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Induction of *in Vitro* Mutation in Chrysanthemum (*Dendranthema grandiflora* Tzvelev) Ray Florets (var. Ravi Kiran) using Gamma Rays and EMS

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ABSTRACT

Chrysanthemum (*Dendranthema grandiflora* Tzvelev) is commercially cultivated as a cut flower, loose flower and pot plant. To develop a novel variety with ornamental value, *in vitro* mutations were induced in var. 'Ravi Kiran' using gamma rays and ethyl methane sulphonate (EMS). The mutagens *viz*., gamma rays (0.5 and 1.0 kR) and EMS (0.1, 0.2 and 0.3%) were used individually and in combination. The treated explants, when cultured *in vitro* on MS medium fortified with 2.0 mg l^{-1} 6-benzyladenine, recorded survival rates ranging from 45 to 68%. The mutagenic treatment combination of 1.5 kR + 0.1% EMS took a maximum of 8.5 days to respond (greening of base of ray florets) while the control required a minimum of 4.3 days. Early shoot initiation (11.3 days) was observed in the control while the treatment (1.5 kR + 0.1% EMS) took most days (18.5) to initiate shoots. The number of shoots proliferated decreased as the dose of mutagens increased. Maximum number of shoots was observed in the control (21.3) while fewest shoots were observed in the 1.5 kR + 0.1% EMS treatment (6.5). Microshoots were elongated on MS medium supplemented with 0.04 mg l^{-1} GA_3. The minimum period for *in vitro* rooting (13.5 days) was observed in the control. In the 1.5 kR gamma rays + 0.1% EMS treatment, delayed rooting was observed. The maximum number of roots per plantlet (15.5) was observed in the control. During hardening, maximum survival of plantlets was observed in the control (90.5%), equivalent to the 0.5 kR gamma ray treatment (86.5%). Minimum survival (51.5%) was observed with the 1.5 kR gamma rays + 0.1% EMS treatment. The putative mutants are under observation for desirable mutations.

Keywords: cut flower, loose flower, survival, regeneration, response
Abbreviations: BAP, 6-benzylaminopurine; **EMS**, ethyl methane sulphonate; **IIHR**, Indian Institute of Horticultural Research; **kR**, kilorad; **MS**, Murashige and Skoog; **SE**, standard error

INTRODUCTION

Chrysanthemum (*Dendranthema grandiflora* Tzvelev) is admired all over the world for its different colours and forms of flowers. Some varieties have the characteristics of both cut and loose flower. Though varied tints and forms are available in chrysanthemum, the ultimate challenge ahead of breeders is to develop novel varieties with a really outstanding bloom of ornamental value. Traditional breeding methods such as cross breeding are not expensive, but involve much time and cannot be always applied for ornamental crops, which are usually heterozygyous, polyploid and vegetatively propagated. Mutation breeding is an easy and relatively inexpensive method of creating new cultivars. However, the challenge associated with mutation breeding is that in the chimeric tissue, mutated cells are present along with normal cells. During subsequent cell division, mutated cells compete with the surrounding normal cells for survival (diplontic selection). If these mutated cells survive in diplontic selection, they are expressed in plants (Datta *et al*. 2005). Hence, *in vitro* mutagenesis has the advantage of creating solid mutants in chrysanthemum. Micropropagation is a proven technique for the rapid multiplication and improvement of many ornamental plants, especially for the species like *Dendranthema grandiflora* (Ben Jaacov and Langhans 1972: Rout and Das 1997; Teixeira da Silva 2004; Panickar *et al*. 2009). Hence, *in vitro* mutagenesis can speed up the breeding programme to create variability and aid in selection and multiplication of desired genotypes.

Perceiving the importance of chrysanthemum as well as the rapid improvement in mutation science, an investigation was taken up at the Department of Floriculture and Landscaping of the Horticultural College and Research Institute, Tamil Nadu Agricultural University, Coimbatore to induce *in vitro* mutation in chrysanthemum.

MATERIALS AND METHODS

'Ravi Kiran', released from the Indian Institute of Horticultural Research (IIHR), was selected for this study. Two types of mutagens *viz*., gamma rays and ethyl methane sulphonate (EMS) were used. After initial disinfection the explants were subjected to gamma irradiation at 0.5 and 1.0 kR and then sterilized with ethanol and mercuric chloride and inoculated in the medium. For EMS treatment, after surface sterilization the ray florets were soaked in EMS solution (0.1, 0.2 and 0.3%) for 1 h and washed thoroughly with sterile distilled water and inoculated in Murashige and Skoog

Table 1 Mutagenic treatments used in this study

T_1	0.50 kR gamma rays
T_2	1.00 kR gamma rays
T_3	1.50 kR gamma rays
T_4	0.1% EMS
T_5	0.2% EMS
T_6	0.3% EMS
T_7	0.50 kR gamma rays + 0.1% EMS
T_8	1.00 kR gamma rays + 0.1% EMS
T_9	1.50 kR gamma rays + 0.1% EMS
T_{10}	Control

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Table 2 Effect of gamma rays and EMS on rate of survival (%) and response to regeneration (rate and time taken) in mutagen -treated ray floret cultures of chrysanthemum variety 'Ravi Kiran'. Figures within parentheses are arc sine transformed mean values ± SE.

Treatments		Survival (%)	Response to regeneration (%)	Days taken for regeneration
T_1	0.5 kR γ rays	67.50 (55.29 ± 1.91)	61.50 (51.67 ± 1.67)	5.00 ± 0.23
T_2	1.0 kR γ rays	56.00 (48.46 ± 1.50)	57.00 (49.03 ± 1.52)	5.30 ± 0.24
T_3	1.5 kR γ rays	48.50 (44.14 ± 1.28)	38.50 (38.34 ± 1.05)	7.00 ± 0.32
T_4	0.1% EMS	62.66 (52.36 ± 1.72)	57.33 (49.23 ± 1.54)	5.50 ± 0.25
T_5	0.2% EMS	58.00 (49.62 ± 1.56)	50.50 (45.29 ± 1.34)	6.30 ± 0.29
T_6	0.3% EMS	51.00 (45.57 ± 1.35)	44.00 (41.55 ± 1.17)	7.50 ± 0.35
T_7	0.5 kR γ rays + 0.1% EMS	60.50 (51.08 ± 1.64)	55.50 (48.17 ± 1.48)	5.00 ± 0.23
T_8	1.0 kR γ rays + 0.1% EMS	54.50 (47.59 ± 1.45)	42.66 (40.77 ± 1.14)	6.50 ± 0.30
T_9	1.5 kR γ rays + 0.1% EMS	45.00 (42.13 ± 1.20)	36.00 (36.86 ± 0.99)	8.50 ± 0.39
T_{10}	Control	73.50 (59.11 ± 2.21)	65.50 (54.07 ± 1.83)	4.30 ± 0.20
	SEd	2.2755	1.9793	0.4051
	CD (0.05)	4.7467	4.1287	0.8451

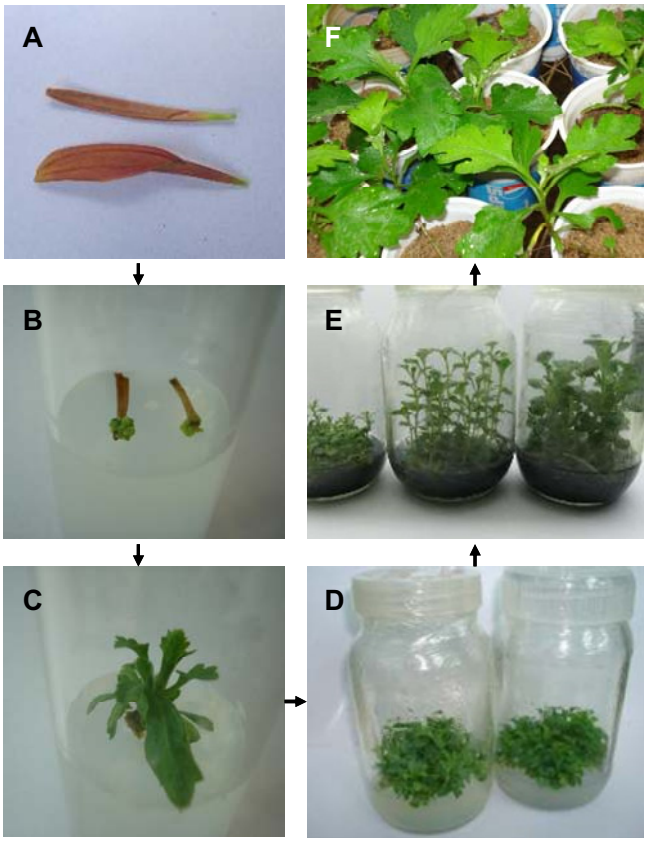

Fig. 1 Direct organogenesis from mutagen treated explants (ray florets) of chrysanthemum var. 'Ravi Kiran'. (**A**) Explant (ray florets); (**B**) Regeneration from ray florets; (**C**) Microshoot formation; (**D**) Multiple shoot induction; (**E**) *In vitro* rooting; (**F**) Hardening.

medium (Chitra *et al*. 2006) fortified with 2 mg l^{-1} 6-benzylaminopurine (BAP) (the protocol was standardized in the preliminary experiment before induction of mutation in 'Ravi Kiran'). The untreated explants served as control. After inoculation of explants in the media, the cultures were incubated in culture room. For combination treatment, the explants were soaked in EMS solution after sterilization and then inoculated on MS medium and the cultures were incubated in the culture room until the initial establishment was noted. Then the culture bottles were exposed to gamma rays and the same were incubated in the culture room for observation (**Fig. 1**). The mutagenic treatments involved in the experiment are listed in **Table 1**.

RESULTS AND DISCUSSION

Rate of survival and response to regeneration

The survival rate decreased with increasing dose of gamma rays and EMS. The survival rate ranged from 45 to 68%. The maximum survival of ray florets was observed in the control (73.50%) (**Table 2**).

The response (greening of base) of ray florets varied significantly among the mutagenic treatments. There was a decrease in the response with an increase in the dose of mutagens (among treatments) from 61.5 to 36%. The maximum response was observed in the control (65.50%). The mutagenic treatment combination of 1.5 kR + 0.1% EMS took a maximum of 8.50 days for greening of the explant while the control recorded the minimum period of 4.30 days.

Gamma irradiation had a negative association with the survival of explants after treatment with both mutagens. 1.5 kR dose resulted in maximum mortality and 0.5 kR exerted minimum mortality. Regeneration (**Table 2**) was reduced at higher dose of 1.5 kR γ rays + 0.1 % EMS and the days taken for response *viz.*, greening of the base of ray florets was longest at higher doses. Irradiation treatment would have suppressed cell division, cell elongation and proliferation of the explants. This is in concurrence with findings of Hewawasam (2004) in *Crossandra infundibuliformis* and Janavi (2005) in *Vanilla planifolia*.

In the EMS treatment the survival rate declined with an increase in concentration (**Table 2**). The maximum mortality of the explants at higher concentrations of EMS might be due to the toxic effect of the mutagen (Datta *et al*. 2005) on the explants. The rate of response to regeneration and days taken for response were also negatively associated with the concentration of EMS. This is in accordance with the findings of Datta *et al*. (2001) in chrysanthemum and Tejaswini *et al*. (2006) in carnation (*Dianthus caryophyllus*). The same trend was noticed in the combination treatment with gamma rays and EMS (**Table 2**).

Days taken for shoot initiation and multiple shoot induction

The number of days taken for shoot initiation (**Fig. 2**) was

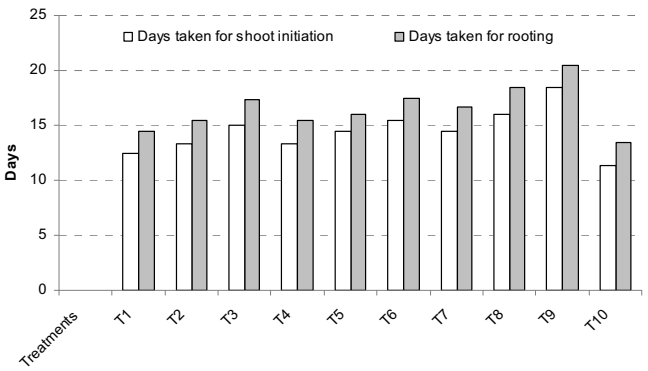

Fig. 2 Effect of gamma rays and EMS on days taken for shoot initiation and *in vitro* rooting in mutagen treated ray floret cultures of chrysanthemum var. 'Ravi Kiran'. For treatments, see **Table 1**.

Table 3 Effect of gamma rays and EMS on number of shoots proliferated, length of shoot (cm) and number of leaves in ray floret derived micro shoots of chrysanthemum var. 'Ravi Kiran'.

Treatments		Number of shoots proliferated/ culture	Length of shoot	Number of leaves per microshoot
T_1	0.5 kR γ rays	18.00 ± 0.83	8.72 ± 0.40	10.50 ± 0.48
T_2	1.0 kR γ rays	16.50 ± 0.76	7.30 ± 0.34	8.50 ± 0.39
T_3	1.5 kR γ rays	14.30 ± 0.66	5.25 ± 0.24	7.00 ± 0.32
T_4	0.1% EMS	15.50 ± 0.72	8.13 ± 0.38	11.66 ± 0.54
T_5	0.2% EMS	15.70 ± 0.73	6.80 ± 0.31	8.00 ± 0.37
T_6	0.3% EMS	14.00 ± 0.65	5.56 ± 0.26	7.66 ± 0.35
T_7	0.5 kR γ rays + 0.1% EMS	14.80 ± 0.68	7.35 ± 0.34	8.50 ± 0.39
T_8	1.0 kR γ rays + 0.1% EMS	12.20 ± 0.56	4.85 ± 0.22	6.50 ± 0.30
T_9	1.5 kR γ rays + 0.1% EMS	6.50 ± 0.30	3.56 ± 0.16	5.50 ± 0.25
T_{10}	Control	21.30 ± 0.98	9.63 ± 0.44	13.00 ± 0.60
	SEd	1.0003	0.4533	0.5852
	CD (0.05)	2.0866	0.9455	1.2206

Table 4 Effect of gamma rays and EMS on rooting of ray floret derived plantlets of chrysanthemum var. 'Ravi Kiran'. Figures within parentheses are arc sine transformed mean values ± SE.

Treatment		Rate of response to rooting (%)	Days taken for rooting	No. of roots per plantlet	Length of roots (cm)
T_1	0.5 kR γ rays	89.28 (70.99 ± 1.52)	14.50 ± 0.67	14.00 ± 0.65	14.00 ± 0.65
T_2	1.0 kR γ rays	85.67 (67.91 ± 2.05)	15.50 ± 0.72	12.50 ± 0.58	12.65 ± 0.58
T_3	1.5 kR γ rays	82.67 (65.45 ± 1.27)	17.33 ± 0.80	9.00 ± 0.42	10.50 ± 0.48
T_4	0.1% EMS	89.00 (71.18 ± 3.52)	15.50 ± 0.72	13.50 ± 0.62	13.75 ± 0.64
T_5	0.2% EMS	84.39 (66.75 ± 0.69)	16.00 ± 0.74	12.50 ± 0.58	11.86 ± 0.55
T_6	0.3% EMS	81.00 (64.17 ± 0.72)	17.50 ± 0.81	10.50 ± 0.48	10.00 ± 0.46
T_7	0.5 kR γ rays + 0.1% EMS	86.98 (68.86 ± 0.49)	16.66 ± 0.77	12.00 ± 0.55	10.65 ± 0.49
T_8	1.0 kR γ rays + 0.1% EMS	81.13 (64.26 ± 0.34)	18.50 ± 0.85	9.33 ± 0.43	8.63 ± 0.40
T_9	1.5 kR γ rays + 0.1% EMS	76.68 (61.19 ± 1.87)	20.50 ± 0.95	7.00 ± 0.32	5.68 ± 0.26
T_{10}	Control	95.77 (78.29 ± 1.41)	13.50 ± 0.62	15.50 ± 0.72	14.70 ± 0.68
	SEd	2.3400	1.0883	0.7737	0.7539
	CD (0.05)	4.8811	2.2702	1.6140	1.5727

significantly influenced by the mutagenic treatments and it was directly proportional to the dose of mutagens. Early shoot initiation (11 days) was observed in control while the treatment 1.5 kR + 0.1% EMS took maximum number of days (18 days) for shoot initiation.

The days taken for shoot initiation increased with increasing dose of mutagens. Shoot initiation was delayed at higher dose of mutagens (**Fig. 2**). Shoot proliferation was affected by the mutagen treatment. Fewest number of shoots proliferated per culture at higher doses of gamma rays, concentration of EMS and also in combination treatments (**Table 3**). The possible reason could be that the mutagens would have disturbed the activities of hormones particularly cytokinin, affecting the shoot proliferation. This is in conformity with the findings of Hewawasam (2004) in crossandra and Janavi (2005) in vanilla. Hewawasam (2004) reported that by increasing dose of gamma rays there was a decrease in the mean number of secondary shoots produced per culture in crossandra during *in vitro* mutation studies. Janavi (2005) reported that the number of shoots proliferated was reduced at higher dose of gamma rays, EMS and oryzalin.

Number of shoots proliferated, length of shoot and number of leaves per shoot

The number of shoots ranged from 6.5 (1.5 kR + 0.1% EMS) to 21.30 (control) (**Table 3**). Invariably in all the mutagenic treatments, the number of shoots proliferated decreased with increasing dose of mutagens in all the stages of sub-culturing. Maximum number of shoots was observed in control (21.30). In mutagen-treated cultures, maximum number of shoots was observed in 0.5 kR gamma ray treatment (18). Minimum number of shoots was observed in the mutagenic combination treatment (1.5 kR + 0.1% EMS) with 6.5 shoots. The length of shoots was maximum in the control (9.63). Among the treatments, 0.5 kR gamma rays produced the longest shoots (8.72 cm) and the shortest shoots (3.56 cm) were observed in 1.5 kR gamma ray + 0.1% EMS treatment. The maximum number of leaves was observed in control (13) and minimum number of leaves (5.5) was observed in the gamma ray and EMS combination of 1.5 kR + 0.1% EMS. The single mutagen dose of mutagen produced more leaves than the combination treatments.

The length of shoot and number of leaves in the shoot obtained were observed to be the lowest at higher concentration of gamma rays and EMS. This is attributed to the physiological disturbances created by the mutagen in the growing shoots. It is in concomitance with the findings of Hewawasam (2004) in crossandra. Barakat *et al.* (2010) reported that the shoot length decreased in chrysanthemum *in vitro* condition as a result of increasing gamma ray treatments in comparison to the control.

In vitro rooting

The rate of response to rooting was highest in control (95.77%) and lowest (76.68%) in 1.5 kR gamma rays + 0.1% EMS (**Table 4**). The rooting was significantly reduced in the treated plantlets compared to control.

A gradual increase in the days taken for rooting was noticed with increasing dose of mutagens. The minimum period for rooting (13 days) was observed in control. In combination treatment, 1.5 kR gamma rays + 0.1% EMS, delayed rooting was observed.

The maximum number of roots/plantlet (15) was observed in control which was on par with 0.5 kR gamma ray treatment (14). Minimum number of roots (7) was recorded in the treatment 1.5 kR gamma rays + 0.1% EMS. Comparaparatively fewer roots were observed in the combination treatments of mutagens than the single dose mutagen treatments.

The length of root decreased with increasing dose of mutagens. The control recorded the longest root of 14 cm. The minimum root length (5 cm) was observed in 1.5 kR gamma rays + 0.1% EMS treatment.

The response to rooting, days taken for rooting, number of roots and length of root were found to be affected by the

Table 4 Effect of gamma rays and EMS on hardening of ray floret derived plantlets of chrysanthemum var. 'Ravi Kiran'. Figures within parentheses are arc sine transformed mean values ± SE.

Treatment		*Ex vitro* survival (%) during hardening
T_1	0.5 kR γ rays	86.50 (68.91 ± 3.47)
T_2	1.0 kR γ rays	75.33 (60.33 ± 2.33)
T_3	1.5 kR γ rays	63.50 (52.86 ± 1.75)
T_4	0.1% EMS	80.50 (64.00 ± 2.73)
T_5	0.2% EMS	73.55 (59.14 ± 2.22)
T_6	0.3% EMS	60.67 (51.18 ± 1.65)
T_7	0.5 kR γ rays + 0.1% EMS	72.50 (58.46 ± 2.16)
T_8	1.0 kR γ rays + 0.1% EMS	63.50 (52.86 ± 1.75)
T_9	1.5 kR γ rays + 0.1% EMS	51.50 (45.86 ± 1.37)
T_{10}	Control	90.50 (73.09 ± 4.51)
	SEd	4.7502
	CD (0.05)	9.9087

mutagenic treatment. The number of days taken for rooting increased with the dose of mutagens. Number of roots and length of roots expressed inverse relationship with the dose and concentration of mutagens. Physiological disturbances especially in relation to auxin and chromosomal aberrations would have hampered *in vitro* rooting of plantlets from the explants. In crossandra it was reported that increasing dose of gamma rays caused root development to be postponed giving poor percentage of rooting and less number of roots per plant (Hewawasam 2004). The rooting time was delayed in microshoots of chrysanthemum and was almost doubled compared to control (Misra and Datta 2006).

Hardening

The maximum survival of plantlets during hardening was observed in control (90.5%) which was on par with 0.5 kR gamma ray treatment (86.5%) (**Table 5**). It was followed by 0.1% EMS treatment (80.5%). The minimum survival (51.5%) was observed in the combination treatment of 1.5 kR gamma rays + 0.1% EMS treatment.

The *ex vitro* survival of the plantlets was affected by the mutagens which exhibited the lowest survival rates in the treatments with higher dose of mutagens. The survival of the plants till maturity depends on the nature and extent of chromosomal damage. Increasing frequency of chromosomal damage with increasing dose of mutagen might have been responsible for the poor survival rates. Datta *et al.* (2005) observed reduced survival of the chrysanthemum plants during hardening which were subjected to higher dose of mutagens.

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