

International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
ISSN Online: 2617-4707
NAAS Rating (2025): 5.29
IJABR 2025; SP-9(12): 912-915
www.biochemjournal.com
Received: 25-09-2025
Accepted: 30-10-2025

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In vitro regeneration from ray florets: An efficient protocol to establish flower mutants in chrysanthemum (*Chrysanthemum morifolium*)

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DOI: <https://www.doi.org/10.33545/26174693.2025.v9.i12Sk.7016>

Abstract

The present study was carried out to standardize an *in vitro* protocol for establishment of flower mutant from ray florets in gamma ray treated variety BC-8-05 (yellowish brown colour). Flower mutant was appeared as brown stripes on yellow florets at 10 Gy. Variant ray florets were detected from flowers and subjected for regeneration through *in vitro* culture. Ray florets washed thoroughly in running tap water for 15 minutes and treated with 0.01% of antifungal (Bavistin) solution for 10 minutes and for another 5 minutes with 0.1% aqueous solution of teepol and then again washed in tap water for 5 minutes. The explants were disinfected with 70 % ethanol for 30 seconds and then rinsed with sterile water for 3-4 times. Surface sterilization with 0.1% mercuric chloride for 4 minutes gave the maximum survival percentage (93.97%) with less contamination (6.03%). Among the various combinations of BAP and NAA with MS medium 1 mg/l BAP + 0.1 mg/l NAA showed cent percent callus induction in less days of 12.93 with highest shooting percentage (97.50 %). Addition of 0.1 mg/l GA3 to this medium resulted maximum number of micro shoots per callus (10.15) with a shoot length of 10.53 cm. Successful root induction was achieved with half MS + 0.5 mg/l NAA. These plantlets were successfully transferred to field after successful primary and secondary hardening.

Keywords: Chrysanthemum, mutants, flower colour, *in vitro* regeneration, hardening, irradiation

Introduction

Increase in urbanization emphasizes the interest in various coloured flowers in urban and semi urban areas where the consumers are preferring for novel types especially in flower crops. Ornamental and flower crops with added advantage of vegetative propagation offers to develop new and novel forms and colours. Mutation breeding is one of the tools for ornamental and flower crops which provides ease in development of novel types especially in chrysanthemum. So far, several mutants have been developed in chrysanthemum as solid and chimeric forms as reported by Mahure *et al.*, 2010 [9]. Though the isolation of these mutants is the bottleneck in chrysanthemum if the variation occurs as chimera, it is difficult to isolate and establish (Mandal and Datta, 2005) [10]. Keeping this in view the present study was taken up to standardize a protocol for rapid multiplication of plants through florets in chrysanthemum.

Material and Methods

The present experiment was conducted at Bidhan Chandra Krishi Viswavidyalaya during 2014-15. The cultivar BC-8-05 which is spray type with dwarf nature good for pots, beds, borders. The flowers are decorative type with yellowish-brown colour florets. Terminal cuttings collected from AICRP on Floriculture, BCKV and treated with different doses of gamma rays i.e. 10 Gy, 15 Gy, 20 Gy, 25 Gy 30 Gy and planted in field. Variations were observed in M₁V₁ generation in terms of flower colour such as chimeric flowers as it appeared as brown stripes on yellow ray floret at 10 Gy. These ray florets were taken as explant for standardization of *in vitro* regeneration. At first the ray florets washed thoroughly in running tap water for 15 minutes and treated with 0.01% of antifungal (Bavistin) solution for 10 minutes and for another 5 minutes with 0.1% aqueous solution of teepol and then again washed in tap water for 5 minutes. Then, the explants were brought under laminar air flow. Later, the explants were disinfected with 70 % ethanol for 30 seconds and then rinsed

with sterile water for 3-4 times. The individual explants were subjected to surface sterilization with different concentrations of mercuric chloride for 2-6 minutes followed by three 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 pH 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 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:

Growth and survival of uncontaminated cultures varied with the concentration and duration of the treatments (Table 1). The explants which were exposed to 0.1% mercuric chloride for 4 minutes gave the maximum survival percentage (93.97%) with less contamination (6.03%). Present findings were in the line of earlier work done by Misra *et al.* (2003) [11]; Ilahi *et al.* (2007) [5]. The lower concentration resulted in contamination due to microbial load in the explants and higher concentration coupled with longer duration caused phytotoxicity and drying of explants leading to death of tissues. Similar results were also reported in chrysanthemum by many workers (Verma *et al.*, 2012) [16].

Table 1: Standardization of surface sterilization of explants of chrysanthemum

Treatment	Percent survival	Percent contamination
T ₁ -HgCl ₂ 0.1 %-2 min	31.37 (34.06) ^d	68.61(55.90) ^d
T ₂ -HgCl ₂ 0.1%-4 min	93.97 (75.79) ^a	6.03(14.21) ^a
T ₃ -HgCl ₂ 0.1%-6 min	24.60 (29.72) ^e	75.40(60.24) ^e
T ₄ -HgCl ₂ 0.2 %-2 min	78.20 (62.18) ^b	21.79(27.81) ^b
T ₅ -HgCl ₂ 0.2 %-4 min	39.63(39.01) ^c	60.37(50.96) ^c
T ₆ -HgCl ₂ 0.2 %-6 min	19.77 (26.39) ^f	80.23(63.58) ^f
SEm $\pm$	0.692(0.488)	0.339 (0.231)
CD @ 1%	2.155 (1.520)	1.055 (0.720)

Values in the parentheses are angular transformed data

Callus induction and callus establishment

MS medium supplemented with different combinations of cytokinin (BAP) and auxin (NAA) for callus induction and shoot regeneration. Various concentrations of BAP and NAA showed significant difference for callus induction as well as callus establishment (Table 2). High percentage of callus induction was observed on MS medium supplemented with different concentrations of BAP and NAA combination as compared to control (MS medium devoid of hormones). Cent percent callus was attained with the ray florets on MS medium supplemented with 1 mg/l BAP + 0.1 mg/l NAA (T₁₁) with minimum number of days to callus induction (12.93). Present findings are in the line with the earlier work done by Datta *et al.* (2001) [3]. It was suggested by Nahid *et al.* (2007) [13] 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) [2].

Table 2: Effect of BAP and NAA on days to callus induction and percentage of callus induction from ray floret explants

Treatments	Days to callus induction	Percentage of callus induction
T ₁ -Basal MS medium	0	0
T ₂ -MS + 3.0 mg/l BAP + 0.1 mg/l NAA	24.13 ^f	34.87 (35.70) ^{ef}
T ₃ -MS+3.0 mg/l BAP+ 0.5 mg/l NAA	18.40 ^g	70.33 (57.17) ^{bc}
T ₄ -MS + 3.0 mg/l BAP + 1.0 mg/l NAA	20.00 ^g	59.00 (50.38) ^{cd}
T ₅ -MS + 4.0 mg/l BAP+ 0.1 mg/l NAA	29.67 ^d	14.00 (21.88) ^g
T ₆ -MS + 4.0 mg/l BAP+ 0.5 mg/l NAA	26.63 ^e	21.13 (27.37) ^{fg}
T ₇ -MS + 4.0 mg/l BAP+ 1.0 mg/l NAA	29.83 ^d	19.60 (26.26) ^{fg}
T ₈ -MS + 5.0 mg/l BAP+ 0.1 mg/l NAA	30.00 ^d	16.67 (23.98) ^{fg}
T ₉ -MS + 5.0 mg/l BAP+ 0.5 mg/l NAA	34.00 ^a	11.80 (20.04) ^{gh}
T ₁₀ -MS + 5.0 mg/l BAP+ 1.0 mg/l NAA	35.00 ^a	9.33 (17.31) ^{gh}
T ₁₁ -MS + 1.0 mg/l BAP+ 0.1 mg/l NAA	12.93 ⁱ	100 (90.00) ^a
T ₁₂ -MS + 2.0 mg/l BAP+ 0.1 mg/l NAA	15.37 ^h	82.22 (65.08) ^b
T ₁₃ -MS + 3.0 mg/l BAP+ 1.5 mg/l NAA	22.57 ^f	43.33 (41.07) ^{de}
T ₁₄ -MS + 4.0 mg/l BAP+ 1.5 mg/l NAA	33.67 ^{ab}	8.00 (9.70) ^h
T ₁₅ -MS + 5.0 mg/l BAP+ 1.5 mg/l NAA	35.33 ^a	7.20 (9.12) ^h
T ₁₆ -MS + 1.5 mg/l BAP+ 0.1 mg/l NAA	14.60 ^{hi}	95.56 (80.35) ^a
T ₁₇ -MS + 2.0 mg/l BAP+ 0.5 mg/l NAA	30.47 ^{cd}	18.67 (25.55) ^{fg}
T ₁₈ -MS + 2.0 mg/l BAP + 1.0 mg/l NAA	32.00 ^{bc}	17.20 (24.50) ^{fg}
SEm $\pm$	0.64	4.99 (3.71)
CD @ 1%	2.48	19.24 (16.05)

Values in the parentheses are angular transformed data

Adventitious microshoot regeneration and proliferation:

Different combinations of cytokinin (BAP) and Auxin (NAA) supplemented with MS media was subjected for shoot proliferation and presented in Table 3. Highest shooting percentage (97.50 %) in minimum days (28.23) was found in MS + 1.0 mg/l BAP+ 0.1 mg/l NAA. For the successful shoot regeneration, a combination of auxin and cytokinin was found effective (Kumari *et al.*, 2013) [7].

Cytokinins accelerate the cell division process in the first step of their application and then large amount of callus was formed followed by differentiation of shoot initials, organogenesis and shoot regeneration (Verma *et al.* 2012) [16]. BAP is the most effective plant growth regulator in relation to shoot proliferation. These findings lend support from the various workers (Rahayu and Prayogi, 2013; Lindiro *et al.*, 2013) [14, 8].

Table 3: Effect of BAP and NAA on days to shoot regeneration and percent shoot regeneration from ray floret explants

Treatments	Days to shoot regeneration	Percent shoot regeneration
T ₁ -Basal MS medium	0	0
T ₂ -MS + 3.0 mg/l BAP + 0.1 mg/l NAA	39.47 ^{gh}	50.00 (40.19) ^{cdefg}
T ₃ -MS+3.0 mg/l BAP+ 0.5 mg/l NAA	34.63 ^{ij}	64.72(54.48) ^{bcd}
T ₄ -MS + 3.0 mg/l BAP + 1.0 mg/l NAA	36.90 ^h	53.93(47.80) ^{cdef}
T ₅ -MS + 4.0 mg/l BAP+ 0.1 mg/l NAA	50.00 ^e	42.22(40.46) ^{defg}
T ₆ -MS + 4.0 mg/l BAP+ 0.5 mg/l NAA	40.77 ^g	57.00(49.32) ^{cde}
T ₇ -MS + 4.0 mg/l BAP+ 1.0 mg/l NAA	42.77 ^f	54.21(48.63) ^{cdef}
T ₈ -MS + 5.0 mg/l BAP+ 0.1 mg/l NAA	47.53 ^{de}	67.22(55.14) ^{abcd}
T ₉ -MS + 5.0 mg/l BAP+ 0.5 mg/l NAA	52.80 ^d	18.48(19.49) ^{gh}
T ₁₀ -MS + 5.0 mg/l BAP+ 1.0 mg/l NAA	55.03 ^c	4.52(9.97) ^h
T ₁₁ -MS + 1.0 mg/l BAP+ 0.1 mg/l NAA	28.23 ^j	97.50(82.63) ^a
T ₁₂ -MS + 2.0 mg/l BAP+ 0.1 mg/l NAA	35.17 ⁱ	76.54(61.47) ^{abc}
T ₁₃ -MS + 3.0 mg/l BAP+ 1.5 mg/l NAA	36.73 ^h	77.78(62.44) ^{abc}
T ₁₄ -MS + 4.0 mg/l BAP+ 1.5 mg/l NAA	56.57 ^b	6.23(14.32) ^h
T ₁₅ -MS + 5.0 mg/l BAP+ 1.5 mg/l NAA	59.50 ^a	8.67(15.40) ^h
T ₁₆ -MS + 1.5 mg/l BAP+ 0.1 mg/l NAA	32.70	90.17(72.93) ^{ab}
T ₁₇ -MS + 2.0 mg/l BAP+ 0.5 mg/l NAA	45.77 ^e	24.94(29.78) ^{fgh}
T ₁₈ -MS + 2.0 mg/l BAP + 1.0 mg/l NAA	47.13 ^{de}	28.58(31.76) ^{efgh}
SEm ±	0.41	10.97(8.18)
CD @ 1%	1.57	31.67 (23.61)

Values in the parentheses are angular transformed data

Number of micro shoots and shoot length:

Cytokinin in combination with auxin and Gibberellin was used for production of micro shoots and was represented in table 4. Among the various treatments, MS + 1.0 mg/l BAP+ 0.1 mg/l NAA+0.1 mg/l GA₃ (T₁₁) gave maximum number of micro shoots per callus (10.15) with a shoot length of 10.53 cm. Influence of both auxin and cytokinin combination on plantlet regeneration in *in vitro* culture is a well-known fact and was confirmed by several researches (Jaime and da Silva, 2014) [6]. Shoot proliferation in tissue culture might be due to the role of optimum dose of BAP, which enhanced auxiliary branching and multiple shoot formation though the significant effect of GA₃ in terms of shoot differentiation and shoot elongation was clear from

this study. This may be due to the significant effect of GA₃ on shoots which made them elongated, with thick, sturdy and strong stem and well developed dark green expanded leaves. These results indicate that not only the balance between auxin and cytokinin for shoot regeneration but also that GA₃ plays a role in shoot differentiation (Chakraborty *et al.*, 2000) [1] by involving in several important biochemical and morphogenetic responses which include the promotion of elongation in axial organ, such as stem and flower pedicels. Similar findings of effect of GA₃ on organogenesis and shoot differentiation in callus culture have been demonstrated in many species (Srivastava, 2005) [15].

Table 4: Effect of BAP, NAA and GA₃ on number of micro shoots and shoot length of *in vitro* regenerated shoots from ray floret explants

Treatments	Number of micro shoots per callus	Shoot length (cm)
T ₁ -Basal MS medium	0	0
T ₂ -MS + 3.0 mg/l BAP + 0.1 mg/l NAA+0.1 mg/l GA ₃	7.33 ^b	5.83 ^{fgh}
T ₃ -MS+3.0 mg/l BAP+ 0.5 mg/l NAA+0.1 mg/l GA ₃	4.58 ^{cde}	7.30 ^{cd}
T ₄ -MS + 3.0 mg/l BAP+ 1.0 mg/l NAA+0.1 mg/l GA ₃	5.38 ^c	7.00 ^{cde}
T ₅ -MS + 4.0 mg/l BAP+ 0.1 mg/l NAA+0.1 mg/l GA ₃	4.10 ^{def}	5.20 ^{hi}
T ₆ -MS + 4.0 mg/l BAP+ 0.5 mg/l NAA+0.1 mg/l GA ₃	4.95 ^{cd}	5.50 ^{gh}
T ₇ -MS + 4.0 mg/l BAP+ 1.0 mg/l NAA+0.1 mg/l GA ₃	5.43 ^c	5.17 ^{hi}
T ₈ -MS + 5.0 mg/l BAP+ 0.1 mg/l NAA+0.1 mg/l GA ₃	3.92 ^{efg}	6.25 ^{efg}
T ₉ -MS + 5.0 mg/l BAP+ 0.5 mg/l NAA+0.1 mg/l GA ₃	3.03 ^g	4.65 ⁱ
T ₁₀ -MS + 5.0 mg/l BAP+ 1.0 mg/l NAA+0.1 mg/l GA ₃	1.03 ⁱ	3.80 ^j
T ₁₁ -MS + 1.0 mg/l BAP+ 0.1 mg/l NAA+0.1 mg/l GA ₃	10.15 ^a	10.53 ^a
T ₁₂ -MS + 2.0 mg/l BAP+ 0.1 mg/l NAA+0.1 mg/l GA ₃	5.53 ^c	7.70 ^{bc}
T ₁₃ -MS + 3.0 mg/l BAP+ 1.5 mg/l NAA+0.1 mg/l GA ₃	3.20 ^{fg}	7.03 ^{cde}
T ₁₄ -MS + 4.0 mg/l BAP + 1.5 mg/l NAA+ 0.1 mg/l GA ₃	1.07 ^h	3.40 ^{jk}
T ₁₅ -MS + 5.0 mg/l BAP + 1.5 mg/l NAA+ 0.1 mg/l GA ₃	1.02 ^h	2.65 ^k
T ₁₆ -MS + 1.5 mg/l BAP + 0.1 mg/l NAA+ 0.1 mg/l GA ₃	7.08 ^b	8.50 ^b
T ₁₇ -MS + 2.0 mg/l BAP + 0.5 mg/l NAA+ 0.1 mg/l GA ₃	1.65 ^h	3.60 ^j
T ₁₈ -MS + 2.0 mg/l BAP + 1.0 mg/l NAA+ 0.1 mg/l GA ₃	5.26 ^c	6.63 ^{def}
SEm ±	0.36	0.29
CD @ 1%	1.38	1.12

Rhizogenesis and acclimatization

Successful root induction on elongated shoots depends on number of factors including strength of basal medium salts, level of sucrose and supplementation of auxins. For this, half strength MS medium was supplemented with different combinations of auxins i.e. NAA and IBA for rooting of micro shoots (Table 5). Among the auxins, earliest root initiation was observed by 7.67 days with maximum root length of 7.33 cm in medium half MS + 0.5 mg/l NAA (T₅) while maximum rooting percentage of 98.30 % was recorded in medium half MS + 1.0 mg/l NAA (T₆) and highest roots per microshoot (13.50) was observed in T₇ i.e. half MS + 2.0 mg/l NAA. This indicates that compared to IBA, NAA was good to initiate earlier roots. Lower

concentration of NAA at 0.5 mg/l produced more number of roots as well as maximum root length as compared to higher concentrations. Auxin is a major player in shaping root systems by regulating growth of primary and lateral roots. Specifically, normal auxin transport and signalling are indispensable for the initiation and development of lateral root. It was in close conformity with the findings of Fukaki *et al.* (2002) [4]. Furthermore, reduction in salt levels in the MS medium particularly macronutrients including potassium during *in vitro* rhizogenesis, utilizes and prevents the accumulation of salt complex by guard cells in the stomata which facilitates for easy rooting as opined by Nagatomi *et al.* (2003) [12].

Table 5: Effect of ½ MS and NAA and IBA on days to rooting and rooting percentage, number of roots and root length of micro shoots regenerated from ray floret explants

Treatments	Days to root initiation	Rooting Percentage	Number of roots/microshoot	Root length (cm)
T ₁ -½MS+No hormones(control)	24.83 ^a	34.67(36.06) ^g	3.67 ^d	3.68 ^c
T ₂ -½ MS + 0.2 mg/l IBA	20.17 ^b	46.37(42.92) ^f	10.00 ^b	4.87 ^{bc}
T ₃ -½ MS + 0.4 mg/l IBA	11.33 ^{cd}	83.20(65.82) ^b	12.42 ^{ab}	5.83 ^{ab}
T ₄ -½ MS + 0.6 mg/l IBA	11.50 ^{cd}	51.90(46.09) ^e	11.75 ^{ab}	4.18 ^{bc}
T ₅ -½ MS + 0.5 mg/l NAA	7.67 ^d	74.87(59.91) ^c	12.93 ^{ab}	7.33 ^a
T ₆ -½ MS + 1.0 mg/l NAA	13.83 ^c	98.30(82.67) ^a	7.03 ^c	4.22 ^{bc}
T ₇ -½ MS + 2.0 mg/l NAA	9.67 ^{cd}	64.90(53.67) ^d	13.50 ^a	4.52 ^{bc}
SEm ±	1.40	0.96	0.98	0.57
CD @ 1%	5.90	4.03(3.04)	4.12	2.41

Values in the parentheses are angular transformed data

Successful micro propagation depends on the ability to transfer *in vitro* plantlets to potting mixture and acclimatized them successfully to the field conditions. Primary hardening, was done with sterilized vermiculite fortified with ½ strength MS plant salt mixture (devoid of growth regulators, calcium, organics and sucrose) then the plantlets were transferred to potting mix consisting of soil + sand + FYM (2:1:1) after 3-4 weeks of acclimatization for secondary hardening. Later these plantlets were successfully transferred to field.

This protocol may be useful for isolation and establishment of chimeric variants in case of florets especially in chrysanthemum where conventional propagation is not possible without losing the valuable mutants.

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