all treatment modes, showed pollen fertility between 97.62 to 97.99%, however, in EMS treatment, it ranged between 71.03 to 49.35%, 72.74 to 54.13% and 78.90 to 68.59%, respectively, in dry, 3h and 6h presoaking treatment modes. Gradual reduction in pollen fertility in all concentrations was noted, except, the median (1.00%) concentration in 3h presoaking treatment mode, had marginally more fertile pollens than the lower (0.50%) concentration of the mutagens. The lethal effect of EMS on pollen fertility was found to be less in all concentrations of EMS, of presoaked treatment. In *R. humilis*, comparative deletorius effects of all the employed mutagnes on pollen fertility was observed. Exposure of seeds to gamma irradiation severely affected the pollen fertility, particularly at higher doses, whereas, among chemical mutagens, SA in both pre-soaking treatment modes and EMS in dry seed treatment mode had severe effect on pollen fertility.

Most of the mutagens are known to interfere with the reproductive potential of the plants in different ways. Several researchers have confirmed the severe damage caused by the mutagens to the reproductive potential of the plants either in the form of alteration in floral structures or reduction in pollen viability. Pollen fertility in a true sense can be used as an index of meiotic behaviour, and is mostly found to be directly associated with the chromosomal aberrations i.e. greater the chromosomal abnormality, greater will be the pollen sterility. According to Tomkins and Grants (1972), the mutagen induced pollen sterility in M_1 generation is mainly caused by small or minute deficiencies. Most of the induced chromosomal abnormalities are eliminated during succeeding cell division, whereas, some persists upto the formation of pollen grains, which results into formation of non-viable pollen grains (Kumari *et al*., 2009).

An inclining tendency of pollen sterility along with increase in dose/concentrations of all the mutagens was registered. However, SA was found to be more effective as compared to other two mutagens. Similar trends of reduction in pollen fertility due to various mutagens, in different plants, was reported by Patil and Bora (1961), Hagberg and Hagberg (1979), Reddy and Rao (1982), Bhat *et al*. (2007), Kumari *et al*. (2009), Elena (2010), Chatterjee *et al*. (2011), Kumar and Dwivedi (2012), Aney (2014), Mishra and Khan (2014), Abubakar *et al*. (2015).

Several workers have advocated different causes of reduction in pollen fertility due to mutagen treatment. Ehrenberg (1961) suggested that the small or minute deficiencies induced by mutagen are mainly responsible for reduction in pollen fertility, and this was also supported by Husain *et al*. (2013), who further elaborated and concluded that the induction of pollen sterility is because of gene mutations or more probably due to invisible deficiencies, whereas, Bora *et al*. (1961) opined that inversions along with the non-orientation of chromosomes at metaphase-II and laggards at anaphase-II are the main causes affecting the pollen fertility. Sinha and Godward (1972b) described that chromosomal aberrations, particularly, translocations to be responsible for decreased pollen fertility. However, Sparrow and Woodwell (1962) considered the gross chromosomal irregularities are the main cause for affecting the pollen fertility. The higher rate of pollen sterility observed in the present investigation, particularly, at higher doses/concentrations of the mutagens, might be due to various types of chromosomal aberrations during meiosis.

Reddy and Rao (1982) observed the pollen sterility as a result of interchanges of segments between non-homologous chromosomes and suggested that the induction of laggards, univalents, micronuclei, stickiness and clumping of chromosomes were closely associated with pollen sterility. Kumar and Dwivedi (2012) observed the colinear association between chromosomal aberrations and the doses of the mutagen and concluded that different chromosomal aberrations induced by the mutagens resulted in the asymmetrical distribution of chromatin material in PMCs that compromised with pollen fertility and resulted in the increase in pollen sterility. However, Rana and Swaminathan (1964) and Ramana (1974) concluded that deviation in karyokinesis and cytokinesis could produce non-viable microspores that ultimately results into pollen sterility. Radiation induced pollen sterility in M_1 generation, was thought to be due to detectable chromosomal aberrations and cryptic deficiencies, while the sterility induced by chemical mutagens might be due to cryptic deficiencies and specific gene mutation (Sato and Gaul, 1967).

Sato and Gaul (1967) classified the EMS induced pollen sterility into three categories such as i. chromosomal, ii. genetical, and iii. purely physiological. According to them, the heritable changes, in the fertility of the plant, may be due to gene mutation or more probably invisible deficiencies and inspite of the elimination of a part of these deficiencies and gene mutation, some part may pass through the sieve of meiosis and induce sterility in offsprings. However, Abubakar *et al*. (2015) in SA and fast neutron treated *Celosia argentea*, observed not only the decreased production of pollen grains but also noted significant reduction in diameter of pollen grains in treated plants, and confirmed the reduction in pollen diameter is due to aberration in microsporogenesis and correlated it with increase in pollen sterility. Similar conclusions were also drawn earlier by Sato and Gaul (1967) in barley, Azad (2012) in *Vigna radiata*, and Mahamune and Kothekar (2012) in *Phaseolus vulgaris*.

The failure in any sort of chromosomal association consequently reduces the pollen fertility. Thus, in *R. humilis*, considering the linear increase in meiotic aberrations with the dose/concentration of mutagens, reduction in pollen fertility might be either due to meiotic abnormalities or due to slight changes in the genetic composition of plant due to mutagenic action. The higher rate of sterility observed at the higher dose/concentration of the mutagen can be presumed to be due to vast array of meiotic aberrations, particularly, the invisible deficiencies. At a gross level, it can be presumed that the reduction in pollen fertility could be a manifestation of cumulative effect of polygenic system and the genes controlling the meiotic behaviour.

CONCLUSION

Thus, in *Rivinia humilis* L. both the chemical mutagens and gamma rays particularly, at higher doses exhibited severe effect on the structural and physiological level of chromosomes in PMCs and consequently resulted in enhacement in aberrant PMCs and thereby significant reduction in pollen fertility. The mutagenic effect of all the mutagens on the genome of the plant under study was clearly observed in the form of induction of various chromosomal aberrations, and the result on the meiotic behaviour of the chromosomes as well as on pollen fertility revealed that the mutagenic effectiveness increased with the increase in dose/concentration of all the mutagens. The genetic variability induced by all these mutagens at the genomic level led to the variation in plant morphology, chlorophyll, sterility and yield can be favourably exploited for the improvement of agronomic characters of the plant. The observation of 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 and Tah, 2013).

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