Indian J. Hort. 71(1), March 2014: 77-81

Studies on ^{60}Co gamma irradiation for inducing *in vitro* mutagenesis in gerbera

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ABSTRACT

***In vitro* shoots of gerbera 'Regiko' and 'South Pacific' were irradiated with 10, 20 and 30 Gy ^{60}Co gamma rays (100 Gy/min.) and sub-cultured for two cycles. After rooting *in vitro* plants were hardened and later transferred in pots for survival and morphological analysis. LD_{50} value for *in vitro* shoots in both cultivars was around 20 Gy. 30 Gy irradiation dose severely impaired proliferation and shoot yield in two *in vitro* cycles. Around 55% shoots initially irradiated with 30 Gy dose were able to root in comparison to above 80% in 20 Gy irradiated shoots. However, plants raised from 20 and 30 Gy irradiated shoots survived equally well in the field. Differences in leaf area and number under 20 and 30 Gy doses after 8 weeks of growth in pots were marginal. Stomata size and number mm^{-2} was significantly reduced in plants raised from 30 Gy irradiated shoots. Under 20 Gy dose mutation frequency calculated on the basis of stomata number mm^{-2} was close to 0.50 in both cultivars, whereas it was above 0.60 with 30 Gy dose. In the present study estimation of mutation frequency on the basis of changes in stomata number per unit area offers an alternative way to assess effects of gamma irradiation even before the appearance of flowers.**

Key words: Gerbera, Gamma irradiation, *in vitro* mutagenesis.

INTRODUCTION

Crop improvement using classical induced mutagenesis through physical mutagens is now a reliable and standardized technique. Numerous cultivars in diverse genera have been evolved by use of physical and chemical mutagens (Maluszynski *et al*., 9). Mutation derived varieties have had a significant impact on the array and choice of genetic resources available in modern agriculture (Ahloowalia *et al*., 1) and more so in improvement of ornamentals propagated through vegetative means (Datta, 4). Modern gerbera (*Gerbera jamesonii* Bolus.) have a complex ancestry and are highly heterozygous (Schiva, 10). Most of the varieties are sterile, hence, necessitating multiplication through tissue culture. Breeding of novel cultivars is mostly carried out through mutagenesis. The current investigation was aimed to standardize gamma irradiation dose for optimal explant survival of ensuing generations propagated in *vitro* and in field. A novel measure of changes in stomata number was used to calculate mutation frequency in the irradiated plants.

MATERIALS AND METHODS

Uniform size (3 cm) shoots of the two cultivars (Rejiko - a medium sized maroon cultivar and South Pacific- a large yellow cultivar) of gerbera were extracted from *in vitro* grown clumps of uniform age and transferred to flasks containing Murashige and Skoog (1962) medium supplemented with BAP 1.0 mgl^{-1} + kinetin 0.25 mgl^{-1}. Shoots were exposed to ^{60}Co gamma irradiation (10, 20 and 30 Gy at 100 Gy per min.) a week later. Irradiation treatment was executed by a Panoramic Batch Irradiator (PANBIT) at Bhabha Atomic Research Station, Zakura, Srinagar. Each treatment comprised three flasks containing 10 shoots each replicated thrice. Untreated shoots were retained as control. Shoots were allowed to proliferate for 5 weeks to raise vM_1 *in vitro* generation. The latter were sub-cultured in similarly constituted fresh medium and allowed to proliferate for another five weeks to constitute vM_2 *in vitro* generation. Observations on *in vitro* shoot survival (%), shoot proliferation and rhizogenesis were recorded. Progeny of irradiated and control plant-lets were run through hardening process and transferred to the field. Data on field survival (%), leaf area and leaf number plant^{-1}, size of stomata, stomata frequency and mutation frequency were recorded.

For stomata studies middle portion of the fifth leaf of each irradiated plant was excised and immediately brought to the laboratory. An epidermal impression of each leaf was made by spreading a thin layer of clear enamel on lower leaf surface that was later peeled off on drying and viewed under a stage and ocular

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microscope at 400X power. Stomata frequency (No. mm^2) and dimensions (width and length of 5 stomata) were recorded in at least 5 microscopic fields on each leaf. Mutation frequency was calculated on the basis of variation observed in stomata frequency (No. per unit area) in irradiated plants. Stomata count mm^{-2} for 50 non-irradiated plants was recorded. The data was used to work out the lower and upper range values of the stomata count for control or non-irradiated plants. The lower and upper range values of the control plants were taken as a cut-off value. Any irradiated plant having stomata frequency less than or more than the upper or lower cut-off value as established in control plants was counted as a mutant. Mutation frequency was calculated as the ratio between such mutants and total number of plants examined under each irradiation treatment. Data was subject to analysis of variance for completely randomized design with three replications using Mini Tab 16.

RESULTS AND DISCUSSION

Post irradiation survival of tissue cultured shoots was recorded at 1 and 2 week intervals (Table 1). Higher gamma irradiation dose resulted in increased plant mortality and LD_{50} value for both the cultivars estimated at the end of 2 weeks was around 20 Gy. Slight difference in LD_{50} value may be due to differential radio sensitivity of the two cultivars. Previous studies on *in vitro* somatic mutagenesis of gerbera have demonstrated that application of 20 Gy X-rays to *in vitro* propagated shoots resulted in 10 per cent morphological mutations (Walther and Sauer, 13). In another study on gerbera, Walther and Sauer (14) used 10-25 Gy gamma irradiations and extimated LD_{50} value at around 20 Gy. Dubec-Lebreux and Vieth (5) concluded that a single dose of 30 Gy at 600 Gy h^{-1} or 50 Gy at 8.4 Gy h^{-1} was optimum for reasonable survival and growth of irradiated shoots.

Shoot proliferation in 'Rejiko' and 'South Pacific' was studied in terms of days to shoot initiation and per cent proliferation (Table 1). There was no significant reduction in days to shoot initiation with 10 Gy treatment. However, 20 Gy treatment resulted in significant delay in shoot initiation over control whereas, exposure to 30 Gy significantly delayed initiation in comparison to all other treatments and control. Shoot proliferation declined significantly with increased gamma irradiation. A 30 Gy irradiation dose resulted in a more than 70 per cent decline in proliferation in both the cultivars. Dubec-Lebreux and Vieth (5) reported a 50% decline in shoot multiplication rate at 30 Gy irradiation at a dose rate 600 Gy h^{-1} or 50 Gy at 8.4 Gy h^{-1}. In another study, Dubec-Lebreux and Vieth (6) put 26 Gy as an optimum dose to ensure 50% multiplication rate. Laneri *et al.* (8) reported only 25 per cent decline in propagation rate of gerbera at 20 Gy exposure. Walther and Sauer (15) also reported interaction between shoot forming capacity of cultivars and X-Ray sensitivity in gerbera.

Influence of gamma irradiation on shoot growth in terms of shoot number $explant^{-1}$ and shoot length (Table 2) was recorded in two successive post irradiation *in vitro* propagation cycles (vM_1 and vM_2). In vM_1, decline in shoot yield in both cultivars with 10 Gy treatment was not significant. However, 20 Gy exposure resulted in a significant decline in shoot number $explant^{-1}$ in comparison to control. The decline in shoot yield continued in vM_2 cycle also. At 30 Gy shoot yield in both the cultivars declined significantly in

Table 1. Influence of ^{60}Co gamma irradiation on survival and proliferation in *Gerbera jamesonii* cvs. Rejiko and South Pacific.

Dose (Gy)	Rejiko				South Pacific			
	Survival (%)		Days to shoot initiation	Proliferation (%)	Survival (%)		Days to shoot initiation	Proliferation (%)
	1st week	2nd week			1st week	2nd week		
0	97.33 (84.01)	97.33 (84.01)	7.56	94.66 (79.72)	98.66 (87.00)	98.66 (87.00)	7.88	95.99 (81.02)
10	71.99 (58.14)	61.33 (51.58)	7.92	51.99 (46.12)	65.33 (53.98)	57.33 (46.91)	8.08	46.66 (43.07)
20	62.66 (52.37)	52.33 (46.33)	8.64	46.66 (43.03)	47.99 (43.83)	47.33 (37.64)	8.96	30.66 (33.59)
30	33.99 (35.67)	19.33 (26.08)	10.56	14.66 (22.18)	29.32 (32.75)	18.66 (25.40)	12.64	12.99 (21.12)
CD (P = 0.05)	6.89	7.42	1.12	8.84	6.56	5.00	0.62	6.39

Data in parenthesis are the Arc sine transformed values of the original percentage data

Table 2. Influence of ^{60}Co gamma irradiation on shoot number and length in successive post irradiation propagation cycles in *Gerbera jamesonii* Bolus. cultivars.

Dose (Gy)	Rejiko				South Pacific			
	vM_1		vM_2		vM_1		vM_2	
	Shoot No. explant $^{-1}$	Shoot length (cm)	Shoot No. explant $^{-1}$	Shoot length (cm)	Shoot No. explant^{1}	Shoot length (cm)	Shoot No. explant $^{-1}$	Shoot length (cm)
0	7.88	3.88	8.11	3.94	8.44	4.11	9.12	4.16
10	7.55	3.61	7.88	3.77	7.22	3.62	7.96	3.50
20	6.22	2.88	6.55	3.33	6.55	2.77	7.81	2.88
30	4.44	2.55	5.44	3.05	4.99	2.38	5.63	2.61
CD (P = 0.05)	0.88	0.28	0.55	0.55	1.38	0.50	1.25	0.40

comparison to control and all other treatments. Laneri *et al.* (8) reported a multiplication rate of 2.1 in vM_1 and 2.5 in vM_2 cycles in gerbera cv. 'Rebeca'. The study also reported a 20% increase in multiplication rate in the second cycle which conforms to results obtained in the present study. Datta (3) reported a reduction of 62.50% in survival during vM_1 cycle in chrysanthemum cuttings and a 40% decline in vM_2 cycle over control. 10 Gy dose had no significant influence on shoot length in 'Rejiko', whereas, suppression of shoot length in 'South Pacific' was significant. Dosage of 20 and 30 Gy significantly suppressed shoot length in both the cultivars. Similar results have been reported by Banerji and Datta (2), and Datta (4) in chrysanthemum.

Decline in per cent rooting under all irradiation doses in cv. Rejiko was significant in comparison to control, the same was not significant for 10 Gy dose in cv. South Pacific (Table 3). However, 20 and 30 Gy irradiation doses resulted in significant decline in per cent shoots that rooted in comparison to control. These effects are also reflected in significant decline in root number with 20 and 30 Gy treatments in both the cultivars. Rhizogenesis *in vitro* involves development of roots *de novo*. It is a process of dedifferentiation of specific pre-determined cells near the vascular bundles. Any damage to cell division ability will have a negative effect on de-differentiation of cells and subsequent reorganization into root primordia. This may result in failure of rooting or delayed emergence of roots.

Field survival of rooted progeny of irradiated shoots of 'Rejiko' and 'South Pacific' was evaluated after 4 and 8 weeks (Table 4). There was no significant mortality with 10 Gy gamma irradiation. However, field survival among plants developed from 20 and 30 Gy irradiated shoots was significantly low in both cultivars. Major reason for mortality in post irradiation propagation generations is due to deleterious chimera load carried by the plants. The impaired cells or tissue sectors on coming to occupy significant portions of

Table 3. Influence of ^{60}Co gamma irradiation on *in vitro* rooting of *Gerbera jamesonii* cultivars.

Dose (Gy)	Rejiko				South Pacific			
	Rooting (%)	Days to root initiation	Root No. shoot^{-1}	Root length (cm)	Rooting (%)	Days to root initiation	Root No. shoot^{-1}	Root length (cm)
0	95.83 (81.60)	16.05	5.63	3.68	97.91 (85.80)	11.90	5.16	4.57
10	83.33 (66.25)	16.85	5.54	3.58	91.66 (75.58)	12.60	4.91	4.46
20	81.24 (64.42)	17.15	5.33	3.21	85.41 (67.73)	13.00	4.66	3.76
30	57.83 (49.36)	17.25	5.08	2.77	53.08 (46..47)	13.22	4.58	2.90
CD (P = 0.05)	9.82	0.80	0.68	0.24	11.22	0.90	0.56	0.40

Data in parenthesis are the Arc sine transformed values of the original percentage data

Table 4. Influence of ^{60}Co gamma irradiation on field survival and growth of plantlets of *Gerbera jamesonii* cultivars.

Dose (Gy)	Rejiko						South Pacific					
	Survival (%)		Leaf area plant^{-1} (cm^2)		Leaf No. plant^{-1}		Survival (%)		Leaf area plant^{-1} (cm^2)		Leaf No. plant^{-1}	
	4th week	8th week	4th week	8th week	4th week	8th week	4th week	8th week	4th week	8th week	4th week	8th week
0	95.00 (81.71)	95.00 (81.71)	20.27	44.02	8.12	6.32	100.00 (90.00)	100.00 (90.00)	24.64	47.10	8.32	6.60
10	92.50 (77.57)	90.00 (73.43)	19.44	41.76	7.92	6.20	95.00 (81.71)	95.00 (81.71)	22.34	45.64	8.04	6.32
20	77.50 (61.85)	75.00 (60.30)	16.21	34.76	7.84	6.00	75.00 (60.00)	75.00 (60.00)	18.44	37.68	7.96	6.20
30	70.00 (57.20)	65.00 (53.89)	15.07	32.60	7.84	5.84	67.50 (55.34)	65.00 (53.79)	17.52	35.33	7.92	6.16
CD (P = 0.05)	13.99	11.93	2.94	3.15	NS	NS	8.49	8.62	2.22	1.70	NS	NS

Data in parenthesis are the Arc sine transformed values of the original percentage data

growing point lead to death or debilitated plants which do not survive more exacting environmental demands in the field. Any impairment in the epidermal skin of the plants gets quickly exposed in the field leading to mortality. Leaf number explant^{-1} was insensitive to irradiation which is expected as it is an entity that is fixed at the time of initiation of bud. However, 20 and 30 Gy irradiation significantly reduced the leaf area plant^{-1} at 4th and 8th week in both the cultivars. Increase in leaf area is a function of the expansion of leaf cells which depends upon the acid growth driven elasticity of cell walls mainly controlled by auxins. Irradiation is known to disturb auxin synthesis and distribution patterns.

There was no significant effect of 10 Gy dose on stomata size (Table 5). However, stomata in plants originating from 20 and 30 Gy irradiated shoots were significantly reduced in size. Literature abounds in reports of increased size of stomata in plants where chromosome number is doubled through irradiation and cholchicine treatment (Usman *et al*., 12). UV irradiation has also been reported to

Table 5. Influence of ^{60}Co gamma irradiation on stomata size, number and mutation frequency in *Gerbera jamesonii* cultivars.

Dose (Gy)	Rejiko				South Pacific			
	Stomata size (µm)		Stomata No. mm^{-2}	Mutation frequency	Stomata size (µm)		Stomata No. mm^{-2}	Mutation frequency
	Width	Length			Width	Length		
0	21.35 (17.50-23.50)	32.02 (26.00-36.00)	42.84	0.00	24.50 (22.50-28.50)	36.70 (30.50-39.00)	45.22	0.00
10	20.28 (15.00-22.50)	30.70 (26.00-33.00)	40.93 [a]	0.22	23.35 (21.00-26.00)	34.98 (27.50-37.00)	41.88	0.26
20	17.70 (13.00-19.50)	26.25 (24.00-28.50)	32.36	0.46	19.32 (17.00-23.00)	29.72 (24.00-33.50)	33.35	0.49
30	14.94 (12.00-16.50)	22.73 (19.50-23.50)	28.56	0.63	16.64 (12.50-19.00)	26.52 (22.00-28.50)	30.73	0.67
CD (P = 0.05)	1.43	1.84	3.20	0.11	1.22	1.92	3.84	0.19

Figures in parenthesis depict the range

increase stomatal size and frequency (Siavash *et al*., 11). However, decline in stomata size in the current experiment is consistent with the general trend of deleterious consequences of gamma irradiation on related growth parameters like leaf size. Mutation frequency in 'Rejiko' ranged from 0.27 to 0.52, whereas, in 'South Pacific' the range was from 0.30 to 0.54. The values are again consistent with the per cent changes recorded in related parameters of leaf size. Epidermis develops from the outer L_1 histogenic layer. The cells in the L_1 layer divide mostly by anticlinal divisions (Hartman *et al.*, 7). The daughter cells ultimately become the outer skin of the plant. Epidermal structures like trichomes and stomata also develop from the cells in the L_1 layer. No L_1 layer cell progeny are pushed into the core of the plant tissue as a result of periclinal divisions. Hence, any change as a result of mutations are preserved and passed down the cell lineage that forms the outer skin of the plant. This makes study of size and frequency of stomata a reliable indicator of the magnitude of mutation as a result of exposure to physical mutagens like gamma rays. Hence, change in stomata size and number per unit area can potentially be used to fix gamma radiation dose levels for reasonable survival of mutagenic progeny in *in vitro* and *ex vitro* systems.

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Received: February, 2013; Revised: September, 2013; Accepted: January, 2014