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In vitro isolation of red coloured mutant from chimeric ray florets of chrysanthemum induced by gamma-ray

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ABSTRACT

Mutation breeding played a pivotal role in generating genetic variation in chrysanthemum. As a result of gamma radiation large numbers of novel mutants appear in the form of chimeras that differ in flower colour, shape and size. Isolation of such types of mutated tissue is impossible through conventional techniques. Therefore, all such novel mutants appear as a result of mutation is lost every year due to lack of suitable means to isolate them through conventional propagation methods. Therefore, an effort was made to develop efficient regeneration system from ray florets of a chrysanthemum mutant in order to isolate, purify and field establish the novel mutant. The maximum survival of cultures were found when the ray florets were per-treated with mancozeb-45 (0.2%) + carbendazim (0.2%) + 8-HQC (200 mg/l) for 3 h followed by surface sterilized with $HgCl_2$ (0.1%) for a duration of four minutes. The callus induction was maximum on Murashige and Skoog (MS) medium supplemented with BAP (4.0 mg/l) and NAA (1.0 mg/l). The maximum regeneration of microshoots (95.56%) from the ray floret induced callus was recorded on MS medium fortified with BAP (4.0 mg/l) and NAA (0.1 mg/l). MS medium supplemented with BAP (5.0 mg/l) + NAA (0.1 mg/l) + GA_3 (0.2 mg/l) gave the highest microshoot proliferation (100.0%). *In vitro* rooting ideal (99.0%) on half-strength MS medium fortified with 0.5 mg/l NAA. The hardening of rooted plantlets was successfully achieved after 3-4 weeks of acclimatization. The isolated mutant produced flowers that were true-to-type to the original red mutant, *i.e.*, solid mutant.

Key words: Chrysanthemum, mutation breeding, growth regulators, *in vitro* regeneration, novel mutant.

INTRODUCTION

Chrysanthemum (*Chrysanthemum morifolium* Ramat.) is an important cut flower and pot plant species commonly known as 'Gul-e-Daudi' or 'Autumn Queen'. Chrysanthemum is one of the important cut flowers in the international market and ranks 2nd in the global cut flower trade after rose (Datta and Gupta, 4). Today, chrysanthemum has earned tremendous popularity due to its wide range of brilliant colour, shapes, size, long lasting floret life and diversity in height and growth. Conventional breeding in chrysanthemum has its own limitations including restricted gene pool, longer ray florets that prevent timely pollination, self-incompatibility, parental ploidy differences etc. Chrysanthemum appear to be ideal plant for mutation breeding as several characters of economic interest, *i.e.*, flower traits or growth habit are easily monitored after mutagenic treatment and can be isolated in υM_1 generation itself. In India too, mutation breeding has been employed in chrysanthemum for improvement of flower colour, size, form, height, growth form and sensitivity to light quality/quantity (Rout and Das, 15). Mutation breeding induced by physical means is one of the efficient methods to evolve new cultivars for the floriculture industry. When irradiation was employed as a means of mutagenesis in chrysanthemum, large number of promising mutants emerged in the form of solid mutants as well as partial chimeras. The main bottleneck in vegetative propagated plants is when the mutation appears as partial chimeras after treatment with physical and/or chemical mutagens. The size of the mutant chimeral sector varies from a narrow streak on a petal, a portion of a flower, in single flower, a portion of a branch to the entire branch. Although it is possible to isolate a portion of a branch or an entire branch if it is completely mutated, but it is difficult to isolate such mutants/chimeras, which are often limited in extent and may only be expressed as strip of colour in a single floret (Mandal and Datta, 9). Similarly, a small sector of a mutated branch or flower cannot be isolated using the available conventional propagation techniques. Therefore, a large number of new flower colour/shape mutants, induced by mutagens, are lost every year due to the lack of an efficient regeneration system from small mutated sectors (Chakrabarty *et al*., 2). Mutation breeding coupled with *in vitro* regeneration would be a useful approach to establish novel mutants in pure form and facilitate production of a wide range of novel coloured chrysanthemum cultivars. Thus, *in vitro* approach opens up new vistas to produce a wide range of new

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flower colour/type mutants for the floriculture trade. Different explants, such as stem, flower receptacle, whole floret, floret segment and epidermis, shoot tip etc. have been used to regenerate plants *in vitro* (Bhattacharya *et al*., 1). The efficiency of recovery of solid colour mutants in chrysanthemum was different due to type of explants used. The ray florets gave the maximum (100%) recovery of solid colour mutants (Mandal *et al*., 10). However, reports on successful establishment of novel mutants by using irradiation and *in vitro* regeneration protocols are limited in case of chrysanthemum (Prasad *et al.*, 14).

MATERIALS AND METHODS

The experiment was conducted at Division of Floriculture and Landscaping and Central Tissue Culture Laboratory at National Research Centre on Plant Biotechnology, IARI, New Delhi. The cuttings of chrysanthemum cultivar Thai Chen Queen, which is one of the popular standard cut flower variety grown under polyhouse conditions after irradiation with gamma rays. The explants used in the isolation of novel mutant were mutated ray florets of red colour solid mutant appeared in υM_1 generation because 100% recovery of solid mutant was reported (Mandal *et al*., 10). The explant tissue was brought to the laboratory and washed thoroughly with running tap water for 30 min. (Bhattacharya *et al*., 1) then whole flowers were washed with Teepol® (0.1%) solution for 5 min. followed by washing under running tap water for 20 min. The explants were then given pre-treatments before inoculation to minimize the microbial contamination in the cultures, using Mancozeb-45 (0.1%), carbendazim (0.1%) and 8-hydroxyquinoline citrate (200 ppm) for 3 h**.** The pre-treated explants shifted to laminar air-flow and whole flower was surface sterilized by agitation of individual ray florets in 0.1% $HgCl_2$ for 4 min. followed by three times rinse with sterilized distilled water. The individual ray florets was pinched with the help of forceps and then inoculated onto MS medium (Murashige and Skoog, 11) supplemented with 0.8% bacto-agar, 3% sucrose and different concentrations of 6-Benzylaminopurine (BAP), α-naphthalene-acetic acid (NAA) and gibberellic acid (GA_3) for different micro-propagation stages, *viz*., callus induction, shoot regeneration, shoot proliferation, elongation and rooting. The pH of the medium was adjusted to 5.6 before autoclaving at 121°C for 20 min. The cultures were incubated in culture room after inoculation and provided with a constant photoperiod of 16/8 h light/ dark regimes at 25 ± 1°C temperature having light intensity of 3,000 lux at plant level provided by cool white fluorescent tubes (Philips India, Kolkata). The temperature of the culture room was maintained by automatic temperature controller.

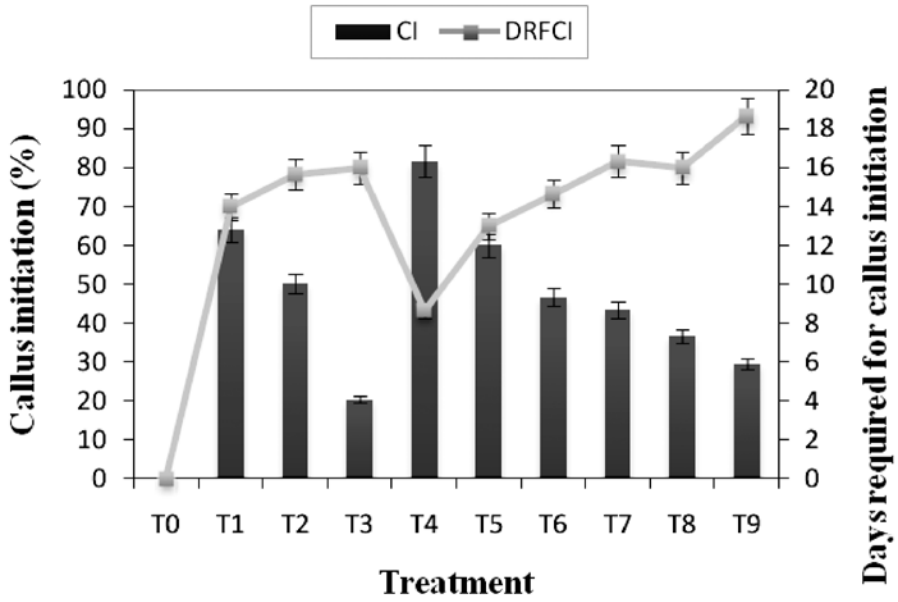
The following concentrations of BAP (3.0, 4.0 and 5.0 mg/l) and NAA (1.0, 2.0 and 3.0 mg/l) were used in different combination for callus initiation from ray florets of chrysanthemum, BAP (3.0, 4.0 and 5.0 mg/l) and NAA (0.1, 0.5 and 1.0 mg/l) for regeneration from ray florets of chrysanthemum, and BAP (3.0, 5.0 and 7.5 mg/l), NAA (0.01, 0.05 and 0.1 mg/l) and GA_3 (0.2 mg/l) for shoot proliferation. Half-strength MS medium supplemented with different concentrations of NAA (0.25, 0.5 and 1.0 mg/l) used for rooting of elongated shoots. The rooted plants were transferred from *in vitro* to *ex vitro* conditions in glass jar with polypropylene cap filled with sterilized peat + Soilrite® (1:1). After 2-4 weeks of hardening, the plants were transferred to greenhouse for flowering.

The experiments were laid out in completely randomized design (CRD). Each treatment had 20-25 units with four replications. All the percentage data was subjected to Arc Sin percentage transformation before calculating ANOVA.

RESULTS AND DISCUSSION

The pre-treatment of ray florets of red colour mutant with mancozeb-45 (0.1%) + carbendazim (0.1%) + 8-hydroxy quinnoline citrate (200 mg/l) for 3 h was found to be the best with regards to minimum microbial contamination (14.44%) and maximum explant survival (85.56%) when compared to the other treatments and control (distilled water dip). Pre-treatment followed by surface sterilization with $HgCl_2$ (0.1%) for 4 min. resulted in lower contamination (11.11%) along with maximum survival (88.89%). Present findings are in the line with the earlier work done by Mandal and Datta (9).

The ray florets first pinched and then culture on medium (Fig. 2a). The wounded parts of ray florets showed faster callusing as compared to those inoculated without wounding. However, some of the wounded ray florets which were not in contact with the culture medium did not show callus initiation and finally dried (Fig. 1). Indicates that among different treatment combinations used, MS medium supplemented with BAP (4.0 mg/l) and NAA (1.0 mg/l) gave the maximum (81.67%) callus initiation in minimum duration (8.67 days) as compared to other treatments. The callus initiation was observed first on wounded parts of the ray florets (Fig. 2b) in the form of greening, which later on got spread on the whole ray florets. It was due to direct exposure of tissue to the culture medium containing higher levels of cytokinin in combination with auxin (NAA), which resulted in rapid cell division and callus formation (Dash *et al.*, 3). It was suggested by Nahid *et al.* (12) that BAP is more crucial for callus induction in



T_0 = MS + No hormone (control), T_1= MS + BAP (3.0 mg/l) + NAA (1.0 mg/l), T_2= MS + BAP (3.0 mg/l) + NAA (2.0 mg/l), T_3= MS + BAP (3.0 mg/l) + NAA (3.0 mg/l), T_4= MS + BAP(4.0 mg/l) + NAA (1.0 mg/l), T_5= MS + BAP (4.0 mg/l) + NAA (2.0 mg/l), T_6= MS + BAP (4.0 mg/l) + NAA (3.0 mg/l), T_7= MS + BAP (5.0 mg/l) + NAA (1.0 mg/l), T_8= MS + BAP (5.0 mg/l) + NAA (2.0 mg/l), T_9= MS + BAP (5.0 mg/l) + NAA (3.0 mg/l).

Fig. 1. Effect of different concentrations of BAP and NAA on days required for callus induction and callusing from ray florets of red colour mutant.

chrysanthemum. The earliest callus induction and vigorous callus growth was noticed in the explants cultured on BAP supplemented MS medium than those on kinetin based medium (Nahid *et al.*, 12). For the successful shoot regeneration, a combination of auxin and cytokinin was found effective (Kumar *et al.*, 6). Regeneration from floral parts of chrysanthemum was also reported by Latado *et al.* (7). When the ray florets were cultured on MS medium supplemented with BAP (4.0 mg/) and NAA (0.1 mg/l) has given the highest shoot regeneration (95.56%), minimum days (31.00) required for shoot regeneration (Fig. 2c & d) and highest number of micro-shoots per callus (5.67) from ray florets over the control (Table 1). These

Table 1. Effect of different growth regulator concentrations on shoot regeneration from ray florets of red colour mutant.

Treatment	Shoot regeneration (%)	Days required for shoot regeneration	No. of micro-shoots per callus
MS + No hormones (control)	6.67 (15.00)*	51.33	1.00
MS + BAP (3.0 mg/l) + NAA (0.1 mg/l)	26.67 (31.11)	51.00	1.33
MS + BAP (3.0 mg/l) + NAA (0.5 mg/l)	48.89 (48.97)	41.67	2.67
MS + BAP (3.0 mg/l) + NAA (1.0 mg/l)	73.33 (58.89)	36.67	4.33
MS + BAP (4.0 mg/l) + NAA (0.1 mg/l)	95.56 (77.75)	31.00	5.67
MS + BAP (4.0 mg/l) + NAA (0.5 mg/l)	68.89 (56.11)	36.67	3.33
MS + BAP (4.0 mg/l) + NAA (1.0 mg/l)	64.44 (53.37)	38.67	3.33
MS + BAP (5.0 mg/l) + NAA (0.1 mg/l)	51.11 (45.63)	40.33	1.67
MS + BAP (5.0 mg/l) + NAA (0.5 mg/l)	42.22 (40.51)	43.00	1.67
MS + BAP (5.0 mg/l) + NAA (1.0 mg/l)	17.78 (25.03)	44.67	0.67
CD at 5%	13.201	4.645	1.085

* Arc sin √% transformed data

Table 2. Effect of different concentrations of BAP, NAA and GA_3 on percentage of shoot proliferation and number of micro-shoots from ray florets of red colour mutant.

Treatment	Shoot proliferation (%)	No. of shoots per microshoot
MS + No hormones (control)	13.33 (21.39)*	4.00
MS + BAP (3.0 mg/l) + NAA (0.01 mg/l) + GA_3 (0.2 mg/l)	26.67 (31.11)	6.00
MS + BAP (3.0 mg/l) + NAA (0.05 mg/l) + GA_3 (0.2 mg/l)	33.33 (35.24)	7.67
MS + BAP (3.0 mg/l) + NAA (0.1 mg/l) + GA_3 (0.2 mg/l)	46.67 (43.11)	10.33
MS + BAP (5.0 mg/l) + NAA (0.01 mg/l) + GA_3 (0.2 mg/l)	53.33 (46.89)	12.00
MS + BAP (5.0 mg/l) + NAA (0.05 mg/l) + GA_3 (0.2 mg/l)	73.33 (58.89)	13.00
MS + BAP (5.0 mg/l) + NAA (0.1 mg/l) + GA_3 (0.2 mg/l)	100.0 (90.00)	15.67
MS + BAP (7.5 mg/l) + NAA (0.01 mg/l) + GA_3 (0.2 mg/l)	73.33 (58.89)	11.00
MS + BAP (7.5 mg/l) + NAA (0.05 mg/l) + GA_3 (0.2 mg/l)	33.33 (35.24)	7.33
MS + BAP (7.5 mg/l) + NAA (0.1 mg/l) + GA_3 (0.2 mg/l)	6.67 (14.65)	5.67
CD at 5%	24.25	2.40

*Arc Sin √% transformed data

observations are quite close to the results of other workers on chrysanthemum (Chakrabarty *et al.*, 2; Jaime and Silva, 5).

Cytokinins are usually added to tissue culture media to stimulate proliferation. Highest (100%) microshoot proliferation (Fig. 2e) and number of shoots per microshoot (15.67) was recorded in the cultures on MS medium supplemented with BAP (5.0 mg/l) + NAA (0.1 mg/l) + GA_3 (0.2 mg/l) (Table 2). These results lend support from the report of earlier workers (Liu and Gao, 8; Park *et al.*, 13; Waseem *et al*., 16). Shoot proliferation in tissue culture might be due to the role of optimum dose of BAP, which enhances axillary branching and multiple shoot formation.

Successful root induction onto elongated shoots depends on number of factors including strength of basal medium salts, level of sucrose and supplementation of auxins (Rout and Das, 15). Auxin NAA was used in rooting of microshoot, since it gave best response (Fig. 2f). Half-strength MS medium supplemented with 0.5 mg/l NAA took minimum days to root initiation (5.75 days), maximum rooting (99%), higher average number of roots/shoot (10.25) and maximum length of root (11.50 cm) (Table 3). These findings are in the line of reports of Waseem *et al*. (16). Plantlets were successfully acclimatized by transferring them in glass jars with polypropylene lids filled with peat + Soilrite® (1:1) and moistened with half-strength MS salts (devoid of growth regulators, calcium, organics and sucrose). After 3-4 weeks of acclimatization, the plants were ready to transfer in the greenhouse (Fig. 2g). These results are in line with the earlier workers (Mandal and Datta, 9; Nahid *et al.*, 12). The hardened plants transferred to greenhouse for flowering. The isolated mutant produced flowers that are true-to-type to the original red mutant in υM_2 generation (Fig. 2h).

Table 3. Effect of different concentrations of NAA on days to root initiation, percentage of rooting, average number of roots/shoot and root length (cm) in red colour mutant.

Treatment	Days to root initiation	Rooting (%)	Av. No. of roots/ shoot	Root length (cm)
½ MS + No hormones (control)	21.75	38 (38.06)*	3.75	3.43
½ MS + NAA (0.25 mg/l)	14.50	81.5 (64.61)	6.25	6.08
½ MS + NAA (0.5 mg/l)	5.75	99.0 (87.16)	10.25	11.50
½ MS + NAA (1.0 mg/l	17.75	80.5 (63.89)	7.75	6.33
CD at 5%	3.091	5.127	2.524	1.962

*Arc sin √% transformed data

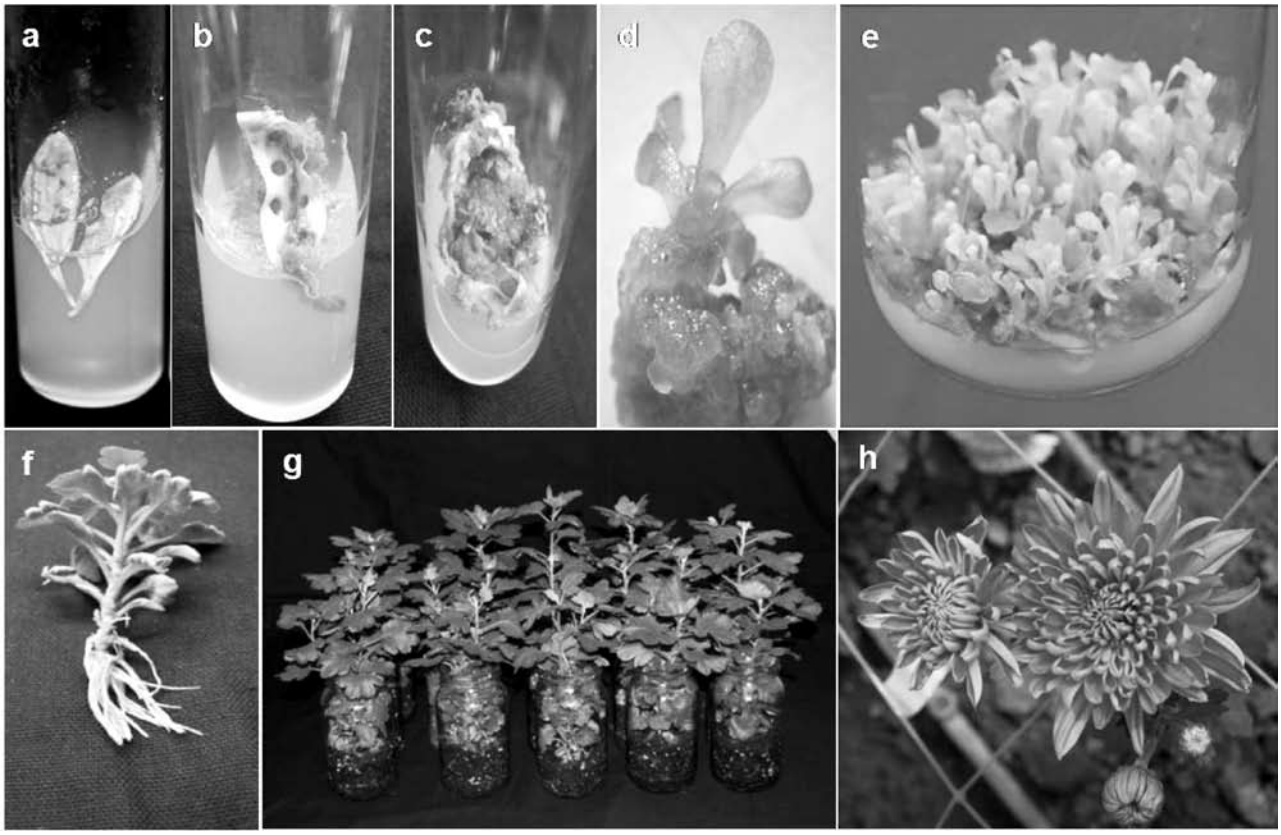


Fig. 2a. Cultured wounded ray floret; b. Ray floret first showing callus on wounded parts; c. Regeneration of micro shoots; d. Stereomicroscopic picture of regenerating shoot; e. Shoot multiplication from regenerated shoot; f. Rooted micro-shoot; g. Acclimatized plants; h. Field established *in vitro* isolated mutant.

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