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Genetic Variability Induction through Gamma Irradiation in Ox-Eye Daisy (*Leucanthemum Vulgare* Lam.) And Their Genetic Relationship

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Abstract: *The present investigation was aimed to review hormesis, morphological and biochemical variability attributes associated to mutation and purification of novel mutants in Ox-eye daisy. The seeds of Leucanthemum vulgare were exposed to gamma rays (Source ^{60}Co) treatments at 20, 40, 60, 80 and 100 Gy. These gamma irradiated seeds in combination with un-irradiated seeds as control were planted in randomized block design. Low doses of gamma irradiation resulted in hormesis and evoked encouraging novelties, whereas the higher doses elicited higher degree of abnormalities and consequently mortality. The M_2 seeds were seeded to look at their characters and mutations in population in every treatment. Three promising mutants viz. Spatulate type (L_1) at 40 Gy, Quilled-Spatulate type (L_2) at 60 Gy and Quilled type (L_3) at 60 Gy gamma irradiation treatment were labelled, screened and checked for stability of characters in M_2 and M_3 generation, for genetic study and feasible uses of the traits. The seeds of M_2 and M_3 generation were seeded to look at their morphological characters and mutants in every population. RAPD molecular marker technique was inclined to study the genetic divergence and establishing distinctiveness or similarity between the mutants developed as a consequence of the mutagen treatment.*

Keywords: *Leucanthemum vulgare, gamma rays, irradiation, mutation, mutants*

1. Introduction

Leucanthemum vulgare Lam. [*Chrysanthemum leucanthemum* Linn.] generally known as Ox-eye daisy or white weed is native to Europe and North Asia. It is a leafy, vigorously growing herbaceous, non-woody perennial. Stem is soft, stout, glabrous, or sparingly pubescent, solitary and unbranched. Capitula are numerous with white ray florets, in dense corymbs. The species is strictly cross pollinated due to self-incompatibility. It can be cultivated as a decorative plant in the garden flower beds or as pot plant.

In floriculture, there is perpetually a craze for developing novelties to substitute the older varieties with novel ones. Since in ornamentals, a specimen cannot maintain interest for a protracted time, people have the desire for newer forms, through varied strategies of breeding. The chances of exploitation of using mutation breeding are favourable for varied reasons in ornamentals, like the usually large heterozygosity of the plant material that permits direct detection of mutations within the irradiated material, with the intention of improvement in visible characteristics [1].

Scientific interest in mutation breeding has considerably shrunk during the last few decades, as the interest in research has shifted towards development and application of molecular techniques as tools in breeding and transformation of plants, since they permit additional directed approach in fulfillment of improvement objectives. Molecular techniques generate high developmental costs and require urbane equipments and a highly trained workforce. The investment in such exclusive procedures does not appear adequate in case of ornamental crops

with their limited economic importance as compared to agricultural crops. Genetically modified ornamental plants do not find market in Europe due to their low acceptance of consumers and the ambiguous legal situation [2]. As very little research work has been carried out on *Leucanthemum vulgare*, the current study was aimed to review hormesis, morphological and biochemical variability attributes accompanied with mutation, creation and purification of novel mutants in *L. vulgare*.

2. Materials and Methods

The experiment was conducted at Model Floriculture Center, G. B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand. The experimental material comprised of the seeds of *Leucanthemum vulgare*. The seeds of the parental line were procured from Thompson & Morgan, Great Britain and were exposed to gamma rays (Source ^{60}Co) at 20, 40, 60, 80 and 100 Gy of gamma rays doses at gamma chamber facility of National Botanical Research Institute (NBRI), Lucknow. The gamma irradiated seeds together with the control (un-irradiated seeds) were seeded on raised nursery beds and transplanted in experimental field in randomized block design with three replications. The plot size was 1800 cm × 1000 cm with 1200 seedlings/plot with plant spacing of 50 cm × 30 cm. All the recommended package of practices was followed throughout the growing period. Morphological and biochemical observations were recorded for thirteen traits from randomly chosen three plants per treatment per replication. The chlorophyll content (chlorophyll *a*, chlorophyll *b* and total chlorophyll) of the leaves was estimated as proposed by [3]. The figures were recorded for both the years and average was computed. Visual observations on totally different characters were prepared and the plants showing modification in type of flowers, florets, etc. were critically determined and any abnormality determined within the plants of M_1 generation in the treatments was labelled, screened and recorded in the present study. At maturity, every mutant plant was separately harvested and also the seeds were labelled for sowing, subsequently in the subsequent generation.

The M_2 and M_3 seeds together with the control (un-irradiated seeds) were seeded on raised nursery beds and transplanted in experimental field in randomized block design with three replications, with a plot size of 180 cm × 100 cm (12 plants per plot) with spacing of 50 cm × 30 cm, following the recommended package of practices. M_2 mutants were sporadically observed right after germination and were labelled for subsequent observations. The figures were recorded for nineteen traits of arbitrarily chosen three plants per treatment per replication and average was figured out. Any abnormality or variation detected in the plants of M_2 generation was screened and tagged for subsequent observations in M_3 generation. At maturity, every mutant plant was individually harvested and the seeds were labelled and screened, for sowing the consequent generation (M_3) and checked for the stability of the characters. The data generated were subjected to the statistical analysis in accordance with the procedure outlined by [4].

The genomic DNA was extracted by using the CTAB method [5] with slight alterations. PCR amplification was performed [6] with random decamer primers. Band sharing data was analyzed to get genetic similarities based on Jaccard's similarity coefficient [7] among the isolates by using Numerical Taxonomy and Multivariate Analysis System (NTSYSpc, version 2.2) [8]. UPGMA (Unweighted Pair Group Method using Arithmetical Averages) algorithm was engaged to determine the genetic relationship of the parent and also the mutants generated in *L. vulgare*.

3. Results and Discussion

The observations made on several un-irradiated plants of *L. vulgare*, raised in the experimental field are presented in Table 1; indicate the different parameters pertaining to morphology. The normal plants of *L. vulgare* grew to a mean plant height of 51 cm with plant spread of 38.17 cm (E-W) and 35.17 cm (N-S). The leaves were numerous and green lacking punctate glandular hairs, basal leaves 9.15 cm long, 4.63 cm wide with leaf area of

22.62 cm^2, long petiolate with linear or oval cuneately narrow lamina, obtusely toothed, less often shallow lobed. Lower leaves were spatulate, short petiolate, but the upper leaves were sessile, gradually smaller and less divided. Each plant had solitary capitula (25) with ray florets (36) and disc florets (339.17). The diameter of the capitula was 6.87 cm, weighing 1.32 g and disc was 2.05 cm across, borne on a thin, long, 0.475 cm thick peduncle. Involucre was glabrous with involucral bracts with light-coloured or brownish, membranous border. The weight of ray florets was 9.20 mg and that of disc florets was 0.92 mg. The ray florets were 2.77 cm long and 0.86 cm broad borne on the flower head which was 2.10 cm high.

The cytological studies, revealed that *L. vulgare* is a diploid with somatic chromosome number 2n (=2x) =18 (Fig 1), which were confirmed by those reported by [9, 10 and 11].

Data pertaining to the effect of gamma irradiation on vegetative characters, biochemical content and abnormalities thus generated is presented in Table 1, Fig 2. The perusal of the data reveals that, the minimum plant survival of 51.60% was observed, when exposed to 100 Gy which was significantly different from all other gamma rays treatments and maximum in control (99.67%) was recorded, which declined with increase in dose of gamma rays. Reduction in plant survival after exposure to gamma rays has been explained to be due to disturbances of auxin synthesis, chromosomal aberration [12]. Similar results were also observed in *C. paludosum* [13]. Reduction in plant height was observed with increase in the dose of gamma rays irradiation. The maximum plant height in control (46.33 cm) and the minimum in 100 Gy (36.67 cm) were recorded. Inactivation of auxin and decrease in auxin content with increase in radiation doses is responsible for reduction of plant height [14]. Similar observations in *C. morifolium* were observed [15]. Among the different gamma rays irradiation treatments, none was found significant for plant spread, but the plant spread (E-W and N-S) reduced with increase in dose in *L. vulgare*.

Significant delay in flowering over the control was observed, with earliest blooms in control (108.03 days), while the maximum days to bloom (118.30 days) were recorded with 100 Gy treatments. The delay in bud initiation ultimately resulted in late blooming, which may be due to reduction in the rate of various physiological processes and inhibition of growth and the plant remained in juvenile stage and thus unable to differentiate flower heads due to gamma irradiation. Due to irradiation, many biosynthetic pathways are altered which are directly and indirectly associated with the flowering physiology [16]. These results also corroborate with the finding of [17] in *C. morifolium*.

The maximum leaf length (8.97 cm) was observed in control, while minimum was observed in 100 Gy (7.67 cm). All the treatments were non-significant with respect to leaf width. Significant reduction in size of the leaf, with increase in dose of gamma irradiation was observed. This may be due to the poor growth of plants due to radiation damage [18]. In an earlier investigation, gamma rays also significantly reduced the leaf length and width in chrysanthemum varieties 'Sonar Bangla', 'Satish Modi' and 'Flirt' [19]. The chlorophyll content was influenced significantly by various gamma rays treatments. Increase in the chlorophyll content [chlorophyll 'a' (*Chl* a), chlorophyll 'b' (*Chl* b) and total chlorophyll content], with increase in the gamma irradiation dose was observed. [20] and [13] also observed similar results and reported the basic cause of abnormalities is associated with physiological disturbances of growth substances, change in enzyme activity, variation in ascorbic acid concentration, breakage of phosphate metabolism, accumulation of free amino acids, etc.; incited by X-rays and colchicine.

The per cent abnormal plants significantly increased with the increase in gamma rays treatment over the control. Among the different gamma rays treatments, maximum deformed plants were recorded with 100 Gy gamma rays treatment and none in control. This significant production of abnormalities may be due to radiation damage of the irradiated plants particularly chromosomal breakage [18], which causes physiological, morphological and cytological disturbance by gamma radiation. [15] also recorded similar trends in chrysanthemum variety 'Pooja'. Per cent abnormal leaves significantly increased with the gamma rays treatment over the control. The different types of leaf abnormalities included change in leaf shape and size, margins, apex,

fission and fusion were recorded after irradiation. There was no dose specific or variety specific abnormalities in leaves. [21] in *Dendranthema* cv. 'Surekha' and [22] in *Glebionis segetum*, observed the similar results. Significant increase was found in per cent plants with flower head fasciation/asymmetrical flower heads due to irradiation and again these were not dose specific. Flower heads became fascinated in different forms (Fig 2). The formation of fascinated heads after irradiation were also observed by [13]. These abnormalities are genotype dependent and mechanism may be involved in the repair of radiation induced damage within the organism [23].

Visual observations on different characters were made and the plants showing change in form of flowers, florets etc. (Fig 2a to 2d), were critically observed and the type of form other than normal were tagged and recorded. The plants were also observed for any chimera which could be used for chimera management through vegetative propagation or through tissue culture techniques. The change in flower form was also recorded by [24] in *C. morifolium*.

3.1. Screening of Mutants

Three mutants *viz*. Spatulate type (L_1) at 40 Gy, Quilled-Spatulate type (L_2) and Quilled type (L_3) at 60 Gy were screened, tagged and checked for the stability of the characters. The observations were recorded on morphological characters of the mutants developed after gamma irradiation in M_1, M_2 and M_3 generations and the pooled mean values are presented in Table 2 and Fig 1. Mutation in flower head shape/size in *Chrysanthemum* have also been earlier reported in annual chrysanthemum (*C. coronarium*), by [25, 26 and 27].

3.1.1 Mutant L_1 (Spatulate Type)

This mutant developed in *L. vulgare* at 40 Gy gamma rays irradiation treatment and differed in many characters than the original *L. vulgare* plants and attained marginally lesser plant height and plant spread. The leaf length was more, while width and area were lesser and the number of flower per plant was marginally lesser. Flowers were of bigger size, flower head diameter, disc diameter was more with more ray florets and disc florets. The flower head weight and ray floret weight were more, while disc floret weight was marginally lesser. The ray floret length was marginally more, while width was lesser but the flower had more flower head height. Change was recorded in flower form to semi-double, along with the change in the shape of the ray florets from ligulate with laciniate shape of tip and keeled upper surface to spatulate.

3.1.2 Mutant L_2 (Quilled-Spatulate type)

This mutant also developed at 60 Gy gamma rays irradiation treatment and was dwarfed than the original species. The plant spread also reduced. The leaf length was more, while leaf width and leaf area were lesser. The number of flowers per plant reduced marginally, but the flowers were smaller in size, more disc diameter and lesser number of ray florets and more number of disc florets and lesser weight. The florets were more in number and weight. The length of the ray floret was more while width was lesser. The flower had more flower head height. No change was recorded in flower form, except for the change in the shape of the ray florets from ligulate with laciniate shape of tip and keeled upper surface to quilled-spatulate.

3.1.3 Mutant L_3 (Quilled type)

This mutant also developed at 60 Gy gamma rays irradiation treatment and differed in many characters from the original *L. vulgare* plants, *viz*. plants were very dwarf with lesser plant spread. The leaf length was more, while leaf width and leaf area were less and the number of flowers per plant also reduced. Mutant L_3 had slightly bigger flower with more flower head diameter, disc diameter and lesser number of ray florets and more number of disc florets. The flower head weight was more, while ray floret weight was lesser and disc floret weight was more. The ray floret size was lesser and flower head height was more than that of the original. No change was recorded in flower form, except for the change in the shape of the ray florets from ligulate with laciniate shape of tip and keeled upper surface to quilled.

From the present scrutiny of aboriginal species of *L. vulgare* and their gamma rays application induced mutants, it has been empirically perceived that in addition to change in flower shape, cogent changes in some morphological characters had occurred in the mutants. Gamma irradiation induced new flower shape appearance mutants, screened in the present investigation may find very advantageous in future practical breeding programmes and can also be used directly for floriculture industry.

PCR amplification of DNA extracted from the mutants and the original species of *L. vulgare* was performed with random primers of which LC-94 and LC-86 showed polymorphism. A total number of 26 loci were amplified (Fig 4). This gave an average of 13 loci per primer. The polymorphism percentage ranged from 93.33% to 90.91%, with an average polymorphic percentage of 92.12%. Based on polymorphism percentage and unique band amplification, all the primers were considered highly informative primers. PIC value was 0.57 for primer LC-94 and 0.59 for primer LC-86 with an average of 0.58 for both the primers.

The dendrogram generated using SAHN cluster analysis and UPGMA method illustrated in Fig 5 and the matrix of the Jaccard's similarity coefficient of the mutants of *L. vulgare* based RAPD markers (Table 4) reveal that the dendrogram separated the original species of *L. vulgare* and its 3 mutants into two major clusters A and B, at the demarcation of approximately 37% genetic similarity. Cluster A consisted of the original species and its 2 mutants while the cluster B had only mutant L_3. Cluster A was further categorized into two sub-clusters I and II, at the demarcation of approximately 58% genetic similarity. Sub-cluster I had the original species and its mutants L_1 with approximately 68% genetic similarity. Sub-cluster II had only one mutant L_2.

The UPGMA dendrogram based on RAPD analysis indicated that the mutants are fairly distant from the parents and also among themselves. Knowledge of the genetic relationship between the two mutants, contributing to genetic diversity can greatly aid the development of competent germplasm utilization and management policies. The marked variability that is introduced by mutation breeding includes flower form variation which is quite divergent when compared to the parent. The percentile variations in the mutated population were studied to decipher the extent of variation the mutagen has brought about at the molecular level. DNA based markers are suggested as key strategy to determine the cultivars purity resulting in improvement of Intellectual Property Right [28].

Our present abstraction clearly indicates that RAPD markers can be efficiently used for genetic diversity studies among the radiation induced mutants and the original species at genomic level, suggesting that by using RAPD molecular marker, the newly evolved mutants can be easily differentiated from their parents. This would be a very advantageous tool in identifying and protecting them from possible infringements and for guarding the Plant Breeders' Rights.

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TABLE I Effect of gamma irradiation on different characters of *Leucanthemum vulgare*

Characters	Control	Gamma irradiation (Gy)					SEm±	CD (5%)	CV
		20	40	60	80	100			
Plant survival (%)	99.67	94.67	85.20	76.90	64.83	51.60	2.27	7.16	4.99
Plant abnormality (%)	0	4.11	7.40	12.46	15.33	20.67	0.52	1.64	9
Plant height (cm)	46.33	45.13	42.50	40.87	39.53	36.67	1.94	6.12	8.04
Plant spread (E-W) (cm)	37.67	34.00	35.00	32.67	31.57	31.20	1.8	NS	9.26
Plant spread (N-S) (cm)	34.00	33.13	32.47	31.00	29.83	28.30	0.96	3.02	5.27
Leaf length (cm)	8.97	8.83	8.47	8.4	7.73	7.67	0.22	0.69	4.54
Leaf width (cm)	4.53	4.13	4.05	3.82	3.53	3.3	0.25	NS	11.07
Days to flowering	108.03	110.80	111.57	113.97	117.63	118.30	1.12	3.54	1.72
Chlorophyll *a* (*Chl a*)	1.782	1.83	1.84	1.599	1.852	1.927	0.02	0.07	2.14
Chlorophyll *b* (*Chl b*)	0.515	0.531	0.55	0.552	0.564	0.583	0.01	0.03	2.74
Total Chlorophyll (*Chl*)	2.282	2.345	2.373	2.134	2.399	2.493	0.03	0.08	1.95
Abnormal leaf (%)	0	3.80	7.30	11.87	15.53	21.93	0.41	1.28	6.99
Abnormal flower (%)	0	6.47	11.23	13.56	16.13	18.4	0.49	1.57	7.85

TABLE II:Morphological characters of original species and its mutants developed after gamma irradiation in *Leucanthemum vulgare*

Characters	*L. vulgare*	Mutants of *L. vulgare*		
		L_1	L_2	L_3
		40 Gy	60 Gy	40 Gy
Plant height (cm)	51.00	39.33	34.67	32.33
Plant spread (E-W) (cm)	38.17	37.33	33.17	32.67
Plant spread (N-S) (cm)	35.17	34.50	33.50	31.50
Leaf length (cm)	9.15	9.77	9.62	8.52
Leaf width (cm)	4.63	3.52	3.35	3.60
Leaf area (cm^2)	22.62	18.39	17.23	16.40
No. of flowers/plant	25.33	21.50	23.00	24.00
Flower diameter (cm)	6.87	7.18	5.83	7.15
Disc diameter (cm)	2.05	2.90	2.47	2.60
No. of ray florets	36.00	39.00	34.00	33.67
No of disc florets	339.17	488.07	415.14	437.58
Head weight (g)	1.32	1.38	1.12	1.37
Ray floret weight (mg)	9.20	10.33	7.55	8.54
Disc floret weight (mg)	0.92	0.91	0.95	1.09
Ray floret length (cm)	2.77	2.80	2.37	2.30
Ray floret width (cm)	0.86	0.43	0.34	0.31
Head height (cm)	2.10	3.04	3.10	3.13
Flower form	Single	Semi-double	Single	Single
Shape of ray florets	Ligulate	Spatulate	Quilled-spatulate	Quilled

TABLE III: Details of RAPD primers used for the molecular characterization of mutants of *Leucanthemum vulgare*

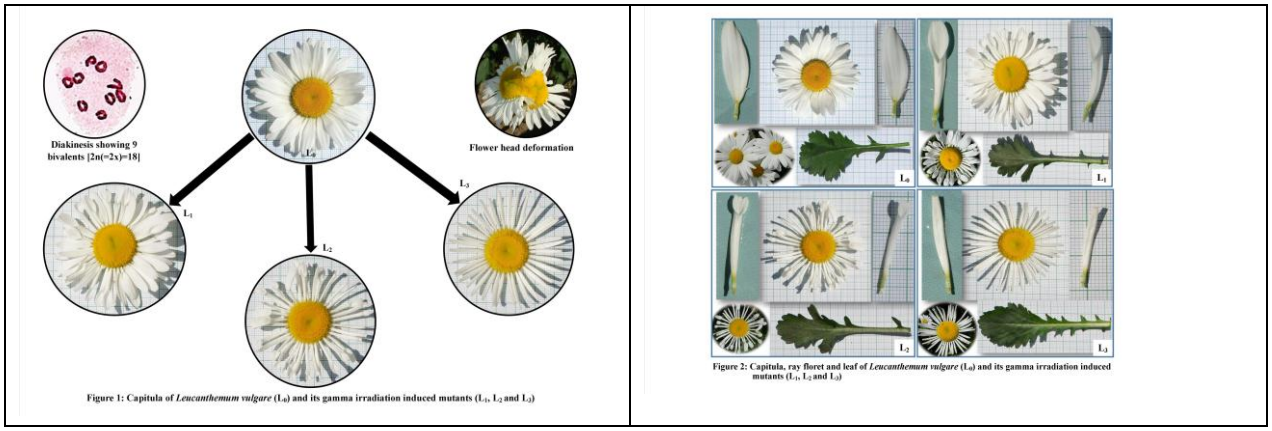
Primer			*Leucanthemum vulgare*							
Code	**Sequence (5′ to 3′)**	**%GC**	**MMB**	**PMB**	**% Poly**	**PIC**	**H_i**	**Rp**	**D**	**D_L**
LC-94	$^{5'}$GTCGCCGTCA$^{3'}$	70	1	14	93.33	0.57	0.30	8	0.63	0.37
LC-86	$^{5'}$GTTGCGATCC$^{3'}$	60	1	10	90.91	0.59	0.33	7	0.66	0.38
Average			**1**	**12**	**92.12**	**0.58**	**0.31**	**7.5**	**0.65**	**0.37**

MMB - Monomorphic bands
PMB - Polymorphic bands
%Poly - Per cent polymorphism
PIC - Polymorphic Information Content
H_i - Average expected gene diversity
Rp - Resolving power
D - Discrimination power
D_L - Discriminating power

TABLE IV. Matrix of Jaccard's similarity coefficient of *Leucanthemum vulgare* and its mutants based on RAPD markers

	1	**2**	**3**	**4**
1	1			
2	0.667	1		
3	0.482	0.667	1	
4	0.407	0.444	0.259	1

1 - *L. vulgare*, **2** to **4** - Mutants of *L. vulgare* L_1 to L_3



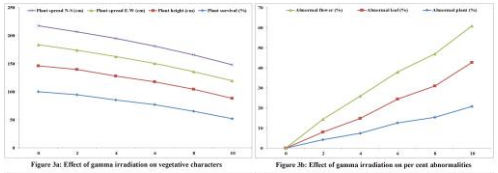


Figure 3a: Effect of gamma irradiation on vegetative characters

Figure 3b: Effect of gamma irradiation on per cent abnormalities

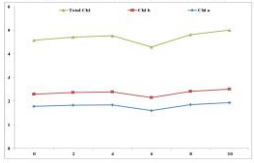


Figure 3c: Effect of gamma irradiation on chlorophyll content

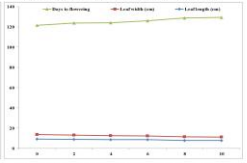
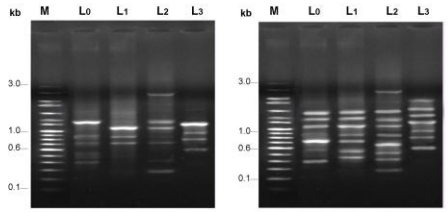


Figure 3d: Effect of gamma irradiation on days to flowering and leaf size



(a) LC-86 (b) LC-94

Figure 4: Molecular diversity generated among *Leucanthemum vulgare* (L_0) and its three mutants (L_1, L_2 and L_3) by RAPD primer LC-86 and LC-94

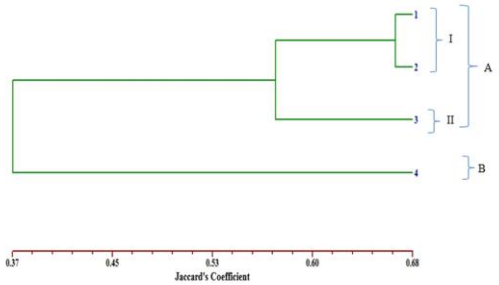


Figure 5: Dendrogram depicting the classification of *Leucanthemum vulgare* and its three mutants based on RAPD. 1 - *L. vulgare* , 2 to 3 - Mutants of *L. vulgare* L_1 to L_3