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Chlorophyll deficiencies induced by physical and chemical mutagens in mutagenized population of *Rivinia humilis* L.

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ABSTRACT

We identified *Rivinia humilis* as a source of natural dye. In order to induce variability in the genome of the plant, primarily for the qualitative and quantitative improvement in dye content, the seeds of the plant were subjected to the treatment of physical (gamma irradiation) and chemical mutagens (SA and EMS). Gamma rays are known to induce gross structural changes in the chromosomes leading to the alteration of various characters, whereas, both the chemical mutagens have already proved their mutagenic effect on induction of variability in different plants. Generally, the chemical mutagens induce point or gene mutations leading to the base pair substitution and thus changing the functions of proteins without abolishing them. Mutagens are known to affect pigment synthesis in the leaves of plant due to alteration in genetic architecture, resulting in the production of various types of chlorophyll deficiencies. Induction and spectrum of chlorophyll mutations is considered as markers for the evaluation of gene action of mutagenic factors in induced mutation studies, and that can be used as a primary index of effectiveness and mutability of the genotypes towards the mutagen which would be useful to generate the wide array of desirable mutations in the treated population. Results obtained, in the present study, on effect of different doses/concentrations of the applied mutagens, indicated differential effect of all the mutagens in induction of chlorophyll abnormalities. Five different types of chlorophyll chimeras, viz., *Albino*, *Xantha*, *Chlorina*, *Viridis* and *Marginata* were reported to be induced by the mutagens in *R. humilis*. Both the chemical mutagens induced five types of chimeras, whereas, no dose of gamma rays was able to induce *Marginata*. Comparatively, treatment of seeds by SA was found to be more effective than EMS and gamma rays. Presoaking treatment modes, in both the chemical mutagens, were found to be more effective than dry seed treatment mode. Wide spectrum of induction of chlorophyll deficiencies clearly indicates the sensitivity of the genome of *R. humilis* to all the three employed mutagens.

Keywords: *Rivinia humilis* L., mutagens, effectiveness, chloprophyll chimeras, sensitivity.

INTRODUCTION

India is rich in natural wealth and there is ample scope to explore and revive application of natural dyes on textiles. Nature has gifted India about 500 colour yielding plants (Gokhale *et al*., 2004). Technologies have been developed for the extraction of natural dye, improvement of natural dyeing methods, yield, optimization of dyeing conditions, and to improve dyeability with natural dyes (Samanta and Agarwal, 2009). Natural dyes have certain inherent qualities that either has not been or cannot be matched by synthetic dyes. Natural dyes are biogenic in origin and are obtained from biodegradable sources. Many of them have pharmacological effects and possible health benefits. Almost all natural dyes are potential antioxidants and thus prevent the oxidative degradation to ensure the shelf-life of the food material. Natural dyes are not only cost-effective but also cheaper than the synthetic dyes and can be produced in bulk quantities.

We identified *R. humilis* as a source of red natural dye obtained from the fruits (berries). The plant is a perennial herb belonging to dicot family Phytolaccaceae. Basically, it is native of tropical America and listed as notorious weed in several countries (Matthew, 1983). The plant is found growing as an occasional weed in Sri Lanka, Malaysia and India. According to Naik (1998), the plant is deliberately introduced in India from Florida, purely for ornamental purpose and now found to be grown mostly in gardens and green-houses. Due to the pearl like appearance of the ripened fruits, the plant is known as 'Motiya' in India. It bears green coloured unripped fruits called berries which turn red when ripened and yield red natural dye. The red colour yielding bioactive compound isolated by Imperato (1975) is structurally very much similar to betanin, the pigment present in beet root. He named it as rivianin or rivinianin. However, it was found to contain red-violet betacyanin derivative, and 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 properties too. 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 (Mitchell and Ahmad, 2006). Salvat *et al.* (2001) reported out the inhibitory effect of methonolic extracts of the branches of the plant against various strains of bacteria such as *Escherichia coli, Salmonella typhimurium, Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus and Enterococcus faecium*. Fathima and Tilton (2012) also confirmed the radical scavenging activities of methonolic leaf extracts, and suggested its potent antioxidant activity. Khan *et al.* (2011) 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^{o} C, without the alteration of product quality. Studies by Joseph and Avita (2013) on antibacterial activities of the root and shoot of the plant against 10 bacterial strains and 4 fungal species reported the inhibitory effect against all the strains.

Induction of variability in the genome is the pre-requisite for selection and varietal development in crop plants. Modification in genetic architecture through induction of mutation has become proven way of creating variability within crop and other useful plant species. Mutations are the tools and being used to study the nature and functions of the genes which are the buiding blocks and the basis of the plant growth and development, thereby producing raw materials for genetic improvement of crops with economic importance (Adamu and Aliyu, 2007). Mutagenesis offers the possibility of inducing desired attributes that either cannot 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). The mutations, may be spontaneous or induced, leads to loss or gain of a 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. Induced mutation, due to modification in genetic architecture, leading to the change in biochemical pathways, are reflected at physiological, morphological and anatomical levels in the first generation (Aney 2013a, b). However, mutagen induced variations in different traits may be beneficial or harmful, sometimes lethal.

Various physical and chemical mutagens were widely used for the induction of genetic variability. The variations induced at morphological, anatomical, physiological and yield attributing level help to identify and isolate the mutants for desirable characters from the mutagenized populations. Physical mutagens, particularly, gamma rays are known to produce gross changes at the structural level of chromosomes, and that may have inhibitory effect on most of the morphological and yield attributing characters. The chemical mutagens, generally induce point or gene mutations, leaing to the base pair substitutions and thus changing the functions of proteins without abolishing them. Mutagenic effects for creating variations of both the chemical mutagens i.e. sodium azide and ethyl methanesulphonate has been already proved in various plants. 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 plant genome subjecting to the treatment of various doses/ concentrations of the mutagens used. The mutagenized population was screened for various morphological and yield attributing characters. Mutagen induced chlorophyll chimeras and chlorophyll mutants is considered as markers for the evaluation of gene action. Present article deals with the effect of mutagens on chlorophyll synthesis as a primary index of effectiveness and mutability of the genotype towards the mutagen which would be useful to generate the wide array of desirable mutants in the mutagenized population. The high dye yielding mutants of the plant can provide an opportunity to be an alternative source of red natural dye which are quite difficult to obtain from the underground parts of the other existing dye yielding plants.

MATERIALS AND METHODS

R. humilis L. was identified as a source of natural dye. Initially, the germplasm 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. Collected seeds were separately grown for the screening of high dye content. Finally, healthy and uniform sized seeds, from the plants raised from the seeds obtained from Paradise Nursery, were used for the treatment of physical mutagen (gamma irradiation) and two chemical mutagens (Sodium azide and Ethyl methansulphonate). Initially, in order to determine LD_{50}, the seeds were exposed to 100, 150, 200, 250, 300 and 350Gy doses of gamma irradiation, in gamma chamber, using ^{60}Co source of gamma radiation. In case of both the chemical mutagens, three treatment modes i.e. dry seeds (DS), presoaking in water for 3h (PSW-3H) and 6h (PSW-6H) were used. 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 of particular mutagen was determined on the basis of 50% reduction in germination count, seedling height and root length, compared to the value in 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 mutagen were selected for the induction of mutation in the genome of the *R. humilis*. Healthy and uniform sized seeds were exposed to 100, 150 and 200Gy doses of gamma rays, whereas, 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, in order to raise the M_1 generation. 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 plants was found to be survived after one month of germination and hence, the dose rate was reduced to 50, 75, 100, 125 and 150Gy.

Chlorophyll deficiencies in M_1 generation

Effect of different doses/concentrations of all the employed mutagens was observed on chlorophyll deficiencies in M_1 generation. Different types of chlorophyll chimeras were recorded in M_1 generation and classified according to the classification proposed

by Gustafsson (1940). Identified chimeras were classified as *Albino* (white), *Xantha* (golden-yellow), *Chlorina* (yellowish-green), *Viridis* (pale green) and *Marginata* (white margin). Number of chlorophyll mutants were isolated from M_2 and M_3 generations.

RESULTS AND DISCUSSION

Mutagen induced chlorophyll deficiencies in M_1 generation:

Different mutagens are known to affect pigment synthesis in leaves of the plants due to the change in genetic material, and that can lead to the production of different types of chlorophyll chimeras. Different doses/concentrations of the mutagen differentially induced chlorophyll chimeras in M_1 generation, in the form of streaks and sectors on the leaves. Five different types of chlorophyll chimeras viz., *Albino* (white), *Xantha* (golden yellow) *Chlorina* (yellowish green) *Viridis* (light green) and *Marginata* (white margin) were recorded (Figs. 1-6).

Exposure of seeds to gamma rays induced four types of chlorophyll chimeras, except marginata, with varying frequencies. *Xantha* were induced by all the doses of gamma rays, with frequency ranged between 1.37-3.65%, whereas, the other types were induced at certain doses, particularly at higher doses of gamma rays. *Albino* was found to be induced by 125Gy dose and above with percent frequency ranged betweern 1.47 to 3.59%, whereas, *Viridis* were recorded only at 125 and 150Gy doses of gamma rays (Table 1). Induction frequency of both *Albino* and *Xantha* was found linearly associated with doses of gamma rays, while *Chlorina* and *Viridis* were induced by certain doses with varying frequencies, the values for *Chlorina* were 0.73, 1.02 and 0.56% at 100, 125 and 150Gy doses, respectively, and for *Viridis* were 0.97 and 0.54% at 125 and 150Gy doses of gamma rays. No chimeras were found to be induced by higher (200Gy) dose of gamma rays (Table 1). *Albino* and *Xantha* were observed at seedling stages and all of them did not survive till maturity. The total percent frequency of all types of chimeras was observed to be increased in dose dependent manner and ranged between 1.37 and 7.24%.

Both the chemical mutagens also induced different types of chlorophyll chimeras. The genome of the plant under investigation responded in a similar fashion to both the chemical mutagens. All five types of chimeras were induced by both mutagens with varying frequencies, but the *Marginata* was more prominently observed in all treatment modes of SA, whereas, it was induced by certain concentrations of EMS, in both presoaking treatment modes only. Comparatively, SA was found to be more effective than EMS. Differential effects of SA was observed in different treatment modes.

In dry seed treatment, no concentration of SA induced the *Albino* chimera, however, all chimeras were induced in concentration depended manner, except *Chlorina* (Table 2). Both *Xantha* and *Viridis* were induced with the same percent frequency by different concentrations of SA, and was ranged between 0.72 and 1.09%, whereas, *Chlorina* and *Marginata* were induced in the range of 0.65-1.17 and 0.24-1.09%, respectively. The lower (0.0075%) concentration was found to be more effective in inducing *Chlorina* than the remaining concentrations, the value being 1.17%. Dry seeds treatment mode induced all types of chimeras with total percent frequencies varied from 2.09 to 4.04% (Table 2).

Table: 1. Frequency of chlorophyll chimeras induced by different doses of gamma (γ) irradiation in *Rivinia humilis* L., in M_1 generation.

Parameters Dose (Gy)	Frequency of different chlorophyll chimeras (%)					
	Albino	***Xantha***	***Chlorina***	***Viridis***	***Marginata***	**Total**
Control	-	-	-	-	-	-
50	-	1.37 ±0.656	-	-	-	1.37 ±0.686
75	-	2.17 ±0.028	-	-	-	2.17 ±0.028
100	-	2.23 ±0.023	0.73 ±0.366	-	-	2.96 ±0.345
125	1.47 ±0.073	2.94 ±0.148	1.02 ±0.512	0.97 ±0.490	-	6.40 ±0.778
150	3.35 ±0.065	2.81 ±0.600	0.56 ±0.563	0.54 ±0.929	-	7.26 ±0.610
200	3.59 ±0.662	3.65 ±0.788	-	-	-	7.24 ±0.736

± = Standard error

Table: 2. Frequency of chlorophyll chimeras induced by different concentrations of SA under variable treatment modes in *Rivinia humilis* L., in M_1 generation.

Parameters Concentration (%)	Frequency of different chlorophyll chimeras (%)					
	Albino	*Xantha*	*Chlorina*	*Viridis*	*Marginata*	Total
Control (DS)	-	-	-	-	-	-
0.0075 (DS)	-	0.72 ±0.554	1.17 ±0.534	0.70 ±0.301	0.24 ±0.313	2.09 ±0.931
0.010 (DS)	-	0.93 ±0.410	0.65 ±0.863	0.93 ±0.410	0.63 ±0.417	3.13 ±0.424
0.020 (DS)	-	1.09 ±0.488	0.76 ±1.010	1.09 ±0.488	1.09 ±0.488	4.04 ±0.457
Control (PSW-3H)	-	-	-	-	-	-
0.0075 (PSW-3H)	0.55 ±0.366	0.81 ±0.357	0.77 ±0.341	0.51 ±0.338	0.50 ±0.660	3.13 ±0.070
0.010 (PSW-3H)	0.33 ±0.436	1.36 ±0.032	1.36 ±0.032	1.36 ±0.032	1.35 ±0.900	5.75 ±0.724
0.020 (PSW-3H)	0.40 ±0.526	1.23 ±0.540	1.67 ±1.110	1.62 ±0.915	0.82 ±1.090	5.73 ±1.077
Control (PSW-6H)	-	-	-	-	-	-
0.0075 (PSW-6H)	-	0.70 ±0.348	1.06 ±0.014	1.39 ±0.696	0.36 ±0.360	3.51 ±0.674
0.010 (PSW-6H)	0.48 ±0.480	1.44 ±0.043	0.98 ±0.492	1.89 ±0.480	0.50 ±0.503	5.30 ±0.608
0.020 (PSW-6H)	0.54 ±0.543	1.11 ±0.553	1.65 ±0.944	1.63 ±0.031	0.53 ±0.526	5.46 ±1.457

Table: 3. Frequency of chlorophyll chimeras induced by different concentrations of EMS under variable treatment modes in *Rivinia humilis* L., in M_1 generation.

Parameters Concentration (%)	Frequency of different chlorophyll chimeras (%)					
	Albino	*Xantha*	*Chlorina*	*Viridis*	*Marginata*	Total
Control (DS)	-	-	-	-	-	-
0.50 (DS)	-	0.70 ±0.350	0.69 ±0.343	0.70 ±0.700	-	2.09 ±0.620
1.00 (DS)	-	-	1.01 ±0.503	1.04 ±0.518	-	2.04 ±0.509
1.50 (DS)	-	0.55 ±0.553	1.61 ±0.028	2.13 ±0.495	-	4.29 ±0.543
Control (PSW-3H)	-	-	-	-	-	-
0.50 (PSW-3H)	-	0.72 ±0.361	-	0.36 ±0.360	-	1.08 ±0.623
1.00 (PSW-3H)	-	1.03 ±0.515	-	1.04 ±0.518	-	2.07 ±0.496
1.50 (PSW-3H)	0.55 ±0.553	1.07 ±0.536	1.07 ±0.535	1.11 ±1.110	0.54 ±0.543	4.35 ±0.594
Control (PSW-6H)	-	-	-	-	-	-
0.50 (PSW-6H)	-	0.36 ±0.360	0.73 ±0.365	-	0.74 ±0.371	1.83 ±0.360
1.00 (PSW-6H)	0.54 ±0.536	0.55 ±0.553	1.09 ±0.545	1.07 ±1.073	-	3.25 ±1.859
1.50 (PSW-6H)	-	0.57 ±0.573	1.15 ±0.573	1.15 ±1.147	-	2.87 ±2.067

Effectiveness of SA was found to be enhanced due to presoaking of seeds, which exhibited concentration dependent incease in induction of most of the chimeras. The *Albino* chimeras were induced more by lower (0.0075%) concentration than others, in PSW-3H treatment mode. All concentrations of SA, induced *Xantha*, *Chlorina* and *Viridis* in the range of 0.81-1.23, 0.77-1.67 and 0.51-1.62%, respectively. The *Marginata* were induced more by lower (0.010%) concentration than the remaining concentrations of SA, the values being 0.50, 1.35 and 0.82% at lower, medium and higher concentrations, respectively. The total frequency of all types of chimeras ranged from 3.13-5.75% with the treatment of mutagen.

PSW-6H treatment of SA differentially induced various types of chimeras. Medium (0.010%) and higher (0.020%) concentrations induced *Albino* chimeras with percent frequency of 0.48 and 0.54%, respectively, whereas, it was not observed at lower (0.0010%) concentration. The *Xantha* was found to be induced with percent frequency ranging between 0.70 and 1.44%, however, it was induced marginally more by medium (0.010%) concentration than by higher (0.020%) concentration, the value being 1.11%. The *Chlorina* was induced in the range of 0.98-1.65%, whereas, *Viridis* in the range between 1.39 and 1.89%, where the highest percent frequency was noted at 0.010%SA. The *Marginata* was also induced by all concentrations of the mutagen, in the range between

0.36-0.53%. Total percent frequency of all types of chimeras, ranged between 3.51-5.46% indicated linear increase in induction of chimeras with increase concentrations of mutagen (Table 2).

In *R. humilis* different concentrations of EMS differentially induced all types of chimeras, however, it was found less effective than SA. The *Albino* and *Marginata* chimeras were not observed at any concentrations of the mutagen in dry seed treatment mode. Dry seed treatment mode induced the *Chlorina* and *Viridis* in concentration dependent manner, whereas the *Xantha* was induced by lower (0.50%) and higher (1.50%) concentrations with frequency 0.70 and 0.55%, respectively (Table 3). The *Chlorina* and *Viridis* were found to be induced by all concentrations, in the range of 0.69-1.61 and 0.70-2.13% frequencies, respectively. *Viridis* were the most abundantly isolated chlorophyll deficient types in dry seed treatment mode. The total percent frequency was found to be in the range of 2.04-4.29%, where, it was almost similar at lower and medium concentrations of EMS (Table 3). PSW-3H treatment mode induced all five types of chimera, where, *Albino* (0.55%), and *Marginata* (0.54%) were induced only by higher (1.50%) concentration of EMS. The *Xantha* and *Viridis* were induced by all concentrations of the mutagen in concentration dependent manner, and were induced in the range of 0.72-1.07 and 0.36-1.11% frequencies, respectively. The total percent frequency of chlorophyll chimeras was found to be ranged between 1.08-4.35% (Table 3). PSW-6H treatment mode differentially induced all types of chlorophyll chimeras. All concentrations were found to be effective in inducing *Xantha* and *Chlorina* chimeras, however, *Albino* (0.54%) and *Marginata* (0.74%) were induced only by medium (1.00%) and lower (0.50%) conentrations, respectively. Increased concentration of EMS increased the induction of *Xantha* and *Chlorina* chimeras, which ranged between 0.36-0.57% for *Xantha* and 0.73-1.15% for *Chlorina*. *Viridis* were induced only by 1.00 and 1.50% concentrations of EMS with 1.07 and 1.15% frequencies, respectively, whereas, *Marginata* with 0.74% were induced by lower (0.50%) concentration of EMS. The total percent frequency of all types of chimeras ranged between 1.83-3.25%, where the maximum of percent frequency was noted at medium (1.00%) concentration of the mutagen.

Data depicted in table 1-3 clearely revealed that, chlorophyll synthesis was found to be affected by all the three mutagens used. Seeds exposed to gamma irradiation could not induce *Marginata*, while *Albino*, *Xantha*, *Chlorina* and *Viridis* were induced by all the mutagens. The *Xantha* was found to be the most commonly induced chlorophyll chimeras, followed by *Chlorina* and *Viridis.* Both the *Albino* and *Xantha* were observed during early seedling stage and resulted early death of seedling. The chlorophyll chimeras such as *Chlorina* and *Viridis* were found to recover at the later stages of growth of plants, whereas, the leaves showing *Marginata* were abscised and fall off from the plants. Few of the induced chlorophyll deficiencies were found to be transmitted to the next generations which were reported as chlorophyll mutants from both M_2 and M_3 generations (Figs. 7-12).

Gene mutations influencing the green colouration of photosynthetically active parts are among the most common spontaneous or induced alterations observed in many plants. Chlorophyll mutations are employed as markers for the evaluation of gene action of mutagenic factors in induced mutation studies (Gaul, 1964), and that can be used as a primary index of effectiveness and mutability of the genotypes towards the mutagen which would be useful to generate the wide array of desirable mutations in the treated population. The spectrum and frequency of chlorophyll deficiencies in M_1 generation was assessed, and were identified as *Albino, Xantha, Chlorina*, *Viridis* and *Marginata*. Earlier workers like Gaul (1964), Savin *et al*. (1968), Burton and Powell (1966), Sato (1966), Yadava and Chaudhary (1974), Dnyansagar and Gaikwad (1977), Chowdhary (1978), Lakshmi *et al*. (1996), Krishnaveni *et al*. (1998), Reddi *et al*. (2001), Shah *et al*. (2006), Srinivas and Veerabadhiran (2010), Kolar *et al*. (2011), Hòa and Giang (2012), Chatterjee *et al*. (2012), Bankar *et al*. (2013), Makeen *et al*. (2013), Patil and Rane (2015) have isolated mutagen induced various types of chlorophyll deficiencies and chlorophyll mutants in different plants.

Gustafsson (1940), at the first time, observed the chlorophyll mutations in barley and classified it on the basis of presence or absence of patches and variegations. Chatterjee *et al*. (2012), considered the chlorophyll mutations as broad morphological changes due to the secondary effects of physiological disturbances. The chlorophyll mutation frequency is

Figures: 1-12: Mutagen induced different types of chlorophyll chimeras in *Rivinia humilis* reported from mutagenized populations: 1-6: Chlorophyll chimeras in M_1 generation: 1. *Albino* seedling (Lethal), 2. *Albino* sector, 3. *Xantha* sector, 4. *Viridis* sector, 5. *Chlorina* sector, 6. *Marginata*, 7-12: Chlorophyll chimeras in M_2 and M_3 generations: 7. *Albino* at early growth, 8. *Albino* at late growth stage, 9. *Xantha* at seedling stage, 10. *Virescence*, 11. *Viridis* sector, 12. *Marginata* sector.

an indicator to predict the frequency of factor mutations and thus is an index for evaluation of genetic effects of mutagen (Gustafsson, 1951; D'Amato *et al*., 1962; Gichner and Veleminsky, 1965 and Walles, 1973). The results obtained in the present study are in agreement with the earlier reports by Ehrenberg and Gustafsson (1957); Ehrenberg (1960a and b); Nilan (1964); Swaminathan (1964); Konzak *et al*. (1965a and b); Ehrenberg *et al*. (1966); Goud (1967) and Wagner *et al*. (1968). These authors have also received dose/concentration dependent increment in the spectrum of chlorophyll mutations. Konzak *et al*. (1965a and b) opined that since, the mutagen induced chlorophyll mutations are of common occurrence and are easily detectable; these can be used for the assessment of effectiveness and efficiency of a mutagen. They proposed that efficiency of a mutagenic agent depends on the reaction of the plant to the mutagen and the degree of physiological damage, chromosomal aberrations and sterility caused by the mutagen.

Auerbach (1967) suggested that the number and types of chlorophyll mutations to be controlled by several secondary steps or 'sieves'. It involves various factors which determines whether a change in DNA will take place and even if it does, whether it will give rise to an observable mutations. In view of Stummann and Henningsen (1980), the chlorophyll mutations occur up to 15 days from the time of seed germination and in them primary leaves will be deficient of chlorophyll. The gamma irradiation induced *Albino*, characterized by drastic deficiency of both chlorophyll and carotenoids, were mostly observed at the seedling stage and did not survive. Similarly, the *Xantha*, also a lethal mutant, mostly survived upto 5-6 leaf stage, where the leaf colour was yellow to whitish yellow with prevailing carotenoid. The gamma ray induced *Albino* and *Xantha*, in the present investigation, is in agreement with the findings by Gustafsson (1963); Gupta and Sharma (1990) and Srinivas and Veerabadhiran (2010) who believed that ionizing radiations produce high frequency of *Albino* and *Xantha* mutations while the chemical mutagens induced other types of chlorophyll mutations in cereal crops. Westergaard (1960) also proposed that radiations produce extreme mutations (*Albino* and *Xantha*) compared to less drastic and recoverable mutations (*Chlorina* and *Viridis*), which are common in chemical mutagen treatment. Gaul (1964), Chekallin (1977) and Singh *et al*. (2005) also observed the occurrence of certain types of chlorophyll mutants more than the others in *Lathyrus sativus*. Reddy *et al*., (1994) hypothesized that the occurance of particular mutants in mutagenic treatment depends on the availability of mutagenic loci to the mutagen.

The induction of chlorophyll deficient mutation is directly related to the function of mutagen (Blixt, 1972). In *Rivinia humilis*, the chlorophyll chimera spectrum was found to be more with higher doses/concentrations of the mutagens. EMS was found to be more effective in inducing highest frequency of chlorophyll deficiencies. The *Albino* and *Xantha* were more with gamma irradiation, whereas, *Marginata* was induced by certain concentrations of both the chemical mutagens. Ryan and Heslot (1964) also observed the randomness in the action of physical mutagen and specificity of EMS to certain loci in barley for the production of chlorophyll chimeras and mutations. Brock (1965) attributed the increased chlorophyll mutation frequency at higher doses to the chromosomal aberrations or the saturation in the mutational events in the cells. D'Amato *et al*. (1962); Natrajan and Shivashankar (1965) and Kulkarni and Moghe (2013) also observed the higher frequency of chlorophyll chimeras induced by EMS treatment. Varguhese and Swaminathan (1968) stated that the chlorophyll mutations induced in wheat by EMS treatment are mainly due to the chloroplast DNA as EMS is more specific to guanine and cytosine bases. Constantin *et al*. (1974) also reported the fast neutrons and EMS as the most effective inducers of chlorophyll mutants. The chloroplast DNA is rich in guanine-cytosine content in spinach, beet, *Chlamydomonas* and *Chlorella* (Chun *et al*., 1963) and EMS is known to react preferentially with guanine (Freese, 1963). According to Chopra (2005), the high frequency of chlorophyll mutations obtained with mutagen, is due to the selective action of chemical and physical mutagens on genes which are responsible for chlorophyll development.

Various authors putforthed several causes to explain the induction of chlorophyll abnormalities due to mutagenic treatment. Sparrow and Woodwell (1962) thought that the chlorophyll abnormalities are induced due to the somatic mutations. However, Goud (1967) suggested that these abnormalities are the result of periclinal chimeras or plastid mutations. He further added that these abnormalities do not breed true in the subsequent generations. The chlorophyll

development seems to be controlled by many genes located on several chromosomes which could be adjacent to centromere and proximal segment of chromosomes (Swaminathan, 1964; Natarajan and Upaddhya, 1964 and Goud, 1967), and the mutation in these genes reflects in M_1 and subsequent generations in the form of various mutations. Ramulu (1970) suggested that there are at least 250-300 loci responsible for the synthesis of chlorophyll in barley. Gustafsson (1963) and Nilan (1964) have reported 125-150 loci concerned with *Albino*, 125 loci for *Viridis* and 15-50 loci for other types of chlorophyll mutations. The difference in the mutation spectrum is due to the difference in the location of genes in relation to the centromere (Ramulu, 1970). Levine (1972), Walles (1973) and Wildman (1973) opined that the extra chromosomal mutations may also results in the induction of chlorophyll deficiency and subsequently the chlorophyll mutants. According to Maclachlan and Zalik (1963) the chlorophyll abnormalities induced by the mutagens in the structure of chloroplast make them inefficient or abnormal lipoprotein carrier, however, Krishnaveni *et al*. (1998) mentioned that the mutagen induced chlorophyll deficiencies could be either due to high rate of chlorophyll destruction or due to partial deficiency in chlorophyll synthesis. This view was further elaborated by Spanol *et al*. (2003) that the loss of photosynthetic competence including breakdown of proteins and destruction of membrane by lipid degradations results in the chlorophyll abnormalities.

The biosynthesis of photosynthetic pigments occurs in sequence of biochemical reactions. The biosynthesis of plastids and the synthesis of chlorophyll are under control of nuclear genes by the product of regulatory genes (Wettestein *et al*., 1971) and the chlorophyll mutations are due to the monogenic inheritance of the nuclear genes. The mutation in nuclear gene can change genetic resistance to cytoplasmic DNA, present in chloroplast or in mitochondria (van Harten, 1998). Nelson (1967) reported the inability of many chlorophyll mutants to retain or accumulate chlorophyll at high intensities and temperature, inspite of its synthesis, in absence of protective carotenoids. Harb (1990) and Khan and Tyagi (2013) have reported drastic reduction in the chlorophyll and carotenoid contents in the gamma rays and EMS induced chlorophyll mutants, whereas, Laksmi *et al*. (1996) have reported the inhibition in synthesis of chlorophyll a, b and carotenoids in the chlorophyll mutants, raised from SA treated population. Henriques (2008) in *Urtica dioica* observed the decreased pigment content and reduced photosynthetic activities in the leaves of chlorophyll mutations. He further noted the significant decrease in chloroplast number and depletion in total chlorophyll and carotenoids content, thereby reduced rate of photosynthesis. The occurrence of chlorina mutants have been attributed to impaired chlorophyll biosynthesis, further degradation of chlorophyll and bleaching due to deficiency of carotenoids (Shah *et al*., 2006). The occurrence of *Xantha* mutations could be attributed to the availability of genes for xanthophylls synthesis for the mutagenic action (Shah *et al*., 2006). Any alteration in the nucleotide components of the genes controlling the synthesis of enzymes involved in the biosynthesis of pigment, as a result of mutagenic action would eventually lead to the observed chlorophyll mutations (Patil and Rane, 2015).

Thus, the occurance of wide spectrum of chlorophyll mutations induced by different mutagens could be due to the availability of different loci present on the genome of *Rivinia humilis*, which might have affected by the mutagens and resulted in the chlorophyll abnormalities either through inhibition of photosynthetic pigments or the destruction of chloroplast structures. The differential effect of the all the mutagens, observed in the present investigation, might be due to the preferential action, efficiency and effectiveness of the mutagens.

CONCLUSIONS

Occurance and increased frequency of chlorophyll abnormalities reported in M_1 generation is an indication of the sensitivity of the *R. humilis* genome to all the applied mutagens. All the three mutagens were able to alter the genomic architecture of the plant that exhibited in the induction of variability in various morphological traits in mutagenized populations. *R. humilis*, is a potential source of natural dye obtained from the fruits. The plant is found to be best suited in the Indian agro-climatic condition and hence the induction of variability leading to the development of plants with desirable characters would help to exploit *R. humilis* as a viable alternative source to the other natural dye yielding plants. 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. Considering the other medicinal properties of the plant, it can also be made available to the Indian farmers as a cash crop to be grown at large scale.

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Conflict of interest

The author declares that there is no conflict of interest.

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