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In vitro isolation of a gamma ray induced mutant in chrysanthemum (*Dendranthema grandiflora*)

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ABSTRACT

In the present investigation, an effort was made to develop an efficient ray floret regeneration protocol in order to isolate, purify and establish a novel gamma ray induced mutant in the form of brick red flowers in chrysanthemum (*Dendranthema grandiflora* Tzvelev.) cv. Tata Century (pink). Maximum survival (60.00%) and callus formation (56.60%) in minimum duration (10.20 days) were recorded when the ray florets were pre-treated with carbendazim (0.2%) + ridomil (0.2%) + 8-HQC (200 mg/l) for 2 h followed by surface sterilization with $HgCl_2$ (0.1%) for a duration of 4 min and cultured on MS medium supplemented with BAP (4.0 mg/l) and NAA (1.0 mg/l). The maximum regeneration of microshoots (66.00%) from the ray floret induced callus was recorded on MS medium fortified with BAP (4.0 mg/l) + NAA (1 mg/l). MS medium supplemented with BAP (4.0 mg/l) + NAA (0.05 mg/l) + GA_3 (0.1 mg/l) was found to be best for highest micro-shoot proliferation (80.00%). Highest rooting (87.60%) was induced after inoculating the microshoots individually on half-strength MS medium fortified with 0.5 mg/l NAA and 60 g/l sucrose. Successful acclimatization of *in vitro* raised plantlets was done in glass jar with polypropylene cap each filled with a mixture of sterilized cocopeat, soilrite and perlite (1:1:1 v/v) supplemented with half-strength MS inorganic salts. After 3-4 weeks of acclimatization the plantlets were successfully transferred to field conditions.

Key words: Chrysanthemum, Growth regulators, *In vitro* regeneration, Mutation breeding, Novel mutant

Chrysanthemum (*Dendranthema grandiflora* Tzvelev.), popularly known as "Autumn Queen", "Queen of East", belongs to the family Asteraceae and occupies a very important position in world flower industry. It is one of the ten most popular traditional flowers in India and one of the four most popular cut flowers in the world. The plants are used for cut flower, pot plant, garland making, decoration and garden display purposes. A wide range of variability in respect of flower form, shape, size and colour, flowering duration and photo-sensitivity exists in this crop. Mutation breeding has played a vital role in the improvement of Chrysanthemum. Chimera formation, reversion of a variant to its parental type etc. are the major problems facing the development of a new variety through mutation. Isolation and purification of such types of mutated tissue is impossible through conventional techniques which result in complete loss of the precious mutants due to lack of suitable techniques. Mutation breeding method using *in vitro* technique has been applied in Chrysanthemum by several researchers (Mandal and Datta 2005, Prasad *et al.* 2008, Arvind *et al.* 2012). Efficiency of the recovery of solid colour mutants in chrysanthemum varies with the type of explants used. The ray florets gave the maximum (100%) recovery of solid colour mutants (Mandal *et al.* 2000). However, reports on successful establishment of novel mutants by using irradiation and *in vitro* regeneration protocols are limited Chrysanthemum (Prasad *et al.* 2008). Hence, the present investigation was carried out to isolate, purify and regenerate clonal plants using ray florets as explants in a gamma ray induced mutant of chrysanthemum cv. Tata Century.

MATERIALS AND METHODS

The present experiment was conducted at ICAR-IARI, New Delhi during 2016–17. Terminal cuttings (6–8 cm) of Chrysanthemum cv. Tata Century were irradiated with different doses of gamma rays, viz. 0 (control), 5Gy, 10Gy, 15Gy, 20Gy, 25Gy and 30Gy and planted in field. The ray florets of solid brick red flowers appeared in 10Gy treated cuttings, these were selected as explants for the present investigation. First, the ray florets were washed thoroughly with running tap water for 30 min and then with a solution containing few drops of liquid detergent (Teepol) for 2 min followed by 3 washings with running tap water and 1 washing with double distilled water. Next, the explants were pre-treated with a solution containing bavistin (carbendazim) (0.2%), ridomil (0.2%), and 8-hydroxyquinoline citrate (200 ppm) for 2 h in an incubator shaker with 80 rpm and

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at 24°C to minimize microbial load. Then, the pre-treated explants were shifted to laminar air-flow chamber and treated with 0.1% $HgCl_2$ for 4 min followed by 3 washings with sterilized distilled water. The individual ray floret was pinched with help of a sterile needle and then inoculated in MS medium (Murashige and Skoog 1962) supplemented with 7% gelling agent (Agar-Agar type-1), 30% sucrose and different concentrations of 6-benzylaminopurine, α-naphthalene-acetic acid and gibberellic acid for different micropropagation stages like callus induction, shoot regeneration, shoot proliferation, elongation and rooting. The *p*H of the medium was adjusted to 5.7–5.8 before autoclaving at 121°C and 20 psi for 20 min. The cultures were incubated under controlled atmosphere in air conditioned culture room provided with a constant photoperiod of 16/8 h light/dark regimes at 24 ± 1°C temperature and light intensity of 3,000 lux at plant level provided by cool white fluorescent tubes. The temperature of the culture room and photoperiod were maintained by automatic temperature and photoperiod control devices. The experiment was laid out in completely randomized design (CRD). Each treatment had 10 units with five replications. All the percentage values were subjected to data transformation before calculating ANOVA.

RESULTS AND DISCUSSION

Culture establishment and callus induction: For callus induction, the surface sterilized ray florets were inoculated on MS medium supplemented with different concentrations of 6-benzylaminopurine (4.0 and 2.0 mg/l) along with α-naphthalene-acetic acid (1.0 and 2.0 mg/l). Brick red ray florets treated with a solution containing bavistin (carbendazim 0.2%), ridomil (0.2%), and 8-hydroxyquinoline citrate (200 ppm) and inoculated on MS medium supplemented with different concentrations of cytokinins and auxin showed good response in respect of contamination free culture establishment/survival (%) and callus induction (%) (Table 1). Data (Table 1) shows that maximum survival (60%) and callusing (56.60%) were observed after 10.20 days when the surface sterilized and pinched (wounded) ray florets were cultured on MS medium supplemented with BAP (4.0 mg/l) + NAA (1.0 mg/l) (T_1) as compared to those cultured on to the other media. Present findings are in the line with the earlier work done by Gunjeet *et al.* (2017). It was suggested by Nahid *et al.* (2007) that BAP is more crucial for callus induction in Chrysanthemum. The callus initiation was first observed on wounded parts of the ray florets turning to green, which later spread over the whole ray florets. Similar findings were reported by Arvind *et al.* (2012) who reported that the wounded parts of ray florets showed faster callusing as compared to those inoculated without wounding. It was direct exposure of tissue to the culture medium containing higher levels of cytokinin in combination with auxin (NAA), that resulted in rapid cell division and callus formation (Dash *et al.* 2000).

Table 1 Effect of BAP and NAA on callus induction in ray floret explants in mutant of chrysanthemum cv. Tata Century

Treatment	Survival (%)		Callusing (%)		Days required for callusing
	Mean	Angular value	Mean	Angular value	
T_0	44.80	41.99	0.48	1.77	29.20
T_1	60.00	50.77	56.60	48.79	10.20
T_2	48.20	43.95	39.80	39.09	13.80
T_3	43.80	41.40	34.60	36.00	17.40
T_4	50.40	45.21	42.80	40.83	14.40
CD (P=0.05)	3.54		3.90		2.27
SE(m)	1.19		1.31		0.76
CV	5.97		8.83		7.11

T_0, MS Blank (Control); T_1, MS + BAP (4mg/l) + NAA (1mg/l); T_2, MS + BAP (4mg/l) + NAA (2mg/l); T_3, MS + BAP (2 mg/l) + NAA (1.0 mg/l); T_4, MS + BAP (2 mg/l) + NAA (2.0 mg/l).

Adventitious microshoot regeneration and proliferation: MS medium fortified with different concentrations of 6-benzylaminopurine (3.0, 4.0 and 5.0 mg/l) along with α-naphthalene-acetic acid (0.5 and 1.0 mg/l) was used for the adventitious regeneration of microshoots from ray florets. MS medium supplemented with different concentrations of 6-benzylaminopurine (3.0, 4.0 and 5.0 mg/l) along with α-naphthalene-acetic acid (0.05 and 0.1 mg/l) and GA_3 (0.1 mg/l) was used for shoot proliferation. Direct adventitious shoot regeneration initiation begins with cells that are located either in epidermis or just below the surface of the explant apparently originating from single cells. However, the response of explants depends on different growth regulators, their concentrations and combinations. It was observed that new shoot apices developed directly on the ray florets having very small creamish-green callus when cultured on MS medium supplemented with different combinations of cytokinins and auxins. Data on the response of ray floret explants to various culture media in respect of regeneration (%), days required for regeneration and number of micro-shoots per explant are presented in Table 2. Highest regeneration (66%), maximum number of shoots per explant (5.20) in minimum duration (36 days) was recorded in the ray florets cultured on MS medium supplemented with BAP (4.0 mg/l) + NAA (1.0 mg/l) as compared to those cultured on the other media. These observations are similar to the results of other workers working on chrysanthemum (Jaime and Silva 2005, Kumar *et al.* 2012). Cytokinin, i.e. BAP was necessary to induce adventitious buds (Cheah *et al.* 1978) and usually added to tissue culture media to stimulate proliferation. The highest microshoot proliferation (80%) and number of shoots per microshoot (160 after 180 days) was recorded in the cultures on MS medium supplemented with BAP (4.0 mg/l) + NAA (0.05 mg/l) + GA_3 (0.1 mg/l) (Table 3). These results lend support from the report of earlier workers (Liu and Gao 2007, Park *et al.* 2007, Waseem *et al.* 2011, Kumar *et al.* 2012). Shoot proliferation in tissue culture might be due to the role of optimum dose of BAP, which enhances axillary branching and multiple shoot formation.

Table 2 Effect of BAP and NAA on callus regeneration in ray floret explants of mutant of chrysanthemum cv. Tata Century

Treatment	Regeneration (%)		Duration required (days)	No. of shoots/ explant
	Mean	Angular value		
T_0	0.00	0	90.00	0.00
T_1	16.00	23.30	43.00	1.80
T_2	24.00	29.21	41.40	3.60
T_3	32.00	34.28	39.20	3.80
T_4	66.00	54.53	36.00	5.20
T_5	44.00	41.52	38.20	4.80
T_6	36.00	36.81	38.00	4.60
CD (P=0.05)	5.96	-	2.04	1.25
SE(m)	2.05	-	0.70	0.43
CV	14.59	-	3.37	28.12

T_0, MS Blank (Control); T_1, MS + BAP (5 mg/l) + NAA (0.5 mg/l); T_2, MS + BAP (5 mg/l) + NAA (1.0 mg/l); T_3, MS + BAP (4 mg/l) + NAA (0.5 mg/l); T_4, MS + BAP (4 mg/l) + NAA (1.0 mg/l); T_5, MS + BAP (3 mg/l) + NAA (0.5 mg/l); T_6, MS + BAP (3 mg/l) + NAA (1.0 mg/l).

Shoot elongation, rhizogenesis, acclimatization and field establishment: Gibberellic acid stimulates the growth of microshoots and makes them elongated, strong and suitable for *in vitro* induction of rooting. The proliferated microshoots were very small, tender, thin, undeveloped and not suitable for the induction of a very strong root system. Hence, these were cultured individually on MS medium supplemented with GA_3 (0.5 and 1.0 mg/l) along with 40 g/l sucrose. Microshoots subcultured individually on MS medium supplemented with GA_3 (0.5 mg/l) and sucrose (40 g/l) showed very good response with regards to optimum shoot length and stem thickness resulting in an optimum increase in shoot length (4.80 cm) over those grown on other media. The microshoots cultured on MS medium supplemented with GA_3 (1.0 mg/l) became very long (5.40 cm), thin and difficult to manage than those on other media. Gibberellins are known to induce stem elongation in a number of crops. The elongation of the stems is not due to increased formation of nodes and internodes but results from rapid elongation of internodes, which is due to both cell division and cell elongation (Krishnamoorthy 1981).

Successful root induction on elongated shoots depends on number of factors including strength of basal medium salts, level of sucrose and supplementation of auxins (Rout and Das 1997). Half-strength MS medium supplemented with different concentrations of auxins, viz. α-naphthalene-acetic acid (0.5 and 1.0 mg/l) or Indole-butyric acid (0.5 and 1.0 mg/l) along with an increased dose of sucrose (60 g/l) was used for *in vitro* rooting on to the well developed and elongated shoots. The data revealed that the microshoots cultured on half-strength MS medium supplemented with 0.5 mg/l NAA took minimum days root initiation (6.40 days)

Table 3 Effect of BAP and NAA on shoot proliferation in mutant of chrysanthemum cv. Tata Century

Treatment	Proliferation (%)		No. of shoots/micro-shoot after		
	Mean	Angular value	60 days	120 days	180 days
T_0	6.00	9.00	1.40	5.20	9.00
T_1	72.00	58.64	12.00	48.40	72.80
T_2	58.00	49.82	13.40	40.20	62.00
T_3	80.00	64.13	18.00	120.00	160.00
T_4	62.00	52.13	13.60	52.00	66.60
T_5	70.00	57.02	15.80	62.20	102.80
T_6	54.00	47.29	10.40	38.00	84.20
CD(P=0.05)	10.88		2.58	6.49	19.63
SE(m)	3.74		0.89	2.23	6.74
CV	17.30		16.37	9.53	18.93

T_0, MS Blank (Control); T_1, MS + BAP (5 mg/l) + NAA (0.05 mg/l) + GA_3 (0.1 mg/l); T_2, MS + BAP (5 mg/l) + NAA (0.1 mg/l) + GA_3(0.1 mg/l); T_3, MS + BAP (4 mg/l) + NAA (0.05 mg/l) + GA_3 (0.1 mg/l); T_4, MS + BAP (4 mg/l) + NAA (0.1 mg/l) + GA_3 (0.1 mg/l); T_5, (MS + BAP (3 mg/l) + NAA (0.05 mg/l) + GA_3 (0.1 mg/l); T_6, MS + BAP (3 mg/l) + NAA (0.1 mg/l) + GA_3 (0.1 mg/l).

and produced maximum rooting (87.60%), higher average number of roots/shoot (16.40) and optimum and manageable length of longest root (5.20 cm). These findings are in line with reports of Waseem *et al.* 2011, Arvind *et al.* 2012). Plantlets were successfully acclimatized by transferring in glass jars with polypropylene lids each filled with cocopeat + perlite + soilrite (1:1:1) and moistened with half-strength MS salts (devoid of growth regulators, calcium, organics and sucrose). After 3-4 weeks of acclimatization, the plants were transferred to the field.

The present findings can be used to efficiently isolate and purify mutated tissues in Chrysanthemum which are otherwise lost due to lack of suitable techniques to isolate them through conventional propagation methods.

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