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Morphomutagenic variations in leaves induced by physical and chemical mutagens in *Rivinia humilis* L.

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ABSTRACT

Mutagenesis is aimed to induce variability in different plants. Induction of variability is the pre-requisite for selection and varietal development in crop plants and the mutation induction using various mutagenic agents has become a proven and well established practice of creating variations within crop and other species. After identification of *Rivinia humilis* L. as a source of red natural dye, the seeds were subjected to the treatment of physical mutagen (gamma rays) and two chemical mutagens (SA and EMS), in order to induce variability in the genome for the qualitative and quantitative improvement in dye obtained from the ripened berries. The mutagenized populations was screened for various morphological, physiological and yield attributing characters. Screening of M_1 population indicated differential effects of all the three mutagens on different traits. The mutagens induced mutations at genetic level encounter with number of biochemical processes leading to the alteration in the plant genome that consequently exhibited in the modifications of certain morphological structures, which are visible in mutagenized populations. Physical mutagens, particularly gamma rays, are known for the gross structural changes in the chromosomes leading to the production of inhibitory effect on most of the morphological and yield attributing characters, whereas, the mutagenicity of both the chemical mutagens used have been earlier proved in various plants. 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. Genome of *R. humilis* was found to be highly sensitive to all the mutagens used, in terms of inducing variations in different morphological traits. Meticulous observation on leaf, as a main photosynthetic structure, reavealed that all the mutagens have not only affected the leaf dimension and leaf index but also severely affected the morphology of the leaves. Treatment of seeds with EMS was found to be more effective than SA and gamma irradiation in inducing leaf modifications. This article deals with the critical analysis of mutagen induced variations in the leaf morphology and justification of the term 'morphomutagenic variations', used at the first time.

Keywords: *Rivinia humilis* L., morphomutagenic, variations, adnation, mutagnes, sensitivity.

INTRODUCTION

Mutations are the tools and being used to study the nature and functions of the genes which are the building blocks and the basis of plant growth and development, thereby producing raw materials for genetic improvement of economic crops (Adamu and Aliyu, 2007). The mutations, may be spontaneous or induced, 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. Primary injury to the plant material due to mutagens is a physiological damage which is mainly restricted to M_1 generation, however, few of the induced variations can be transmitted and handed over to the subsequent generations. The alterations in the genetic architecture, due to induction of mutations, are reflected at physiological, morphological and anatomical levels as well as at biochemical levels in the first generation (Aney 2013a, b). However, mutagen induced variations in different traits may be beneficial or harmful, sometimes lethal.

Variability is the pre-requisite for selection and varietal development in crop plants and the induction of mutations through mutagenesis has become a proven way of creating variations within crop and other species. Variability in plants, at various level, can be brought about by induction of mutations through the alteration in genetic architecture of the plant by using different mutagenic agents. Mutagenesis offers the possibility of inducing desired attributes that either can not be found in nature or have been lost during evolution (Kharkwal, 1998). The mutant obtained can become an important genetic resource for breeding, gene discovery and functional analysis of various genes (Patial, *et al.*, 2015). Treatment of germplasm and plant material by physical and chemical mutagen is widely used practice to induce mutations at genetic level which encounter with number of biochemical processes leading to the alteration in the plant genome that consequently exhibited in the modifications of certain morphological structures, which are visible in mutagenized populations. Gamma irradiation reported to induce gross structural changes in the chromosomes and produce inhibitory effect on most of the morphological and yield attributing characters. The mutagenicity of both the chemical mutagens i.e. sodium azide and ethylmethane sulphonate have been earlier proved in various plants. 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. Different types of variations induced by the mutagens help to identify and isolate various types of morphological, physiological and yield attributing mutants for the desirable traits. After identification of *Rivinia humilis* as a source of natural dye, obtained from the ripened berries (fruits), and in order to improve dye content at qualitative and quantitative levels, we conceived an idea of incorporating variations in the genome of the plant by induction of mutations subjecting to the treatment of various doses/ concentrations of the aforementioned mutagens.

R. humilis, is a perennial herb belongs to pokewood family Phytolaccaceae. It yields natural red dye from the ripened berries. The plant is native of tropical America and has been listed as a notorious weed in several countries (Matthew, 1982). He mentioned the plant growing as an occasional weed in Sri Lanka, Malaysia and India. The plant was deliberately brought to India from Florida, purely for ornamental purpose and now found to be grown mostly in gardens and greenhouses (Naik, 1998). In India, the plant is popularly known as 'Motiya' because of the pearl like appearance of the ripened berries produced on the recemose inflorescence. It bears green coloured unripped fruits called berries which turn red when ripened and yield red natural dye. Imperato (1975) isolated the bioactive compound yielding red dye, and named it as rivianin or rivinianin, which was found to be structurally very much similar to betanin, the pigment obtained from beet root. It contains red-violet betacyanin derivative, confirmed as betanin 3'-sulphate (Imperato, 1975) and orange yellow betaxanthin derivative named as humilaxanthin by Strack *et al.* (1987).

R. humilis has reported to possess many medicinal properties too. According to Mitchell and Ahmad (2006), in Jamaica, the natural products obtained from different parts of the plant is traditionally used as an antidote to poisoning, headache, cold, diarrhea, inflammation and marasmus. Salvat *et al.* (2001) reported out the inhibitory effect of methonolic extracts of the branches of the plant against different strains of bacteria viz. *Escherichia coli, Salmonella typhimurium, Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus and Enterococcus faecium* whereas, Fathima and 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. Studies on antibacterial activities of the root and shoot of the plant against 10 bacterial strains and 4 fungal species have been carried out by Joseph and Avita (2013) and reported the inhibitory effect against all the strains.

Basically, our investigation was aimed at the improvement of dye content of the plant through mutation breeding, however, while studying the effect of all the mutagens on various parameters, we have prominently observed the modifications in shape and size of the leaves in mutagenized population. The morphological structure of leaves have been reported to be drastically disturbed at certain doses/concentrations of all the mutagens used. We have meticulously studied and analysed the effect of all the mutagens on induction of morphological variations in the normal leaf of the plant. Leaf is the major photosynthetic area of the plant that governs the growth and development of the plant. Through this article, at the first time, we use the term **'Morphomutagenic variations'** to the morphological variations induced by the mutagens in the leaves and other vegetative as well as reproductive structures of the plant. In a true sense, the term refers to the morphological variations induced as a consequences of application of various mutagens leading to the development of plants with modified morphological structures. Although *R. humilis* is deliberately introduced as non-native naturalized plant in India, but it is found best suited in Indian agronomic climate. *Rivinia humilis*, natural dye yielding plant, can provide an opportunity to be an alternative source of natural red dye which are quite difficult to obtain from the underground parts of the other existing dye yielding plants. Present article deals with the critical analysis of the effect of all the employed mutagens on induction of various morphological forms in the leaves of *R. humilis*.

MATERIALS AND METHODS

Germplasm of *R. humilis* was collected from five 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. After testing the seed viability, healthy and uniform sized seeds were selected for the treatment of physical mutagen (Gamma irradiation) and two chemical mutagens (SA and EMS). Initially, in order to determine LD_{50}, the seeds were exposed to 100, 150, 200, 250, 300 and 350Gy doses of gamma irradiation, at a dose rate of 9 Gy/min, in gamma chamber, using ^{60}Co source of gamma radiation. Desired concentrations were prepared from the freshly prepared stock solution of both the chemical mutagens i.e. sodium azide (SA) and ethyl methanesulphonate (EMS). Three treatment modes, such as dry seeds (DS), presoaking in water for 3h (PSW-3H) and 6h (PSW-6H) were used for both the chemical mutagens. Initially, the seeds were treated with both the chemical mutagens for 18h with 0.0010, 0.0025, 0.0050, 0.0075, 0.010, 0.020% of freshly prepared SA and 0.05, 0.10, 0.25, 0.50, 1.00 and 1.50% concentrations of EMS. The treatment was terminated by decanting the mutagen solutions, and then the treated seeds were thoroughly washed several times with distilled water in order to remove the traces of mutagen. The treated seeds were soaked to dryness with filter paper and then kept in distilled water for an hour.

LD_{50} dose/concentration for the particular mutagen was determined based on 50% reduction in germination count, seedling height and root length, compared to the value in untreated (control) seeds. Based on the 50% reduction in the values of these parameters, 150Gy, 0.010% and 1.00% concentrations were found to be LD_{50} dose/concentration, for gamma irradiation, SA and EMS, respectively. Finally, three doses/concentraions (one lower and one higher to LD_{50}) of the mutagens were selected for the induction of mutation in the genome of the experimental plant. Finally, in order to raise the M_1 generation, the healthy and uniform sized seeds were exposed to 100, 150 and 200Gy doses of gamma rays. For chemical mutagens, the seeds were treated with 0.0075, 0.010 and 0.020% concentrations of SA and 0.50, 1.00 and 1.50% concentraions of EMS. M_1 generation was raised in greenhouse, by sowing the treated and untreated seeds, separately in the pots filled with soil. The effect of different doses/concentrations of the mutagens on various parameters was noted and arithmetic mean computed and tabulated. In case of gamma irradiation, no plant was found to be survived after one month of germination and hence, the dose rate was reduced to 50, 75, 100, 125 and 150Gy.

Leaf modification:

While counting total number of leaves per plant (leaf index) and measuring leaf dimension, we observed certain modifications in leaf morphology, particularly in

leaf shape in the mutagenized population. Initially, different forms (shapes) of leaves in control plant were identified first and then number of plants with modifications in leaf, in terms of leaf apex, base, margin and lamina, was counted. Data on percent frequency of number of plants with leaf variation as well as number of variant leaves was recorded, averaged and presented in tabular form.

RESULTS AND DISCUSSION

Leaf, as a photosynthetic organ and is concerned with the synthesis of food, the number of leaves per plant as well as the surface area of the leaf lamina are directly related with the growth and development of plants and subsequently, governing the biomass production. While screening the M_1 population for the leaf index and leaf dimension, it was observed that, all the mutagens used have modified the leaf morphology. Some of the leaves were completely modified in one or the other way, due to mutagenic action, and subsequently changed their normal shape. The leaves in control plants were alternate, remote, 4-12 x 1.5-4cm in size with ovate-oblong or ovate-lanceolate shape of lamina, obtuse, sub-cordate base, entire margin, acute or acuminate apex (Fig. 1). The leaf modifications were noted and categorized into four types, viz. modified apex, modified base, modified margin and modified lamina. The modified forms of the leaves were counted and their dose/concentration wise frequencies were calculated with resect to the total number of leaves counted per dose/concentration of all the mutagens and also calculated the percent frequency of the plants bearing leaf variants.

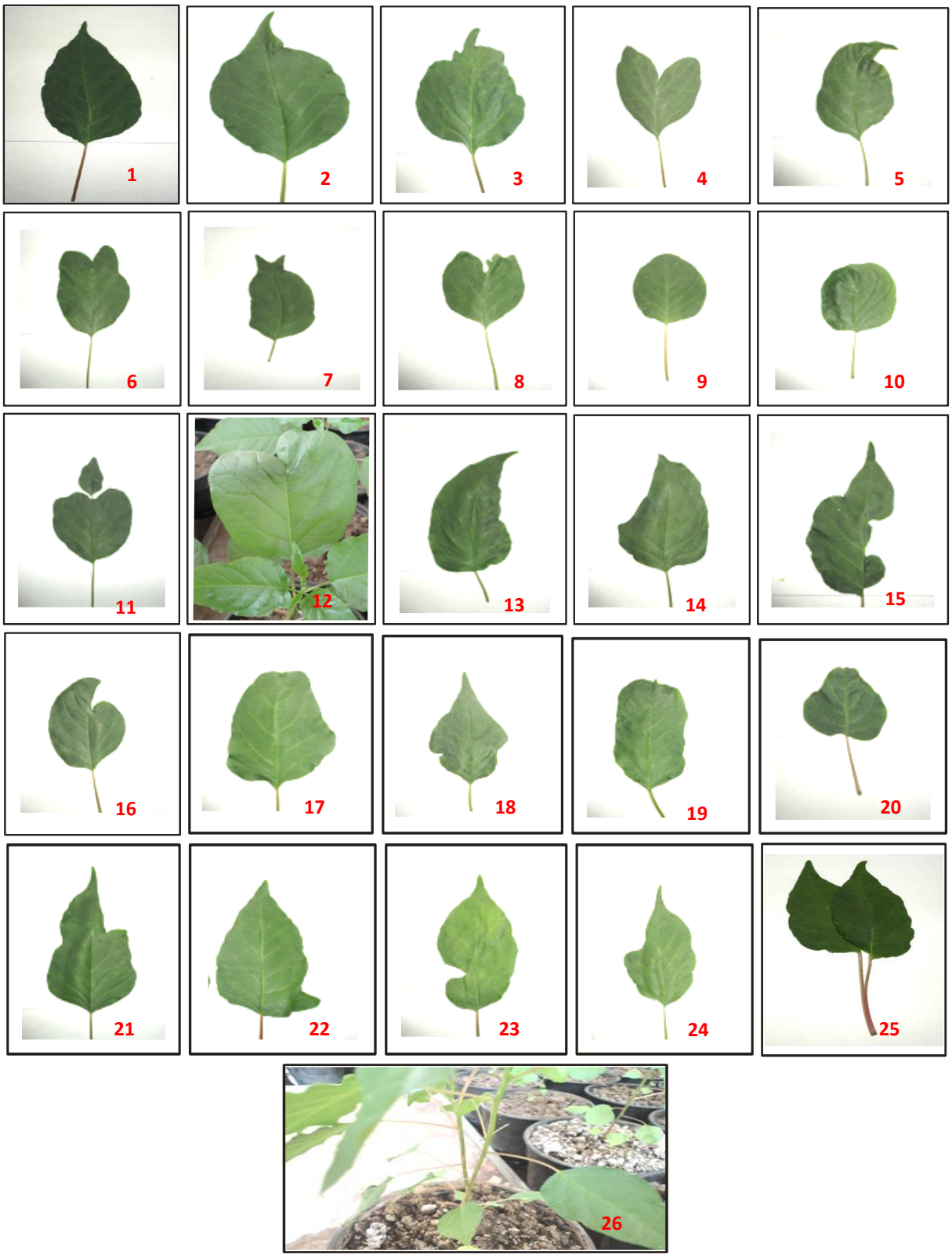
Effect of mutagens on leaf morphology:

Different doses/concentrations of all the mutagens have reported to affect the normal sub-cordate base into swollen, asymmetrical and rectangular, while the normal acute apex was observed to be modified into curved, obtuse, emarginate, semi-obtuse, beaked, retuse and incised apex (Figs. 2-24). Most of the modified leaves exhibited highly dissected and distributed margin, whereas, the normal ovate shape of the lamina was modified into various shapes and became bilobed, curved lamina, asymmetrical, imbalanced, expanded, irregular, rectangular, semi-cordate and pinched (Figs. 2-24). Mutagenized M_1 population of chemical mutagens also induced very rarely occurring adnation of petiole (Fig. 25), and leaf with forked and acme apex (Fig. 11). Bifurcation of stem (Fig. 26) was also reported from M_1 generation raised from EMS treated seeds.

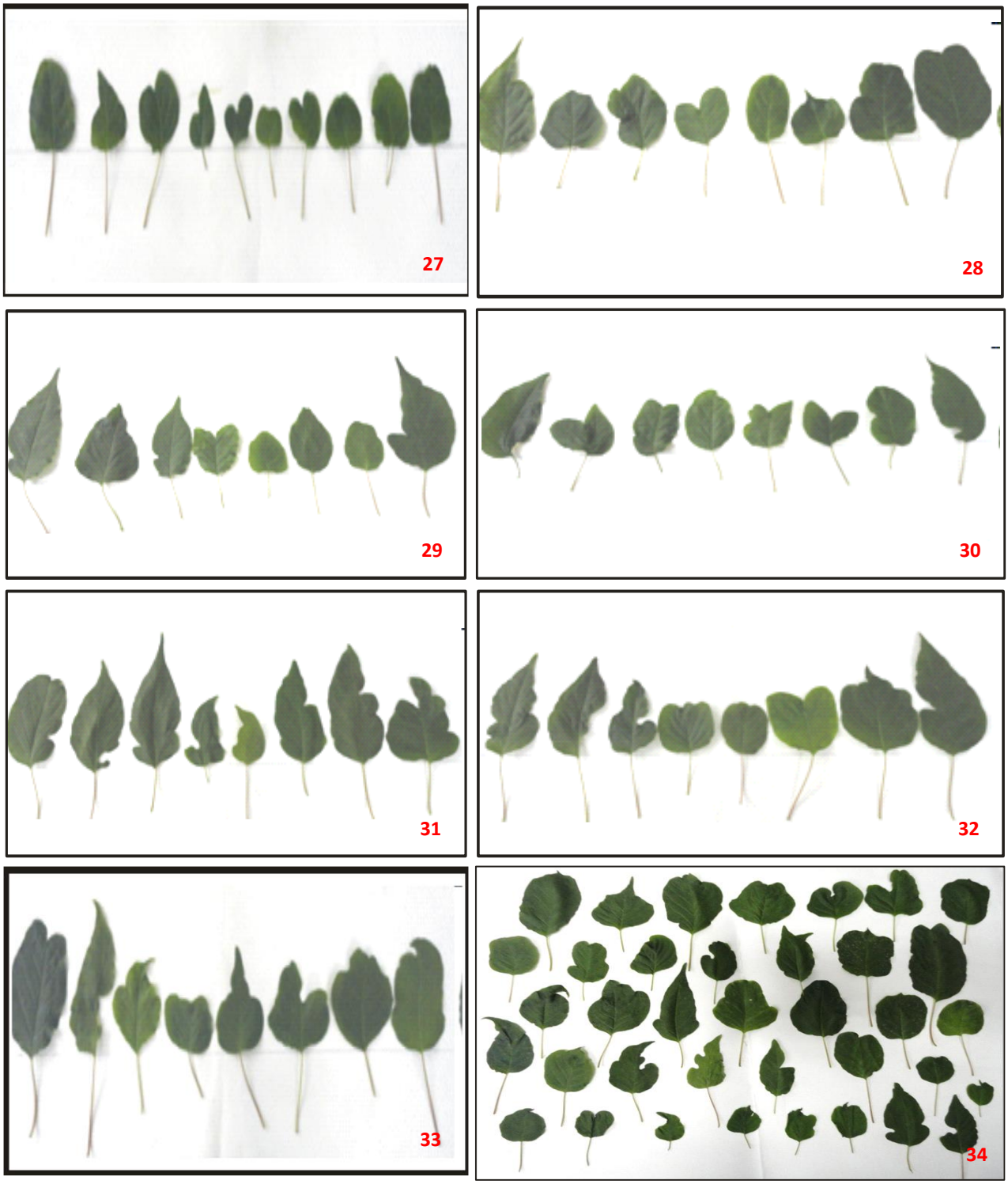
Data on leaf modification (Table 1-3) clearely revealed that, the leaf morphology was differentially affected by different doses/concentrations of the mutagens. Various types of modifications in the leaf morphology also revealed that all the mutagens have interfered with the ontogeny of leaf development by affecting the leaf primordial cells in *Rivinia humilis* L. The control plants were devoid of any prominent modifications in leaf morphology, whereas, gamma irradiation had deleterious effect on the leaf morphology and it was induced in dose dependent manner (Table 1). Progessive enhancement in plants with leaf variants was reported from 50 to 125Gy dose of gamma rays and ranged between 10.95-21.24% (Table 1). Plants with leaf variants was found to be maximum (21.53%) at 125Gy dose, but it was relatively less (20.83%) at higher (150Gy) dose of gamma rays.

Gamma irradiation modified leaves in dose dependent manner. The leaves in control plants were normal in morphology, however, percent frequency of modified apex ranged between 0.70-1.33 percent, due to gamma irradiation. Lower doses (50, 75 and 100Gy) of gamma rays was found to have no effects on leaf base, whereas, it was modified only by 125Gy (0.90%) and 150Gy (1.17%) doses of gamma rays (Table 1). Leaf margin and leaf lamina were found to be modified by all doses of gamma rays with percent frequency for leaf margin ranged between 0.90-2.97%, and that of leaf lamina between 1.16-3.58%. Total percent frequency was found to be gradually enhanced in dose dependent manner (Table 1), the values being ranged between 2.76-9.05%.

In terms of affecting leaf morphology, genome of *R. humilis* behaved more or less similar way in response to the treatment of both the chemical. Percent frequency of plants with leaf variants noted to be gradually enhanced in concentration dependent manner, in all treatment modes (Table 2, 3). However, EMS exhibited more pronounce effect than its counterpart mutagen. Percent frequency of plants with leaf variants in seeds treated with SA ranged between 7.50-10.83, 9.17-14.14 and 5.83-10.83% in dry seed, presoaking in water for 3h and 6h, respectively (Table 2). Effectiveness of SA was found to be enhanced in both presoaking treatment than dry seed treatment mode, however, 3h presoaking had more pronounce effect than 6h presoaking treatment mode indicating marginal reduction in effectiveness due to prolonged duration of presoaking of seeds in water.



Figures:1.-24: Mutagen induced modifications in the leaves of *R. humilis*, in M_1 generation. 1. Leaf in control plant (Ovate, acute, crenate), 2. Acuminate apex, 3. Beaked apex, 4. Bilobed apex, 5. Curved apex, 6. Emarginate apex, 7. Forked apex, 8. Incised apex, 9. Obtuse apex, 10. Semi-obtuse (Rounded) apex, 11. Acme apex, 12. Lobed apex, 13. Curved lamina, 14. Asymmetrical lamina-1, 15. Asymmetrical lamina-2, 16. Asymmetrical lamina with incised apex, 17. Expanded lamina with retuse apex, 18. Pinched lamina with acute apex, 19. Rectangular lamina with pointed apex, 20. Semi-cordate lamina, 21. Disturbed margin-1, 22. Disturbed margin-2, 23. Incised margin, 24. Asymmetrical lamina with irregular margin, 25. Adnation of petiole, 26. Bifurcation of stem.



Figures: 27.-34: Different types of modifications in leaf morphology in *R. humilis* recorded from M_2 and M_3 generations. 27. Mutagen induced modifications in leaves observed from gamma irradiated M_2 generation. 28. Modified leaves in M_2 generation of dry seed treatment mode of SA, 29. Modified leaves in M_2 generation of 3h presoaking treatment mode of SA, 30. Modified leaves in M_2 generation of 6h presoaking treatment mode of SA, 31. Modified leaves in M_2 generation of dry seed treatment mode of EMS, 32. Modified leaves in M_2 generation of 3h presoaking treatment mode of EMS, 33. Modified leaves in M_2 generation of 6h presoaking treatment mode of EMS, 34. Modified leaves in M_3 generation of all mutagens.

Table 1: Effect of different doses of gamma irradiation on leaf morphology forms in *Rivinia humilis* L., in M_1 generation.

Parameters Dose (Gy)	No. of plants screened	No. of leaves screened	Frq. of plants with leaf variants (%)	Frequency of variation in leaf form and shapes (%)				Total frequency (%)
				MA	MB	MM	ML	
Control (DS)	**40**	**477**	**-**	**-**	**-**	**-**	**-**	**-**
50	33	516	10.95 ±0.513	0.70 ±0.096	-	0.90 ±0.095	1.16 ±0.292	2.76 ±0.191
75	26	149	13.06 ±0.326	0.98 ±0.245	-	1.31 ±0.398	1.31 ±0.077	3.60 ±0.165
100	21	262	16.37 ±1.948	1.27 ±0.169	-	1.39 ±0.099	1.87 ±0.946	4.53 ±0.852
125	8	23	21.53 ±7.233	0.90 ±0.900	0.90 ±0.900	2.42 ±1.678	2.75 ±1.179	6.97 ±1.975
150	7	93	20.83 ±7.349	1.33 ±0.672	1.17 ±0.633	2.97 ±2.082	3.58 ±2.975	9.05 ±0.647
200	-	-	-	-	-	-	-	-

MA= Modified apex, MB= Modified base, MM= Modified margin, ML= Modified lamina; ± = Standard error

Table: 2. Effect of different concentrations of SA on leaf morphology, under variable treatment modes in *Rivinia humilis* L., in M_1 generation.

Parameters Concentration (%)	No. of plants screened	Frq. of plants with leaf variants (%)	No. of leaves screened	Frequency of variation in leaf forms and shapes (%)				Total frequency (%)
				MA	MB	MM	ML	
Control (DS)	**40**	-	451	-	-	-	-	-
0.0075 (DS)	40	7.50 ±1.443	388	1.20 ±0.020	0.55 ±0.283	0.86 ±0.085	1.46 ±0.030	4.07 ±0.249
0.010 (DS)	40	9.17 ±0.833	381	1.40 ±0.026	1.05 ±0.048	1.32 ±0.060	1.58 ±0.072	5.35 ±0.163
0.020 (DS)	40	10.83 ±1.667	358	1.76 ±0.206	1.30 ±0.118	1.60 ±0.312	1.88 ±0.271	6.55 ±0.659
Control (PSW-3H)	**40**	-	**432**	-	-	-	-	-
0.0075 (PSW-3H)	40	9.17 ±0.833	379	1.75 ±0.072	1.06 ±0.078	0.96 ±0.055	1.94 ±0.075	5.71 ±0.127
0.010 (PSW-3H)	40	13.33 ±0.833	368	1.90 ±0.169	1.45 ±0.113	1.27 ±0.201	2.16 ±0.114	6.78 ±0.237
0.020 (PSW-3H)	40	14.17 ±0.833	364	2.12 ±0.114	1.64 ±0.035	1.64 ±0.035	2.58 ±0.136	7.97 ±0.141
Control (PSW-6H)	**40**	_	**565**	-	-	-	-	-
0.0075 (PSW-6H)	40	5.83 ±0.833	505	1.24 ±0.145	0.79 ±0.017	0.59 ±0.011	1.57 ±0.077	4.19 ±0.194
0.010 (PSW-6H)	40	8.33 ±0.833	483	1.46 ±0.176	0.89 ±0.035	0.75 ±0.052	1.87 ±0.073	4.97 ±0.199
0.020 (PSW-6H)	40	10.83 ±0.833	466	1.57 ±0.183	0.99 ±0.076	1.07 ±0.130	2.07 ±0.076	5.70 ±0.299

Table: 3. Effect of different concentrations of EMS on leaf morphology, under variable treatment modes in *Rivinia humilis* L., in M_1 generation.

Parameters Concentration (%)	Total number of plants screened	Freq. of plants with leaf variants (%)	Total number of leaves screened	Frequency of modification in leaf morphology (%)				Total freq. (%)
				MA	MB	MM	ML	
Control (DS)	**40**	-	**467**	-	-	-	-	-
0.50 (DS)	40	10.00 ±0.184	485	0.95 ±0.142	0.62 ±0.121	1.59 ±0.172	2.13 ±0.232	5.28 ±0.163
1.00 (DS)	40	11.67 ± 0.285	459	1.08±0.744	0.87 ±0.077	1.74 ±0.224	2.41 ±0.245	6.10 ±0.243
1.50 (DS)	40	13.33 ±0.124	448	1.25 ±0.265	1.04 ±0.231	1.93 ±0.066	2.69 ±0.066	6.90 ±0.201
Control (PSW-3H)	**40**	-	**495**	-	-	-	-	-
0.50 (PSW-3H)	40	11.67 ±0.014	498	1.20 ±0.028	0.79 ±0.268	1.67 ±0.274	2.61 ±0.249	6.26 ±0.192
1.00 (PSW-3H)	40	14.17 ±0.283	470	1.34 ±0.329	1.06 ±0.035	1.91 ±0.126	2.97 ±1.021	7.29 ±0.082
1.50 (PSW-3H)	40	15.00 ±0.886	439	1.44 ±0.054	1.21±0.118	2.27 ±0.088	3.33 ±0.689	8.24 ±0.244
Control (PSW-6H)	**40**	-	**560**	-	-	-	-	-
0.50 (PSW-6H)	40	15.00 ±0.175	600	1.60 ±0.128	1.32 ±0.226	2.04 ±0.055	2.98 ±0.342	7.95 ±0.545
1.00 (PSW-6H)	40	18.33 ±0.148	521	1.72 ±0.722	1.47 ±0.012	2.17 ±0.169	3.38 ±0.452	8.74 ±0.344
1.50 (PSW-6H)	40	19.17 ±0.036	532	1.94 ±1.029	1.57 ±0.168	2.75 ±0.055	3.43 ±0.072	9.69 ±0.642

Treatment of SA found to alter all the morphological features of leaf, in all treatment modes. Leaf apex was found to be affected by dry seed treatment in the range of 1.20-1.76%, while, 3h and 6h presoaking treatment modes affected it in the range of 1.75-2.12 and 1.24-1.57%, respectively. Concentration dependent increase in modification of leaf base, margin and lamina was observed in all treatment modes of SA. Leaf base was modified in the range of 0.55-1.30, 1.06-1.64 and 0.79-0.99% in DS, PSW-3H and PSW-6H treatment modes, respectively, whereas, the percent frequency for the modification in leaf margin was found to be between 0.86-1.60% in DS, 0.96-1.64% in PSW-3H and 0.59-1.07% in PSW-6H. Leaf lamina was found to be the most affected feature of leaf, which was modified into various shapes. Gradual enhancement in modification of leaf lamina noticed in all treatment modes of SA. The values of percent frequencies of modification in leaf lamina ranged between 1.46-1.88, 1.94-2.58 and 1.57-2.07%, respectively in DS, PSW-3H and PSW-6H treatment modes. Concentration dependent enhancement in modification of all features of leaf due to treatment of SA consequently resulted in gradual increase in total percent frequency, which ranged between 4.07-6.55% for dry seeds, 5.71-7.97 and 4.19-5.70% for 3h and 6h presoaking treatment modes, respectively (Table 2).

Deteriorating effect on overall leaf morphology was found to be more due to treatment of EMS as compared to SA. Leaf morphology was found to be affected by all concentrations of EMS, in all treatment modes and consequently, the percent frequency of plants with leaf variants increased with the increment in mutagen concentration (Table 3). Dry seed treatment mode of EMS increased the percent frequency of plants with leaf variants in the range of 10.00-13.33%, whereas, the same was ranged between 11.67-15.00 and 15.00-19.17% in PSW-3H and PSW-6H modes, respectively. Enhancement in deletorious effect and drastic alteration in leaf morphology was observed with the increase in presoaking duration of EMS treatment. EMS treatment was found to be more effective than SA and gamma irradiation in inducing leaf modifications. Leaf modifications was found to be linearly related with concentrations of EMS in all treatment modes. The frequency of modification in leaf apex, in dry seeds, ranged from 0.95 to 1.25%, while the variation frequency for leaf base, in the same treatment mode, ranged from 0.62 to 1.04%. Dry seeds treatment mode of EMS modified leaf margin and leaf lamina in the range of 1.59-1.93% and 2.13-2.69%, respectively (Table 3). Effectiveness of EMS was reported to be enhanced not only with the presoaking of seeds but also found to be more with the increase in presoaking duration. Modification in leaf apex and leaf base, ranged between 1.20-1.44% and 0.79-1.21%, respectively, in PSW-3H mode, whereas, leaf margin and leaf lamina was modified with frequencies between 1.67-2.27% and 2.61-3.33%, respectively. Increased presoaking duration from 3 to 6h increased the effectiveness of mutagen. All the features of leaf were found to be modified in concentration dependent manner; however, the leaf lamina is found to be more prone to the effect of EMS than other morphological features. The modification frequencies of leaf apex, leaf base, leaf margin and entire leaf lamina, due to EMS treatment, in 6h presoaking treatment mode, ranged between 1.60-1.94%, 1.32-1.57%, 2.04-2.75% and 2.98-3.43%, respectively (Table 3). Gradual and concentration related increase in all modification frequencies of all features of leaves have consequently resulted in concentration dependent enhancement in total percent frequencies, the values being ranged between 5.28-6.90 in dry seeds, 6.26-8.24 in 3h and 7.95-9.69% in 6h presoaking treatment mode.

The comparative analysis of the data on morpho-mutagenic variations in leaf morphology clearly revealed that, the leaf character in *Rivinia humilis* L. is drastically affected and altered by all the mutagens used. EMS was observed to be more effective than other mutagens, SA and gamma rays. Presoaking of seeds in water for 3h had exhibited more effectiveness of SA than dry seed and presoaking for 6h, whereas, in case of EMS treatment, both presoaking treatment modes proved to be more effective on induction of morphomutagenic variations in morphology of leaf. Few of the leaf modifications were also reported from M_2 (Figs. 27-33) and M_3 generations (Fig. 34) of all the employed mutagens.

Leaves, the lateral green colored appendages of the plants, are photosynthetically important organ of the plant. The initiation of leaves and number of leaves (leaf index) are under strong genetic control and hence these quantitative characters are also genetically defined. The mutagenic screens in model systems have identified genes and the molecular mechanisms regulating initiation, development and shape of leaves are well established (Drost *et al*., 2015). The reduction in

different quantitative characters of the plants, due to the treatment of mutagens, have been attributed to various reasons by different workers. It may be due to inactivation and/or disturbances in auxin synthesis (Gordon, 1957), and extent of chromosomal aberrations (Sparrow and Evan, 1961). Misra *et al*. (2009) and Kapadiya *et al*. (2014) also documented similar evidences in *Chrysanthemum*. Neary *et al.* (1957) mentioned that the damage caused to genetical material and the arrest of cell division by the higher doses of gamma rays may be the probable cause of reduction in quantitative traits. Moh (1962); Abraham and Nilan (1968); Mickaelsen *et al*. (1968) stated that, the plants get killed at higher doses of radiations because of the severe negative effects of the physical mutagens on the tissues and many mutations are lethal. These authors opined that, the primary injuries in the form of retardation or inhibition of cell division are harmful, and cell death affects the growth habit and induce changes in plant morphology. They further stated that these effects are induced by cytological changes, such as chromosomal damages, inhibited mitotic division, degeneration of nuclei, cell enlargement.

Various workers (Rai and Das, 1978; Rao and Rao, 1983; Odeigah *et al*., 1998 and Naik and Murthy, 2009) have earlier documented the reduction in quantitative characters due to the mutagenic effect and has been attributed it to the physiological and other disturbances at the genetic level like chromosome damage, disturbed coiling and failure or restricted pairing. Many aspects of plant development is regulated by auxin (Vanneste and Friml, 2009; Wolters and Jurgens, 2009), and the distribution of auxin in the cell is mediated by PIN protein, that determines the direction of auxin intercellular flow and therefore, influence growth and development of plant (Winsniewska *et al*., 2006).

The size and symmetry of leaves in *R. humilis* has been greatly altered to a considerable extent due to the treatment of all the three mutagens. Alteration in size and shape of leaves in different plants, due to mutagens, have been documented earlier by Mensah *et al*. (2007), Warghat *et al*. (2011), Adelanwa *et al*. (2011), Akhtar (2014), Mshembula (2012), Kuchanova *et al*. (2012), Ramesh *et al*. (2013), Nura *et al.* (2013), Laskar and Khan (2014) and Islam *et al*. (2015), etc. Plants raised from untreated seeds showed normal leaves, however, different doses/concentrations of the mutagens have alterered the normal leaf architecture in one or the other way. The percent frequency of plants carrying leaf modification was reported to be increased with the increase in dose/concentration of all the mutagens. Hazama (1968), Katagiri (1970), Kuchkarov and Ogustove (1987), Datta and Banerjii (1993), Kashid and Salve (2014) also documented the mutagen induced severe modifications in the leaf morphology. Moh (1962), Abraham and Nilan (1968), Mickaelsen *et al*. (1968), Raghuvanshi and Singh (1974) and Karpate and Choudhary (1997) thought that the irregularities in leaf morphology may be induced by the disturbances in phytochromes due to chromosomal aberrations, disruption of auxin synthesis and mineral deficiencies, enlargement of palisade and spongy tissues and mitotic inhibition due to mutagenic treatments. Joshua *et al*. (1972) attributed it to the pleiotropic action of mutated genes. However, Rai and Das (1978) were in support of the mutagen induced physiological damages responsible for inducing deviation in leaf morphology. Pakorn *et al*. (2009) demonstrated the decrease in leaf area with the increasing intensity of irradiation due to the destruction in genetic material and reduction in rate of cell division. Kashid and Salve (2014) related it to the changes in physiological and metabolic activities of the developing primordial as a consequence of mutagenic treatment.

Burssen *et al*. (2000) suggested that the expansion of leaves is a result of cell expansion which is governed by various biochemical factors. According to Tsukaya (2006) leaf shape can be regulated by differential cell elongation (polarity) or differential cell proliferation, whereas, Drost *et al*. (2015) after confirmation of the expansion of leaf lamina by auxin flux, suggested the key role of auxin in determining the shape of leaf lamina. Molecular studies have confirmed the role of auxin in leaf initiation (Scanlon, 2003; Reinhardt *et al*., 2008), lamina margin elaboration (Hay and Tsiantis, 2006; Barkoulas *et al*., 2008) and leaf vasculature patterning (Scarpella *et al*., 2006). Number of genes and networks have been described that affect initial leaf development and pattern of formation in both simple and complex leaves (Hay and Tsiantis, 2006; Barkoulas *et al*., 2008 and Drost *et al*., 2015). Similarly, mutagenic screens have been identified genes that regulate leaf blade shape, width, length and their ratios (Tsuge *et al*., 1996; Narita *et al*., 2004). The quantitative trait loci (QTL) analysis in several plants revealed a polygenic control of leaf trait. Quantitative trait loci have been identified for leaf morphological traits in several species such as *Poplar* (Wu *et al*., 1997), maize (Mickelson *et al*., 2002), tomato (Holtan and Hake, 2003), *Arabidopsis* (Juenger *et al*., 2005), and oak (Gailing, 2008).

Recently, Drost *et al*. (2015), while studying the genomics of *Populus* leaf variation identified a potential candidate gene (PtARF1) as important regulator of leaf shape variation, and hypothesized that variation in lamina shape is a product of inter-specific polymorphism within the leaf QTL. These workers identified 161 transcripts regulating the leaf trait. Of which 25 were found associated with either lamina width, length: width ratio, or both. Whereas, only two out of that 25 genes were identified regulating the lamina shape. It was further reported that the dynamics of endocytosis-mediated PIN protein localization in leaf cell is governed by PtARF1 transcripts that affect the lateral auxin flux and subsequently leaf lamina expansion. The accumulation of PIN in intracellular compartments, abolishing polar localization and disrupting normal auxin flux is responsible for leaf lamina variation (Geldner *et al*., 2001; Xu and Scheres, 2005). The PIN-FORMED (PIN) proteins are the plant-specific family of transmembrane proteins that transport plant signal molecule. It was first described in model plant *Arabidopsis thaliana*, where the loss-of-function mutation in the gene develop floral organ properly and generate naked, pin-like inflorescence which gave the name PIN-FORMED **(**Krecek *et al*., 2009). ADP-ribosylation factors (ARF proteins) have been shown to control the localization of auxin efflux carriers, which function to establish auxin gradients and apical-basal cell polarity in developing plant organs. Decreased abundance of ARF1 transcript and subsequently ARF1 protein slows the process of PIN polarization through the endocytic pathway during development and expansion. Decreased PIN polarization results in increased lateral auxin flux and, subsequently, increased lamina expansion in the leaf width direction. More rapid PIN polarization increases auxin flux and expansion of the lamina in the leaf length direction, decreasing lateral leaf expansion. Thus, the drastic modifications observed in leaf morphology, in the present investigation, might be the result of differential expression of leaf trait regulating transcripts, due to mutagenic treatment, that might have affected the localization of PIN protein and subsequently the auxin flux. Morphomutagenic variations induced by the employed mutagens in *R. humilis* may be the result of mutagenic action that might have caused the inactivation or disturbances in the auxin synthesis as well as cyto-toxic effects of the mutagens induced in terms of chromosomal aberrations or gene mutations.

In *R. humilis* treatment of EMS also induced the forking of stem and leaf apex as well as petiole adnation. The rarely occurring abnormalities like stem bifurcation, forked leaf apex and adnation of petiole, due to mutagenic action in *R. humilis*, could be due to death of apical cells, disturbances in auxin formation and destruction of terminal meristem followed by development of two apices in the stem shoot and leaf apical meristem. Similar observation were also noted by Chandramouli (1970) in EMS treated maize and Broertjes and Van Harten (1998).

CONCLUSION

Morphological variations observed in all the mutagenized populations have confirmed the sensitivity of the *Rivinia humilis* genome to all the employed mutagens. These mutagens were able to induce variability in the genetic architecture of the plant that has exhibited in different traits in the mutagenized populations. *R. humilis*, though brought to India purely for ornamental purpose, it is found best suited to the Indian agro-climatic condition. The variability induced by the mutagens can be favourably exploited for the improvement of agronomic characters, more particularly, the dye yielding potential of the plant can be commercialized in textile, pharmaceutical, cosmetic, food, and paint industries. More scientific inputs are the need of an hour to analyse various medicinal properties of the plant. Mutagen induced morphological variability due to the mutations caused at genetic and molecular is sound reason to coin the term 'morphomutagenic variations' introduced through this article.

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